**Characterization of NfsB,
the minor oxygen-insensitive nitroreductase of *Escherichia coli*.**

by

Jacqueline A. Whiteway

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

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the minor oxygen-insensitive nitroreductase of *Escherichia coli***

Submitted by

Jacqueline A. Whiteway, B.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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ABSTRACT

Nitro-substituted compounds constitute a wide range of chemicals whose potent biological activity has significant human health implications. The biological activity of nitro-substituted compounds is derived from reductive metabolism of the nitro moiety, a process catalyzed by a variety of nitroreductase (NR) activities that can be classified on the basis of oxygen sensitivity. The recent accumulation of genomic data has helped lead to the recognition of the oxygen-insensitive NRs as a widespread family of enzymes that now includes an estimated 140 sequences in organisms throughout the three main domains of life. The physiological role of these enzymes remains a mystery. *Escherichia coli* has two oxygen-insensitive NRs, NfsA and NfsB. NfsB, the focus of this study, is a FMN-containing flavoprotein with a monomeric molecular mass of approximately 24 kDa, and can use either NADH or NADPH as a source of reducing equivalents.

The research reported in this thesis contributes to the characterization of *E. coli* NfsB in four main ways. First, in a combined effort to elucidate the development of 5-nitrofur resistance in *E. coli*, it was determined that the increased resistance associated with second step nitrofur-resistant mutants is a consequence of mutational inactivation of the *nfsB* gene. An unusually high proportion of mutational events in *nfsB* involved IS elements. Second, it was demonstrated that NfsB is expressed in response to induction of the SoxRS and MarRAB regulons, key components of the *E. coli* global response to environmental stress. Third, an affinity purification scheme for NfsB was developed and the protein was kinetically characterized. Detailed kinetic analysis using nitrofurazone as a substrate shows that NfsB has a catalytic specificity (k_{cat}/K_m value) of approximately $200 \mu\text{M}^{-1}\text{sec}^{-1}$ for NADH and NADPH. Mechanistically, the kinetic data are consistent

with the notion that NfsB uses a ping-pong bi-bi mechanism. Seventeen individual mutations were introduced by PCR site-directed mutagenesis. One mutation, G158S, resulted in a decreased specificity for NADPH, while another, F70S, increased the turnover number (k_{cat}) for nitrofurazone without affecting cofactor specificity. Fourth, the active site of NfsB was characterized by computer analysis, leading to suggestions for protein pockets that could be targets for drug development.

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LIST OF ABBREVIATIONS

Å	Angstroms
BLAST	Basic local alignment search tool
bp	Basepair
°C	Degrees Celsius
CFU	Colony forming units
Cnr	Classical nitroreductase (in <i>Salmonella typhimurium</i>)
dATP, dCTP, dTTP, dGTP	deoxynucleotide triphosphates (adenine, cytosine, thymidine, guanine)
ddATP, ddCTP, ddTTP, ddGTP	dideoxynucleotide triphosphates
ddH ₂ O	distilled, deionized water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra-acetate
EtBr	Ethidium bromide
FAD	Flavin adenine dinucleotide
FMN/ FMNH ₂	Flavin mononucleotide/ fully reduced form
Hfr	High frequency of recombination
hr	hours
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IS	Insertion sequence

kb	kilobasepair
k_{cat}	Turnover number, or catalytic velocity
kD	kilodaltons
K_m	Michaelis constant
mA	milliampere
min	Minutes
MW	Molecular weight
NAD(P)H/NAD(P) ⁺	Nicotinamide adenine dinucleotide (phosphate), reduced form/oxidized form
NEC	NfsB homologue in <i>Enterobacter cloacae</i>
NF	Nitrofurazone
NFR	Nitrofurazone reductase
Nfs	Nitrofurazone sensitivity
NfsA	Major NR of <i>E. coli</i>
NfsB	Minor NR of <i>E. coli</i>
NIO	Nicotinic acid
NR	Nitroreductase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TE	Transposable element

TNT	Trinitrotoluene
UV	Ultraviolet
V	Volts
V_o	Initial velocity of an enzyme-catalyzed reaction
V_{max}	Maximal velocity of an enzyme-catalyzed reaction

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Nitro-containing compounds

Nitroheterocyclic and nitroaromatic compounds constitute an enormous range of chemicals that are characterized by the presence of one or more nitro groups on a heterocyclic or aromatic nucleus. For many years nitroaromatic compounds have been used in the industrial production of explosives, pesticides, pharmaceuticals, plastics, and dyes (Drzyzga et al., 1995; Hein, 1988; Spain, 1995). Nitropolyaromatic compounds such as the nitropyrenes have been identified as byproducts of cigarette and food combustion (Salmeen et al., 1982; de Serres and Matsuchima, 1986) and as the principal genotoxic components of diesel, jet fuel, and municipal incinerator emissions (Bond et al., 1985; Eddy et al., 1986; Tokiwa and Ohnishi, 1986), carbon black and toners (Rosenkranz et al., 1980), and urban air particulate (Epstein et al., 1966; Moller et al., 1993).

Human health concern for nitroaromatic compounds has resulted from the identification of several nitro derivatives as potent bacterial mutagens (Lee et al., 1994; Moller et al., 1993; Rosencranz and Mermelstein, 1985; Tokiwa et al., 1981), as mammalian cell mutagens and clastogens (Bauchinger et al., 1988; Li and Dutcher, 1983), and as carcinogens (Cohen, 1978; Howard et al., 1985; King, 1988; Moller et al., 1993; Rosenkranz and Mermelstein, 1983; Tokiwa and Ohnishi, 1986). A further consideration is the potential for synergistic effects on mutagenicity when these compounds are components of complex mixtures (Hass et al., 1987).

Following the Second World War, recognition of the increasing resistance of bacteria to antibiotics led to the investigation of synthetic nitroheterocyclic compounds as

antimicrobial agents. In particular, the 5-nitrofurans generated interest, as these broad-spectrum agents were bactericidal and stable in the presence of serum, pus and blood. Moreover, the development of bacterial resistance to such agents appeared to be infrequent, was rarely complete (Paul and Paul, 1964), and was not transmissible to other bacteria (Asnis, 1957; McCalla et al., 1978; Sastry and Jayarman, 1984). For these reasons the 5-nitrofurans experienced widespread use as antimicrobial agents in human and veterinary medicine, and as food additives in the swine and poultry feed industry (Bryan, 1978; McCalla, 1979; Tazima et al., 1975).

The mechanism of activation of nitrofurans is similar to that of the nitroaromatics (i.e. via nitroreduction, discussed in Section 1.2.1), and thus it is not surprising that the nitrofurans have mutagenic and carcinogenic effects as well. Indeed, most nitrofurans are teratogenic, mutagenic, toxic, immunosuppressive and/or carcinogenic (reviewed in Cohen, 1978; McCalla et al., 1971; McCalla, 1979, 1980, 1983), which has stalled their widespread use as antimicrobial agents. However, many nitrofurans are still used as antimicrobial agents for treatment of infections in which the causative organisms have developed resistance to alternative drugs (Debnath et al., 1993; Johnson et al., 1999; Kwon et al., 2001; Townson et al., 1994). The much more rapid metabolism of nitrofurans by microorganisms (McCalla, 1983) compared to humans makes treatment with these drugs an option when there is no known alternative. Thus, these compounds still represent a promising class of antimicrobial agents that can be developed for the ongoing battle with pathogenic organisms.

1.2 Nitroreductases

The biological activity of nitroheterocyclic and nitroaromatic compounds is derived from reductive metabolism of the parent compound's nitro moiety, a process catalyzed by a variety of nitroreductase (NR) enzymes. Initially, nitroreductases (NRs) were recognized for their ability to reduce the nitro moiety of nitroaromatic and nitroheterocyclic compounds to form biologically reactive products. No other function was ascribed to these enzymes, which distinguished them from other enzymes that exhibited some NR activity in their established physiological roles, such as xanthine oxidase (Wang et al., 1974), NADPH-cytochrome P-450 reductase (Harada and Omura, 1980), NADPH-cytochrome c reductase (Orna and Mason, 1989), and aldehyde oxidase (Wolpert et al., 1973). Genetic characterization of a few established NRs and the advent of genome projects facilitated the identification of the oxygen-insensitive NRs as a family of enzymes.

1.2.1 Nitroreduction

NR activity can be classified on the basis of oxygen sensitivity, a consequence of the enzyme using either one or two electrons to reduce the nitro moiety of the parent compound (Bryant and Deluca, 1991; Eddy et al., 1986; McCalla, 1979; Peterson et al., 1979). In bacteria, reactions catalyzed by type I NRs are oxygen-insensitive, while those mediated by type II NRs are inhibited by the presence of oxygen (Figure 1.1) (Asnis,

Figure 1.1 (A) The metabolic activation of nitrosubstituted compounds via nitroreduction. (B) Reduction of 5-nitrofurans yields an additional intermediate, the open chain acrylonitrile, which can interact with DNA (refer to text for details) (adapted from Bertenyi and Lambert, 1996).

1957; Djuric et al., 1986; Mason and Holtzman, 1975; McCalla et al., 1975; Peterson et al., 1979).

Oxygen-insensitive nitroreduction appears to occur according to a ping-pong Bi-Bi reaction mechanism (see Section 3.4.1.3), a double displacement reaction that involves the release of a product between the addition of two substrates (Segel, 1975).

Metabolism of nitroaromatic compounds proceeding through two-electron reduction of the nitro moiety yields nitroso and hydroxylamine intermediates, and finally an amine product (Gavin et al., 1966; Biaglow, 1981). Metabolism of 5-nitrofurans compounds produces an open-chain nitrile isomer in addition to the aminofuran derivative (Debnath et al., 1993; McCalla, 1979). The endproducts of metabolism of both nitroheterocyclic and nitroaromatic compounds are biologically inactive; the biological activity of these chemicals is conferred by the reactivity of short-lived intermediates with protein, RNA and especially DNA (Djuric et al., 1986; Fifer et al., 1986; Heflich et al., 1986; McCalla et al., 1971; Mermelstein et al., 1981; Novak et al., 1995; Tatsumi et al., 1986; Tu and McCalla, 1975; Wentzell and McCalla, 1980). Nitroreduction that proceeds through single electron increments yields the nitro-anion free radical, at the first step. This radical intermediate can be reoxidized in the presence of oxygen to give the parent nitro substituted substrate and superoxide anion. The futile cycle ensuing from partial reduction and reoxidation of the nitro substituent accounts for the oxygen sensitivity of some biological NRs (Biaglow, 1981; Mason and Holtzman, 1975; McCalla, 1979; Peterson et al., 1979). Figure 1.1 illustrates this mechanism.

1.2.2 Characterization of nitroreductases

Initial investigation of *E. coli* oxygen-insensitive NRs was accomplished through the characterization of nitrofurazone resistant mutants (Bryant *et al.*, 1981; McCalla *et al.*, 1978). Resistance to elevated concentrations of nitrofurazone develops in two distinct steps (McCalla *et al.*, 1970), a characteristic shared by other nitrofurans such as the widely used antimicrobial agents nitrofurantoin (Breeze and Obaseiki-Ebor, 1983; Sastry and Jayarman, 1994) and furazolidone (Whiteway *et al.*, 1998). Similarly, in *Helicobacter pylori*, resistance to the nitroimidazole compound, metronidazole, occurs through a two step process (Jeong *et al.*, 2000). McCalla and coworkers proposed the existence of two oxygen-insensitive NR enzymes in *E. coli*, encoded by two genes that were referred to as *nfsA* and *nfsB*, and speculated that the stepwise resistance was the result of the sequential loss of the NR activities due to mutations in these genes (McCalla *et al.*, 1978; Breeze and Obaseiki-Ebor, 1983). In keeping with this speculation is the observation of cross-resistance of mutants between these chemicals (Breeze and Obaseiki-Ebor, 1983; Whiteway *et al.*, 1998).

Further biochemical characterization led researchers to identify one major (Type IA) and two minor (Types IB₁ and IB₂) oxygen-insensitive nitroreductase components (Bryant *et al.*, 1981). Approximately three-quarters of the cellular nitrofurazone (NF) reductase activity in *E. coli* is attributable to IA, and the remaining one-quarter of cellular activity is ascribed to IB₁; IB₂ contributes negligible NF reductase activity (Bryant *et al.*, 1981; McCalla *et al.*, 1975). Mutants lacking IA and IB₁ were easily obtained, and appeared to be encoded by the genes *nfsA* and *nfsB*, respectively. The gene encoding IB₂

has not yet been identified. Apart from a study by McCalla *et al.* (1975) that described a crude gel filtration separation which yielded 700 kDa and 120 kDa fractions containing oxygen-sensitive NR activity, no isolation or characterization of bacterial oxygen-sensitive NR activity has been accomplished. No mutants have been described for the oxygen-sensitive species, although extensive attempts have been made to isolate such mutants (McCalla *et al.*, 1975). The difficulty that has been encountered with obtaining type II mutants suggests that the gene(s) encoding this oxygen sensitive activity might be essential. Similarly, it has been shown that several enzymes in *H. pylori* probably have NR activity but appear to be essential for cell survival, as mutants cannot be obtained (Kwon *et al.*, 2000).

A hiatus of nitroreductase research spanned most of the 1980's, but the explosion of genetic and genomic data in the 1990's, particularly the genetic characterization of *nfsB* in *Escherichia coli* (Michael *et al.*, 1994), *cnr* in *Salmonella typhimurium* (Watanabe *et al.*, 1990a), and *nfnB* in *Enterobacter cloacae* (Bryant and DeLuca, 1991), formed the foundation upon which other NRs could be identified by means of sequence comparison. Zenno *et al.* (1996b) first proposed that this family of enzymes existed, and that it intriguingly included the flavin reductases FRaseI from *Vibrio fischeri* and Frp from *V. harveyi*. Sequence similarity searches and alignments with the available genomic data yielded putative members of this family in *Helicobacter pylori* and *Bacillus subtilis* (Whiteway *et al.*, 1998), which were later confirmed (Jenks *et al.*, 1999; Jorgensen *et al.*, 2001; Sisson *et al.*, 2000; Zenno *et al.*, 1998). Indeed, the NR family is emerging as a widely conserved group of enzymes that has members (either confirmed or putative) in

10 phyla and 69 species of the three main domains of life: Bacteria (53 species), Eukaryota (9 species), and Archaea (7 species) (Table 1.1). As is the case in *E. coli*, many of these organisms appear to contain more than one NR. According to the most recent update (version 7.1, March 2002) of the Pfam Protein Families Database (<http://pfam.wustl.edu/>), which detects family members in genomic data based on a profile hidden Markov model (Bateman et al., 2002), the nitroreductase/flavin reductase family contains 123 proteins, including four in Eukaryota, 12 in Archaea, and 107 in Bacteria (Table 1.1). Interestingly, the Pfam Protein Families Database identifies four genes in *E. coli* as encoding NR enzymes: *nfsA*, *nfsB* and two genes with uncharacterized protein products of approximately 20kDa each. One or both of these proteins may correspond to the remaining oxygen-insensitive component IB₂. It may also be that these genes are paralogs of *nfsB*, serving a different function but arising from past gene duplication (Chen and Jeong, 2000).

The Pfam database includes one human hypothetical protein as a NR family member. In addition to this hypothetical protein, humans express NAD(P)H:quinone reductase (NQO1), also known as DT-diaphorase, which is biochemically similar to the oxygen-insensitive NRs of *E. coli* (see Section 1.2.2.1), but does not share any sequence similarity with the bacterial NRs. Including NQO1, there are 19 putative homologous enzymes that may be added to the Pfam list of proteins in the NR family on the basis of biochemical evidence. The organisms in which these proteins have been identified are noted in bold in Table 1.1 and include fungi (McCoy et al., 1984; Rieble et al., 1994) and three species of marine invertebrates (Hetherington et al., 1996).

Table 1.1 Taxonomic distribution of oxygen-insensitive nitroreductases. Most proteins are identified in the Pfam Protein Families Database (version 7.1, March 2002) (refer to text for details). The total number of proteins is indicated in parentheses below the domain name.

Domain	Kingdom/Phylum/class ¹	Species	No. of members	Reference and/or protein name, if available
Bacteria (120)	Thermus/deinococcus:	<i>Thermus aquaticus</i> subspecies <i>thermophilus</i>	1	NOX* (Hecht et al., 1995)
		<i>Deinococcus radiodurans</i>	3	
	<i>Firmicutes (Gram positive):</i>			
	<u>Actinobacteria</u>	<i>Mycobacterium tuberculosis</i>	3	
	(high GC content)	<i>Mycobacterium leprae</i>	2	
		<i>Mycobacterium</i> sp. strain <i>Pyr-1</i>	1	Rafii et al., 1994
		<i>Streptomyces coelicolor</i>	7	
		<i>Streptococcus pneumoniae</i>	2	
		<i>Streptococcus pyogenes</i>	2	
		<i>Lactococcus lactis</i>	4	
		<i>Lactobacillus delbrueckii</i>	1	
		<i>Selenomonas ruminantium</i>	1	'flavoprotein'* (Anderson et al, 2002)
		<i>Phytoplasma</i> sp.	1	
		<i>Clostridium acetobutylicum</i>	11	
		<i>C. perfringens</i>	1	Rafii&Cerniglia (1993); Rafii&Hansen (1998)
		<i>C. leptum</i>	1	Rafii&Hansen (1998); Rafii et al., (1991)
		<i>C. paraputrificum</i>	1	Rafii&Hansen (1998); Rafii et al., (1991)
		<i>Clostridium</i> spp. NR8&9 (human intestine)	1	Rafii&Hansen (1998); Rafii et al., (1991)
		<i>Staphylococcus aureus</i>	3	
		<i>Paenibacillus</i> sp.	1	
		<i>P. polymyxa</i>	1	
		<i>Bacillus halodurans</i>	6	BnrB1*,B2*,B3* (Lambert, unpublished results)
		<i>B. subtilis</i>	7	NfrA1* (Zenno et al. 1998)
		<i>B. megaterium</i>	1	
		<i>B. amyloliquefaciens</i>	1	YwrO* (Anlezark et al. 2002)
		<i>Bacteroides fragilis</i>	4	Kinouchi&Ohnishi (1983)
		<i>Helicobacter pylori</i>	2	FrxA*, RdxA* (Kwon et al., 2001)
		<i>Haemophilus influenzae</i>	2	
		<i>Pasteurella multocida</i>	4	
		<i>Vibrio fischeri</i>	1	FRasel* (Zenno et al., 1994&1996b)
		<i>V. harveyi</i>	1	FRP* (Lei et al., 1994; Tanner et al., 1996)
	Bacteroides:			
	Proteobacteria:			
	<u>Epsilon</u>			
	<u>Gamma</u>			

Domain	Phylum/group	Species	No. of members	Reference and/or protein name, if available
Eukaryota (10)	<u>Gamma</u> (cont'd)	<i>V. orientalis</i>	1	
		<i>V. cholerae</i>	3	
		<i>Xylella fastidiosa</i>	1	
		<i>Shewanella hanedai</i>	1	
		<i>Pseudomonas aeruginosa</i>	4	Oh et al., 2000
		<i>P. putida</i>	2	
		<i>P. pseudoalcaligenes</i>	1	Somerville et al., 1995
		<i>Pseudomonas</i> sp. Strain G-1	1	CR(VI) reductase
		<i>P. picketti</i>	1	Yabannavar & Zylstra, 1995
		<i>Salmonella typhimurium</i>	2	Cnr* (Watanabe et al., 1998), NfsA* (Yamada et al., 1997)
	<u>Beta</u>	<i>Enterobacter cloacae</i>	2	NEC* (Bryant&DeLuca, 1991)
		<i>Morganella morganii</i>	1	Kitts et al., 2000
		<i>Yersinia pestis</i>	1	
		<i>Escherichia coli</i>	4	NfsA*, NfsB* (Bryant et al., 1981)
		<i>Neisseria meningitidis</i>	2	
		<i>Neisseria meningitidis neisseria</i>	1	
		<i>Ralstonia pickettii</i>	2	
		<i>R. eutropha JMP134</i>	1	Schenzle et al., 1999
		<i>Caulobacter crescentus</i>	4	
		<i>Rhodobacter capsulatus</i>	1 ²	BluB (Pollich&Klug, 1995)
Archaea (12)	<u>Cyanobacteria:</u> <u>Thermotogae:</u>	<i>Mesorhizobium loti</i>	3	
		<i>Synechocystis</i> sp.	1	DrgA* (Elanskaya et al., 1998)
		<i>Thermotoga maritima</i>	3	
	<u>Metazoa:</u> <u>Nematoda</u> <u>Arthropoda</u>	<i>Caenorhabditis elegans</i>	1	
		<i>Drosophila melanogaster</i>	1	
		<i>Carcinus maenas</i>	1	Hetherington et al. (1996)
		<i>Asterias rubens</i>	1	Hetherington et al. (1996)
		<i>Mytilus edulis</i>	1	Hetherington et al. (1996)
	<u>Echinodermata</u> <u>Mollusca</u> <u>Chordata</u>	<i>Homo sapiens</i>	2 ³	NQO1 (Li et al., 1995)
		<i>Mus musculus</i>	1	
		<i>Saccharomyces cerevisiae</i>	1	McCoy et al. (1984)
	<u>Fungi:</u> <u>Ascomycota</u> <u>Basidiomycota</u>	<i>Phanerochaete chrysosporium</i>	1	Rieble et al. (1994)
		<i>Sulfolobus solfataricus</i>	1	
	<u>Crenarchaeota:</u> <u>Euryarchaeota:</u> <u>Halobacteria</u>	<i>Aeropyrum pernix</i>	2	
		<i>Halobacterium</i> sp.	1	

Domain	Phylum/group	Species	No. of members
	Methanobacteria	<i>Methanothermobacter thermotrophicus</i>	1
		<i>M. thermoautotrophicum</i>	1
	Thermobacteria	<i>Pyrococcus abyssi</i>	3
	Archaeoglobi	<i>Archaeoglobus fulgidus</i>	3

Bold type indicates that the enzymes have been biochemically characterized but genetic/genomic data are not available yet.

* There is genomic/genetic *and* biochemical/protein evidence that the enzyme is part of the nitroreductase family.

¹ The taxa are assigned and organized according to the NCBI taxonomy database at

www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html (Wheeler et al., 2000). For Bacteria and Archaea, the two major divisions are phylum and class, while for the Eukaryota they are kingdom and phylum. A third level of taxonomic grouping is included only if there are putative members in more than one group within the phylum/class.

² Another sequence in *Rhodobacter capsulatus* was identified, but the two sequences are identical except for one stretch of 11 residues. Interestingly, these residues align with a putative integrase/recombinase, which leads to speculation on the contribution of transposable elements and horizontal transfer to the evolution of the apparently widespread nitroreductase family. Some corroboration of this notion is found in the IS element hotspots of the *E. coli nfsA* and *nfsB* genes (Chapter 2 of this thesis; Whiteway et al., 1998).

³ One gene encodes a hypothetical protein and is identified by the Pfam Database, but the other encodes human NAD(P)H:quinone oxidoreductase (NQO1), previously known as DT diaphorase. There are many biochemical similarities between bacterial nitroreductases and NQO1, but no sequence identity; thus, it is not clear that they are members of the same family. However, NQO1 is included in this list due to similarities with bacterial oxygen-insensitive nitroreductases in structure, mechanism, substrate/cofactor specificity, inhibitor sensitivity and role in the bioactivation of some nitro substituted compounds (Bailey et al., 1998; Chen et al., 1999; Faig et al., 2000; Hasspieler et al., 1997; Li et al., 1995).

The flavin reductases within the NR family provide reduced FMNH₂ for the luminescence reaction in *Vibrio* species, but the physiological role(s) of the nitroreductases remains a mystery. There is some evidence that NfsA and NfsB of *E. coli* (Liochev et al., 1999; Paterson et al., 2002), and the NfsA-like protein of *Salmonella typhimurium* (Nokhbeh et al, in press; Pomposiello and Demple, 2000) are involved in the cellular response to oxidative stress as part of the SoxRS regulon. NfsA and NfsB also appear to be members of the MarRAB regulon, which suggests that they are part of the global stress response that is typically associated with multiple antibiotic resistance (Barbosa and Levy, 2000). Interestingly, the putative homologues of the *E. coli* oxygen-insensitive NRs in *Caulobacter crescentus* have a distinct cell cycle expression profile, with the highest expression occurring during G1 (Grünenfelder et al., 2001). This is similar to several other proteins that are employed in the oxidative stress response, which the researchers suggest is a mechanism to suppress DNA damage during replication.

Recently, structural characterization of NRs has gained momentum. Three-dimensional (3D) structures have been resolved by X-ray crystallography for six of the bacterial enzymes (refer to Chapter 4, Table 4.1 for the PDB identifiers of these structures): *E. coli* NfsA (Kobori et al., 2001) and NfsB (Parkinson et al., 2000; Loverling et al., 2001), *E. cloacae* NEC (Hecht et al., to be published), *V. fischeri* FRaseI (Koike et al., 1998), *V. harveyi* FRP (Tanner et al., 1996), and *T. thermophilus* NOX (Hecht et al., 1995). Furthermore, some of these structures have been resolved with cofactor analogues and inhibitors.

1.2.2.1 Characteristics of the NR family proteins

A protein family groups together proteins of similar sequence and domain structure (Murzin et al., 1995) that usually have the same function in various organisms, and are assumed to share evolutionary origin (Barker et al., 1996). According to the Structural Classification of Proteins (SCOP) version 1.59 (released May 2002) (Murzin et al., 1995), NRs belong to the NADH oxidase/flavin reductase family. However, in the literature and other sources such as the previously mentioned Pfam Database, this family is more often referred to as the NR family.

Enzymes in the oxygen-insensitive NR family are flavoproteins, homodimers of approximately 50 kDa, with one tightly bound molecule of FMN (sometimes FAD) per monomer. NAD(P)H is utilized as the source of reducing equivalents, in the form of hydrides, which reduce the bound flavin molecule. In an obligatory 2-electron transfer mechanism, the reduced flavin subsequently reduces the nitro containing substrate. No semiquinone state of the bound flavin has been observed for enzymes in the NR family. This type of reaction mechanism is referred to as ping-pong, as it is a displacement reaction in which a product is released (i.e. oxidized NAD(P)⁺) between the addition of substrates (refer to Section 3.4.1.3 for more details).

Enzymes in the NR family largely share substrate specificities, with the notable exception of the flavin reductases of *V. fischeri* and *V. harveyi*. Neither NfsA nor NfsB is an effective flavin reductase. However, Zenno et al. (1996a & b) demonstrated that a single missense mutation in NfsA and NfsB resulted in each enzyme being an efficient flavin reductase, which corroborates an orthologous relationship for these proteins.

In addition to the biochemical and kinetic characterization of proteins in the NR family, six 3D structures of bacterial NRs have been resolved (see Section 1.2.2). All six proteins are classified as $\alpha+\beta$ type proteins in the SCOP database, a classification that refers to the segregated α -helix and mainly antiparallel β -sheet regions that comprise the proteins. The 3D structure of human NQO1 (see Section 1.2), which is not included in the SCOP NR family, has also been resolved. NQO1 is also a 60 kDa homodimer, with a bound FAD molecule in each monomer, and is classified as an $\alpha+\beta$ type protein. It uses NAD(P)H as a source of reducing equivalents for the obligatory 2-electron reduction of nitro containing compounds according to a ping-pong reaction mechanism.

1.2.2.2 The NR family can be divided into two subgroups

Within the NR family the enzymes can be divided into two main groups. Here, these will be referred to as NfsA-like and NfsB-like, according to the *E. coli* enzymes. There is little sequence identity between these two groups of enzymes; NfsA and NfsB share approximately 16% identity, although due to some highly conserved residues and regions (discussed in more detail in Chapter 4) sequence identity can be used to define them as part of the NR family. Biochemically, the primary distinction between the NfsA-like and NfsB-like enzymes is cofactor usage. The NfsA-like proteins are NADPH-dependent, while the majority of the NfsB-like enzymes can use both NADH and NADPH.

Despite overall sequence dissimilarity and the difference in cofactor specificity, the structures of NfsA and of FRP, the NfsA-like protein of *V. harveyi*, are similar to

those of NfsB and the NfsB-like proteins. There are a few revealing differences that can be used to distinguish them, however, which are described in Chapter 4 (Section 4.4.4).

1.2.3 *NfsB*

The *nfsB* gene (also referred to as *nfnB*, *nfsI*, *ntr* and *dpra*) in *E. coli* maps to 13 min. on the chromosome (Blattner et al., 1997; McCalla et al., 1978). Little is known about the regulatory control of *nfsB*. There are putative Shine-Delgarno, -10 and -35 sequences in the 5'-untranslated region of the gene, but these have not been experimentally confirmed. The *nfsB* gene appears to be regulated by MarA, the transcriptional activator of the multiple-antibiotic-resistance (*mar*) locus in *E. coli* (Barbosa and Levy, 2000), and is possibly part of the *soxRS* regulon (Liochev et al., 1999; Chapter 2) which suggests that regulatory sites are present within this region, even though a consensus sequence for a 'soxbox' or 'marbox' has not been identified. Some regulatory control of the *E. cloacae nfnB* gene, encoding the NEC protein, is also apparent, as the protein is induced up to ten-fold by exposure to 2,4,6-trinitrotoluene (TNT) (Bryant and DeLuca, 1991).

The *nfsB* gene encodes NfsB, a FMN-containing flavoprotein that corresponds to the minor oxygen-insensitive nitroreductase component IB₁ previously characterized in *E. coli* (Bryant et al., 1981). NfsB is composed of 217 amino acids with a monomeric molecular mass of approximately 24 kDa and can use either NADH or NADPH as a source of reducing equivalents. NfsB shares 88% amino acid sequence identity with homologous NRs isolated from *Salmonella typhimurium* (Watanabe et al., 1990a) and *E.*

cloacae (Bryant et al., 1991), 32% identity with FRaseI, the major flavin reductase of *V. fischeri* (Zenno et al., 1996b), and at least 20% identity with NfsB-like bacterial orthologues. The identification of NR homologues is largely based on the identity of conserved regions in NfsB, especially those spanning Arg-10 to Asp-17, Ser-37 to Val-50, and Gly-158 to Asp-168 (refer to Figure 1.2). To varying degrees each of these regions is involved in FMN binding, but some are also unique to the NfsB-like NRs, particularly the P⁴⁵WHFIV⁵⁰ sequence. While the conserved residues in NRs are informative, the region from approximately Phe-108 to Glu-137 in NfsB is interesting for its lack of conservation. This region most likely contributes to the flexibility of the enzyme that is necessary to accommodate the wide variety of nitro-substituted substrates. Indeed, a point mutation of Phe-124 to Ser significantly increases the catalytic specificity of NfsB for FMN, to a level comparable to that of FRaseI, the NfsB homologue in *V. fischeri* (Zenno et al., 1996c).

1.3 Applications of NfsB

The characterization of NfsB is useful for many applications including drug development (Section 1.3.1.1), cancer therapy (Section 1.3.1.2), and genetic toxicology (Section 1.3.2.1). A thorough understanding of the structure-function relationships of NfsB can be an important element in the optimization of these applications. Knowledge of essential residues for enzyme activity and specificity, and an understanding of the mechanism(s) of nitroreduction will maximize the biotechnological potential of NfsB. The growing number of patents that include nitroreductases and/or their gene sequences exemplifies the commercial potential of nitroreductases: since 1996, the US Patent

Figure 1.2 Alignment of characterized NfsB-like nitroreductases. The boxes noted as 'conserved' indicate the three conserved regions spanning Arg-10 to Asp-17, Ser-37 to Val-50, and Gly-158 to Asp-168 in NfsB that are mentioned in the text (Section 1.2.3). The 'not conserved' box outlines a region that is not well conserved in homologous enzymes and is proposed to be involved in substrate binding (Section 4.4.2.3 this thesis; Haynes et al., 2002; Koike et al., 1998; Loverling et al., 2001; Zenno et al., 1996b). The black line beneath the alignment from Pro-45 to Val-50 of the NfsB sequence indicates the NfsB specific region, especially the Trp-46 residue that is completely conserved in all characterized NfsB-like proteins, but is not present in the NfsA-like proteins.

and Trademark Office lists 75 patents involving nitroreductases; the European Patent Office and the Canadian Intellectual Property Office list 35 and 28 patents, respectively. Most of these patents involve the directed enzyme prodrug approach for cancer therapy (Section 1.3.1.2).

1.3.1 Therapeutic Applications

1.3.1.1 Antimicrobial mechanisms and drug development

The characterization of NfsB and other NRs has promoted a greater understanding of bacterial pathways of resistance to antimicrobial agents (Debets-Ossenkopp et al., 1999; Elanskaya et al., 1998; Goodwin et al., 1998; Jenks et al., 1999; Jorgensen et al., 2001; Kwon et al., 2000, 2001a&b; Sisson et al., 2000, Tankovic et al., 2000; Whiteway et al., 1998). Mechanistic knowledge of nitroheterocyclic drug metabolism is significant as it could lead to the design of new highly effective drugs with minimal side effects (Biaglow, 1981) and less chance for development of resistance. Furthermore, structure-function knowledge can lead to the design of drugs that are more specific for an enzyme, or, conversely, an enzyme that is more compatible with a drug (discussed in Chapter 4). Indeed, some investigations into alternative drugs that are activated by NfsB have been undertaken in order to obtain an optimal prodrug for use in directed enzyme prodrug therapy (DEPT) cancer therapy approaches (see Section 1.3.1.2) (Atwell et al., 1996; Bailey et al., 1996).

1.3.1.2 Cancer Therapy

The toxicity of anticancer drugs is primarily due to their lack of specificity for malignant cells. An anticancer agent can only be effective if a sufficiently high

concentration is achieved at the target site; therefore a great deal of research has focused on selectively targeting tumour cells. Directed enzyme prodrug therapy (DEPT) is a promising method for the selective delivery of cytotoxic anticancer agents to cancer cells. This approach separates the targeting and cytotoxic functions of an anticancer agent by first delivering a foreign enzyme to the target site where it can bioactivate a subsequently administered non-toxic prodrug. NfsB is an excellent candidate for the local generation of a cytotoxic drug as the nitro group of a number of compounds can act as a 'trigger' for drug bioactivation. In addition, since NfsB is a bacterial enzyme, it is unlikely that NfsB substrates will be prematurely activated by a homologous host enzyme.

The most popular choice of prodrug for NfsB has been the DNA crosslinking agent 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) (Anlezark et al., 1992; Knox et al., 1993). CB1954 is preferred to other widely used prodrugs that target DNA synthesis, as it is not dependent on a cell-cycle phase for its cytotoxicity. Furthermore, CB1954 is a poor substrate for human NQO1 (see Sections 1.2 and 1.2.2.1), but is rapidly reduced by NfsB (Anlezark et al., 1992).

Two main approaches are being explored to deliver NfsB, and other suitable bioactivating enzymes, to solid tumours. One is antibody-directed enzyme prodrug therapy (ADEPT), which uses an antibody-enzyme conjugate for delivery. The other is the so-called 'suicide' gene therapy approach, generally referred to as gene-directed enzyme prodrug therapy (GDEPT), in which the gene that expresses the foreign enzyme is delivered to the tumour site. ADEPT was initially explored as the delivery system for

NfsB (Anlezark et al., 1995; Bailey et al., 1996), but GDEPT has now been adopted as the preferred approach for NfsB delivery (Bailey et al., 1996; Bridgewater et al., 1995; Djeha et al., 2001; Green et al., 1997; Nishihara et al., 1998; Weedon et al., 2000). Table 1.2 lists the studies that have investigated the NfsB/CB1954 GDEPT pair, and the vectors that have been used to deliver *nfsB*.

In vitro studies favourably compare NfsB/CB1954 to alternative prodrug activating pairs (Grove et al., 1999). In vivo, the NfsB/CB1954 pair has been shown to be effective against a variety of cancers and tissue types (refer to Table 1.2), with a clear bystander effect (i.e. the ability to kill surrounding cells) and even regression of tumours leading to increased survival and some apparent cures in mice (McNeish et al., 1998; Wilson et al., 2002). DEPT is a relatively new approach in cancer therapy and until 1999 only two enzyme/prodrug pairs had been evaluated in clinical trials (Aghi et al., 2000). In the UK, five applications for clinical trials involving nitroreductase have been approved since 1998, although only two are actually ongoing (see Table 1.2) (Chung-Faye et al., 2001; Djeha et al., 2001).

1.3.2 Detection and analysis of nitro-containing compounds

The specificity of NfsB and other nitroreductases for ubiquitous nitroaromatic environmental contaminants makes them well suited for applications that involve the assessment of nitro compounds. Currently, NRs are widely exploited in genotoxicity assays (Section 1.3.2.1), and they also appear to have potential for the routine detection and quantification of TNT (Section 1.3.2.2).

Table 1.2 Studies of gene directed enzyme prodrug therapy (GDEPT) that use the NfsB/CB1954 enzyme/prodrug pair.

Target cancer or tissue	Vector	Tissue specific expression element and/or special feature	Level of study ¹	Reference
Gastrointestinal	Adenovirus	N/S	#Clinical I/II ²	Chung-Faye et al., 2001
Colorectal	Adenovirus	Synthetic promoter CTP1 that is dependent on beta-catenin/T cell factor for expression	In vivo	Lipinski et al., 2001a; Cobra Therapeutics Ltd.
Melanoma	Retrovirus	N/S	In vitro	Bridgewater et al., 1995
Thyroid	Retrovirus	N/S	In vitro	Nishihara et al., 1998
Lymphoma	Epstein-Barr virus (EBV)	Expressed in EBV positive tumours	In vivo	Westphal et al., 2000
Pancreatic	Retrovirus	N/S	In vivo	McNeish et al., 1998
Ovarian	Retrovirus	N/S	In vitro	Bridgewater et al., 1995
Ovarian	Retrovirus	N/S	In vivo	McNeish et al., 1998
Ovarian	Adenovirus	CMV promoter	In vivo	Weedon et al., 2000
Ovarian	Adenovirus	N/S	Clinical, 03/98	GTAC; Cobra Therapeutics Ltd.
Prostate	Adenovirus	CMV promoter	#Clinical, 04/01 ³	GTAC; Cobra Therapeutics Ltd.
Prostate	Adenovirus	Prostate-specific antigen (PSA) promoter/enhancer	In vitro	Djeha et al., 2001
Breast	Clostridial spores	In hypoxic environment they switch to reproductive state.	In vitro	Latham et al., 2000
Breast epithelial	Retrovirus	Sheep β -lactoglobulin promoter (BLG)	In vivo	Lenmon et al., 1997
p53 null	Adenovirus	p53 repressed promoter	In vivo	Clark et al., 1997
Liver	Adenovirus	N/S	Clinical, 07/99	Cui et al., 1999; Lipinski et al., 2001b
Fibrosarcoma	Adenovirus	Hypoxia inducible elements	In vivo	GTAC; Djeha et al., 2001
Various tumours	Adenovirus	Telomerase promoters hTERT&hTR	Clinical, N/S	Shibata et al., 2002
Solid tumours	Lentivirus	N/S	In vivo	Plumb et al., 2001
Feline (thyroid)	Adenovirus	Thyroglobulin promoter	In vivo	Oxford BioMedica ⁴
				Blackwood et al., 2001

Notes: N/S = not specified; GTAC = Gene Therapy Advisory Committee, Department of Health, United Kingdom.

¹ Represents the highest level of study: in vitro (low), in vivo, and clinical (high). The approval date is indicated for clinical trials. # = ongoing clinical trial.

² Phase I trial is almost complete.

³ The first gene therapy trial for prostate cancer in the UK (Press release, June 26, 2001, International Union Against Cancer).

⁴ Company website (www.oxfordbiomedica.co.uk) claims that in vivo studies are ongoing with a number of novel nitroreductases for which they have licensed the genes. More details are not available.

1.3.2.1 Genetic toxicology

Bacterial genotoxicity assays have been widely used for over two decades to identify genotoxic hazards and elucidate mechanisms of mutagenesis. A number of assays exist, including commonly used *Salmonella typhimurium* *his*⁻ reversion assay, or Ames test (Ames, 1979), and the *E. coli* *lacZ* reversion mutagenicity assay (Cupples and Miller, 1989). The Ames test has been made much more sensitive in the detection of certain carcinogens through the construction of *S. typhimurium* nitroreductase (Cnr) overproducing strains (Watanabe et al., 1989). Recently, several *S. typhimurium* tester strains were created that express the *E. coli* NfsA and NfsB proteins, and sensitivity to some compounds has been shown to be up to two orders of magnitude greater compared to strains containing only the *S. typhimurium* genes (Carroll et al., 2002). The flexibility afforded by a number of different NRs of varying activities is key to the development of sensitive and informative tester strains. The value of sensitive tester strains is threefold. First, heightened sensitivity facilitates detection of mutagens thereby requiring less sample for the testing process (Hagiwara et al., 1993; Josephy et al., 1997; Watanabe et al., 1989). Second, the combined application of these strains with isogenic tester strains of various metabolic capacities provides a means for the initial classification of mutagens (Einisto et al., 1991; Josephy et al., 1997; Jurado and Pueyo, 1995). It should be noted that some commonly used *S. typhimurium* tester strains (TA1535, TA1538 and their derivatives) are not isogenic with respect to NRs, as was previously thought (Yamada et al., 1997), and that the recent flood of genome data should help improve the precision of current genotoxicity assays. Finally, the metabolic activation pathway of a chemical

agent can be elucidated through the application of particular sets of strains (Hatcher et al., 1995; Josephy et al., 1997; Watanabe et al., 1989; 1990b). The characterization of NfsB and the ability to manipulate its sensitivity could be a means to contribute to the development of more informative tester strains. Furthermore, as evident from the new *S. typhimurium* tester strains that use *E. coli* NRs (Carroll et al., 2002), homologous enzymes from other organisms are a resource for the development of additional strains. For example, the expression of the *H. pylori* homologue RdxA in the naturally metronidazole-insensitive *E. coli* leads to metronidazole sensitivity (Sisson et al., 2000).

1.3.2.2 Biosensors

Biosensors, where biological materials are used as sensing components, are gaining attention as a means to specifically and sensitively detect compounds of environmental or health concern. Recently, an amperometric biosensor was constructed using the NEC enzyme of *E. cloacae* to detect low levels of TNT (Naal et al., 2002). The rapid quantification of TNT and its degradation products was also achieved by means of a classical assay that utilizes partially purified NR(s) from *Pseudomonas aeruginosa* (Oh et al., 2000). Both of these techniques represent alternatives to the HPLC method that is currently used, which is unsuitable for large numbers of simultaneous samples.

NRs, with their characteristically broad substrate specificity, are well suited for the detection of nitro compounds. Furthermore, it is possible that, as described in Section 1.3.3 for TNT, the NRs can be subsequently employed to detoxify the contaminant and hence remediate the polluted environment.

1.3.3 Bioremediation

In 1998, the world market for remediation was between \$US15 and 18 billion (Watanabe, 2001). An expensive and time-consuming process, remediation efforts usually focus on ground water and soil, and involve moving the contaminant in its unchanged form to another site. Phytoremediation, a rapidly growing approach that uses plants to remove contaminants (Meagher, 2001), addresses this challenge. Indeed, plants serve as both pump and treatment system: the extensive root systems of plants can extract from the soil and groundwater compounds which can then be metabolized within the plant.

TNT and its natural breakdown products are toxic and fairly stable, thereby representing a significant environmental contaminant. Recently, Bruce's lab constructed transgenic tobacco plants bearing the *nfsB*-like gene from *E. cloacae* (Hannick et al., 2001). As mentioned in Section 1.2.3, in *E. cloacae*, the NfsB-like protein (NEC) is induced up to 10 times in the presence of TNT (Bryant and DeLuca, 1991), a property that is arguably (Koder et al., 2001) ascribed to the isolation of the bacterium from a munitions dump. The NR-expressing plants displayed an increase in TNT uptake and detoxification, and the lab is now investigating the use of this gene in more suitable phytoremediation plants such as poplars, which are deep rooted and fast growing (Hannick et al., 2001).

The broad substrate specificity of NRs, particularly with ubiquitous environmental contaminants, lends itself to bioremediation efforts. Thorough

characterization of the NR proteins, their substrate specificity, and mechanism can aid in the development of specific bioremediation strategies.

1.4 Rationale and Research Objectives

At the initiation of this study, there was little research pertaining to NRs: some biochemical characterization was achieved prior to 1980, and three bacterial *nfsB* gene sequences were available. NRs were known to be key activating enzymes in the Ames test, and NfsB was being explored as a possible candidate for directed enzyme prodrug therapy. The rationale of this study was to use a molecular biology approach to elucidate the role of the *nfsB* gene in the development of resistance to nitroheterocyclic antimicrobial agents and to gain an understanding of the structure-function relationships of the NfsB protein.

The project is divided into four main research objectives and hypotheses.

1.4.1 How does resistance to 5-nitrofurans develop in E. coli?

1.4.1.1 Hypothesis

The observation that bacterial resistance to antimicrobial nitroheterocyclic agents occurs in a stepwise manner is most consistent with the notion that increased resistance is associated with sequential mutagenic inactivation of multiple NRs (Bryant et al., 1981; McCalla et al., 1978). The hypothesis of this study was that mutations in the *E. coli* genes encoding NfsA and NfsB result in the development of *E. coli* resistance to 5-nitrofurans.

1.4.1.2 Objectives

Several nitrofurazone resistant mutants that were well characterized in earlier studies (Bryant et al., 1981; McCalla et al., 1978), and several furazolidone resistant

mutants isolated in our lab were used to investigate this hypothesis by two approaches. First, biochemical characterization of the wild-type and mutant strains was achieved by means of a cellular extract assay for nitrofurazone reductase activity; and second, DNA sequence analysis was used to identify mutations in the *nfsB* gene.

1.4.2 Is NfsB part of the Sox/Mar response to environmental stress?

1.4.2.1 Hypothesis

In *E. coli*, the *soxRS* regulon is the key mediator of the oxidative stress response (Liochev et al., 1999), while the closely related *marRAB* regulon can lead to enhanced resistance to multiple antibiotics (Barbosa and Levy, 2000; Martin et al., 1999). There is a substantial overlap of genes regulated by the *soxRS* and *marRAB* regulons (Martin et al., 1999). Indeed, the *nfsA* gene is a member of the *soxRS* regulon (Liochev et al., 1999; Paterson et al., 2002), and is activated by the MarA protein of the *marRAB* regulon (Barbosa and Levy, 2000). The MarA protein also activates the *nfsB* gene (Barbosa and Levy, 2000), but it is not known whether *nfsB* is a member of the *soxRS* regulon. The hypothesis of this study was that *nfsB* is a member of both the *marRAB* and *soxRS* regulons.

1.4.2.2 Objectives

To test this hypothesis, the induction of NfsB-specific NR activity was analyzed in cellular extracts after treatment of the bacterium with paraquat and salicylate, which specifically stimulate the SoxRS and MarRAB regulons, respectively. A comparison of NADH-associated NR activity in four strains of *E. coli* with differing *nfsAnfsB* genotypes identified NfsB-specific NR activity.

1.4.3 What are the determinants of cofactor specificity in NfsB?

1.4.3.1 Hypothesis

NfsB utilizes NAD(P)H as a source of reducing equivalents, but while the 3D structure of NfsB has been resolved (Parkinson et al., 2000), the binding site for NAD(P)H was not determined and there is no obvious consensus sequence. The seven residue region of NfsB spanning Gly-153 to Leu-159 (GVAALGL) shares some similarity with known NAD(P)H binding sites. It was hypothesized that missense mutations introduced to this region may alter the cofactor specificity of NfsB, thereby indicating its involvement in NAD(P)H binding. The fact that NfsB has the unusual property of using both NADH and NADPH as cofactors was considered an asset, as any disparity in the relative specificities could be attributable to the 2'-phosphate of NADPH.

1.4.3.2 Objectives

Four primary experimental objectives were designed to test this hypothesis. First, a proficient system of protein expression and purification was developed using the IMPACT™ I system from New England Biolabs Ltd.. Second, the wild-type enzyme was kinetically characterized to determine its reaction mechanism and kinetic parameters for NADH, NADPH and nitrofurazone. Third, a PCR mutagenesis approach was used to introduce missense mutations to each position in this region. Finally, the mutant enzymes were purified and kinetically analyzed and the cofactor specificities were compared with those of wild-type NfsB.

1.4.4 What can computer analysis reveal about the active site of NfsB?

1.4.4.1 Hypothesis

Computer analysis of 3D protein structures is becoming increasingly more useful and routine with the growing number of powerful applications that are accessible to the average user. It was hypothesized that a comparison of the 3D structure of NfsB with homologous structures can elucidate fundamental aspects of the NfsB active site, particularly in conjunction with the kinetic and mutant analysis obtained from preceding studies. This notion was extended to predict regions of NfsB that could be recruited for drug or ligand development.

1.4.4.2 Objectives

A four-step approach was used to test this hypothesis. First, a structural alignment of the six proteins for which 3D structures are available was generated. Second, known ligand-protein contacts were determined for each enzyme and their positions in the 3D structures was compared. The structural contacts of other key residues in NfsB were also determined. Third, protein pockets were identified and the main active site pockets of NfsB were defined. The residues in these pockets not involved in FMN binding were considered likely candidates for NAD(P)H and substrate binding. Fourth, some of the mutant enzymes generated from previous studies were modeled and the available kinetic and structural data were harmonized with the computer analyses.

CHAPTER TWO: THE DEVELOPMENT OF 5-NITROFURAN RESISTANCE IN *E. COLI*

2.1 Introduction

2.1.1 Nitroheterocyclic drugs

Since their discovery in the 1940's, nitroheterocyclic drugs have been used extensively in clinical and veterinary medicine (McCalla, 1979) and as food preservatives (Aoki et al., 1975; De Serres, 1974). Thousands of nitroheterocyclic antimicrobial agents have been synthesized, though only a few have been used therapeutically (Hamilton-Miller and Brumfitt, 1978). Of these, the 5-nitroimidazoles and 5-nitrofurans have been especially successful as antimicrobial agents due to broad spectrum activity, high biological stability and a low acquisition of resistance by microorganisms (Bryan, 1978; Hof et al., 1987; McCalla, 1979, 1983) that is apparently not transferred to other microorganisms (Asnis, 1957; McCalla et al., 1978; Sastry and Jayarman, 1984). Nitroheterocyclic drugs can be effective against Gram negative and positive bacteria, protozoa, fungi and even worms (Hof et al., 1987).

The hope that nitroheterocyclic drugs would be the answer to the growing spectre of microbial antibiotic resistance was diminished by the discovery of the toxic, mutagenic, clastogenic, and carcinogenic effects of many of these compounds (Debnath et al, 1993; McCalla, 1983; Tu and McCalla, 1975). Nitrofurans are usually more mutagenic than nitroimidazoles (Tocher, 1997). There are a number of commonly used nitroimidazole drugs (e.g. metronidazole), but only three of the original 5-nitrofuran derivatives are currently used as antimicrobial agents. Nitrofurazone, the first compound to be introduced, is used for bladder irrigation and as a preventive agent against catheter-associated urinary tract infections (Johnson et al., 1999). Nitrofurantoin is a routine

treatment for urinary tract infections (Debnath et al, 1993). Furazolidone is often used for the treatment of gastrointestinal infections, including those due to protozoa (e.g., giardiasis, trypanosomiasis), as a treatment for swine and poultry, and as an antimicrobial agent for diseases that have developed multi-drug resistance to other antibiotics (Townson et al., 1994).

Despite the evident mutagenicity and carcinogenicity of many nitroheterocyclic antimicrobial agents, research and synthesis of novel nitro-substituted compounds, including nitrofuran derivatives, continues. Their effectiveness as broad spectrum antimicrobial agents provides the motivation for such efforts, and the large variety of compounds that can be synthesized through variation of the location and number of nitro group substitutions, allows for the design of numerous candidate drugs that may exhibit reduced toxicity and mutagenicity (Townson et al., 1994). Furthermore, in eukaryotic cells the mutagenicity of many nitro-substituted derivatives is often weak and occurs primarily under anaerobic conditions, suggesting metabolic activation by oxygen-sensitive enzymes. Indeed, exploitation of the anaerobic activation of nitro-substituted compounds such that they can serve as hypoxic-specific cytotoxins and as hypoxic indicators is being explored (Tocher, 1997).

2.1.2 Metabolic activation of nitroheterocyclic drugs

The biological activity of nitroheterocyclic drugs depends upon reductive metabolism of the nitro group attached to the imidazole or furan ring, although the metabolism of these drugs was thought to be via different pathways: the nitroimidazoles through reduction by ferredoxin or flavodoxin, and the nitrofurans by nitroreductase

activity (Townson et al., 1994). However, recent studies on the development of resistance to the nitroimidazole compound metronidazole (Goodwin et al., 1998; Jeung et al., 2000) suggest that nitroreductase activity is also involved in the biological activation of this compound.

In *E. coli*, oxygen-insensitive nitroreductase activity can mostly be ascribed to NfsA and NfsB, with some negligible activity associated with a third uncharacterized component (refer to Chapter 1, Section 1.2.2). Approximately three-quarters of oxygen-insensitive nitroreductase activity is attributable to NfsA, and the remaining one-quarter to NfsB (Bryant et al., 1981). NfsB can use either NADH or NADPH as a source of reducing equivalents, while NfsA can only utilize NADPH as a cofactor.

2.1.3 Microbial resistance to 5-nitrofurans

Many of the early insights regarding nitroreductase enzymes were obtained through the biochemical characterization of nitroreductase mutants (Bryant et al., 1981; McCalla et al., 1970, 1978). The observations that bacterial resistance to antimicrobial nitroheterocyclic agents occurs in a stepwise manner is most consistent with the notion that increased resistance is associated with the sequential inactivation of multiple nitroreductases. Although *E. coli* genes encoding the NfsA and NfsB nitroreductases have now been identified, the direct link between mutation in these genes and the development of bacterial resistance has yet to be established. To address this point, the DNA sequence alterations in the *nfsA* and *nfsB* genes of a large number of first and second step furazolidone resistant mutants and previously isolated nitrofurazone resistant mutants were analyzed in this study, and the cellular nitroreductase activities of the

mutants were determined. The results suggest that sequential resistance to nitrofurans in *E. coli* is associated with a first step event resulting in the inactivation of the *nfsA* gene, and a subsequent mutational event within the *nfsB* gene.

While inactivation of these genes leads to nitrofuran resistance, the physiological roles of NfsA and NfsB still remain a mystery. However, the reductive metabolic activity of the nitroreductase enzymes suggests a protective role. Indeed, recent research demonstrates that *nfsA* and *nfsB* are probably upregulated members of the multiple antibiotic resistance regulon *marRAB* (Barbosa and Levy, 2000), and that *nfsA* is a member of the closely related oxidative stress response regulon *soxRS* (Liochev et al., 1999; Paterson et al., 2002). Here, the inclusion of *nfsB* as part of these protective responses is investigated by assaying the activity of NfsB after induction of each regulon.

In this chapter the results pertaining to *nfsB* will be the primary focus. Characterization of the *nfsA* gene in the nitrofuran resistant mutants was accomplished by Peter Koziarz (Koziarz, 2000), and his contribution is noted and included whenever necessary for clarification. J. Veall isolated the furazolidone resistant mutants as part of a fourth year honours project. Contributions to this work were also made by B. Hoecher (directed studies project), N. Sandhu and P. Kumar (technical assistance). A large part of the work detailed in this chapter was published as "Oxygen-insensitive nitroreductases: analysis of the roles of *nfsA* and *nfsB* in development of resistance to 5-nitrofurans derivatives in *Escherichia coli*" in the Journal of Bacteriology 180:5529-5539 (Whiteway et al., 1998).

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids and growth conditions

E. coli K-12 strains AB1157, NFR402, NFR502, and SIL41 were provided by Dr. D. Bryant, McMaster University. AB1157 [*F*⁻ *thr-1 leu-6 thi-1 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 λ*⁻ *sup-37*] (Bachmann, 1972) is the parent strain of the nitroreductase mutants NFR402, and NFR502 (McCalla et al., 1978). Strain SIL41 was derived from a cross between AB1157 and an HfrH strain lacking NfsA and NfsB activity (Bryant et al., 1981). DH5α [*supE44 lacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] is a standard laboratory strain used for most plasmid manipulations. INVαF' [*F'**endA1 recA1 hsdR17 (r_k⁻,m_k⁺) supE44 thi-1 gyrA96 relA1 ϕ80lacZΔM15 Δ(lacZYA-argF)U169 λ*⁻] was included in the Original TA Cloning[®] Kit (Invitrogen Corp., San Diego CA). The vectors used in this study were pUC118 (Viera and Messing, 1982), pKK232-8 from Pharmacia Biotech Inc. (Brosius, 1984), and pCR[®]2.1 from the Original TA Cloning[®] Kit. Bacterial strains were routinely grown at 37°C in Luria-Bertani (LB) medium, or on LBA (LB medium with 1.5% agar), obtained as a premixed powder from Canadian Life Technologies Inc. (Burlington, Ontario). When appropriate, media was supplemented with antibiotics in the following concentrations: 100 µg/ml ampicillin, 30 µg/ml chloramphenicol and 50 µg/ml kanamycin. Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) and furazolidone (3-(5-nitrofurfurylideneamino)-2-oxazolidinone) were kind gifts from Dr. D. Bryant, McMaster University, Hamilton; stock solutions were prepared in dimethylsulfoxide (DMSO).

2.2.2 Isolation of furazolidone resistant strains.

Furazolidone resistant mutants were obtained as described previously using furazolidone instead of nitrofurazone (McCalla et al., 1975b). Briefly, furazolidone gradient plates were prepared as previously described (Szybalski, and Bryson, 1952) by applying a 10 ml layer of furazolidone-containing LBA upon a slanted 10 ml layer of LBA; twenty-four hours was allowed for diffusion of the drug prior to plating. Multiple cultures were grown to exponential growth phase and spread on the furazolidone gradient plates (0-1.25 µg/mL) which were incubated overnight. Several colonies were selected (first step mutants), subcultured and grown on gradient plates containing higher concentrations of the drug (0-2.5 µg/mL or 0-4 µg/mL). Isolated colonies on these plates represented second step mutants.

2.2.3 Determination of nitrofurazone sensitivity

Overnight cultures of each mutant strain were streaked with a sterile cotton swab onto a gradient plate containing 0-50 µg/mL nitrofurazone. This provided a standard measure of resistance and allowed the new mutants to be compared with previously isolated strains (McCalla et al., 1975b). Absolute concentrations of nitrofurazone tolerance were determined as described by McCalla's group (McCalla et al., 1975b): 10 µl of overnight cultures were inoculated in a grid pattern onto plates containing 5-45 µg/mL concentrations of nitrofurazone.

2.2.4 Preparation of clarified cellular extracts.

Overnight cultures (6 ml) were harvested by centrifugation; the cells were washed and resuspended in 0.25 ml 50mM Tris-HCl (pH 7.0). The suspensions were chilled on

ice, sonically treated with a Kontes Micro-Ultrasonic Cell Disruptor (power setting 3.5) for two periods of 30 sec each, and centrifuged at $14,000 \times g$, 4°C for 30 minutes. The extracts typically contained 5-10 mg of protein per mL.

2.2.5 Determination of protein concentration.

Protein concentration was determined as described by the Bio-Rad Protein Assay (Richmond, California) using a bovine serum albumin standard curve.

2.2.6 Nitrofurazone reductase assay.

Nitrofurazone reductase activity was assayed by measuring the decrease in absorbance of nitrofurazone at 400 nm (molar extinction coefficient $12,960 \text{ M}^{-1}\text{cm}^{-1}$) at 22°C on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.0), 0.1mM nitrofurazone and 0.1 mM NADPH or NADH.

2.2.7 Manipulation of DNA.

Genomic DNA was isolated from 4 ml overnight cultures as described by Rodriguez and Tait (1989) with the exception that the precipitated DNA was centrifuged at $12,000 \times g$, 4°C for 10 min., and the pellet was washed with 70% ethanol, dried and resuspended in 100 μl TE. Plasmid DNA was isolated by the boiling miniprep method described by Holmes and Quiggley (1981) as follows. Cultures (5 ml) were centrifuged in a bench top centrifuge for 5 minutes at full speed (3000 rpm) and the pellet was resuspended in 400 μl of STET buffer (8% sucrose, 5% TritonX-100, 50 mM EDTA, 50 mM Tris, pH 8.0). Lysozyme (0.5 mg/ml in 0.25M EDTA, pH 8.0) was added and the suspension was immediately boiled for 40 seconds with subsequent centrifugation for 5

min at 4°C in an angle rotor Eppendorf centrifuge. The gelatinous pellet was removed with a toothpick, and the DNA was precipitated with 10M ammonium acetate and 95% ethanol. The pellet was washed with 70% ethanol, dried and resuspended in 20 µl of TE (10mM Tris-HCl, 1mM EDTA, pH 8.0). If further purification was necessary, it was accomplished using a GENECLAN[®] Kit (Bio 101, Inc.). Standard procedures were used for molecular cloning.

2.2.8 Amplification of the *nfsB* gene.

Amplification of the *nfsB* gene (Figure 2.1) from genomic DNA was accomplished by PCR using the Expand[™] High Fidelity PCR System (Boehringer Mannheim Canada). Prior to amplification, genomic DNA was digested with *Hind*III (New England Biolabs). Reaction mixtures (100 µl) included the Expand[™] High Fidelity buffer with 15 mM MgCl₂, 0.2 mM of each dNTP, 300 nM of appropriate primers (Table 2.1), 1.7 units of Expand[™] High Fidelity enzyme mix, and 20-750 ng of template DNA. Amplification was performed through 30 repetitions of a [denaturation, 15 sec at 94°C; annealing 30 sec at 55°C; elongation, 1 min at 72°C] cycle that included a 4 min preincubation at 94°C and a 7 min postincubation at 72°C. Prior to further analysis the products were purified using a *High Pure* PCR Product Purification Kit (Boehringer Mannheim Canada). Amplification products and restriction endonuclease analyses were analyzed using agarose gel electrophoresis (1%) in the presence of ethidium bromide.

2.2.9 Cloning of *nfsB*.

Cloning of amplified *nfsB* into pCR[®] 2.1 was carried out according to the Original TA Cloning[®] Kit instructions with the following changes. The Expand[™] High Fidelity

Figure 2.1 Nucleotide sequence of the PCR-generated fragment containing the *nfsB* gene from *E. coli* AB1157 with putative -35, -10, ribosomal binding site and transcription terminator indicated. NFR502 and SIL41 refer to the mutants isolated by McCalla and coworkers (McCalla et al., 1978). Mutations for the 50 mutants isolated in this study are indicated. Altered bases and amino acids are underlined in the wild-type sequence, with the substituted bases shown above the sequence. IS elements are represented by boxes above the sequence, and the insertion point is indicated with an arrow. Deletions and insertions are indicated below the sequence. Horizontal arrows represent the PCR primers, and lowercase letters indicate the synthetic endonuclease sites added onto the PCR primers. S.D., Shine-Dalgarno sequence.

Table 2.1 Primers used in this study. Refer to the *nfsB* sequence in Figure 2.1 for primer locations.

Primer	Sequence 5' → 3' ^a	Strand ^b
E1	Ggaattc ⁻⁸⁶ GCATTTTCTCGCTTACCA	nt
E2	Cgggatc ⁺¹⁰⁸ cGTCTCCTTTATACAACAGCA	t
EBup1	⁻⁹⁰ TCTGGTGGTTGATGGTCTGG	nt
ET2	²¹⁶ GCGTAAAATGCTTGATG	nt
ET3	⁴⁹⁹ TTTGACGCCGCCATCCT	nt
EN2	⁵⁹² GGATGGCGGCGTCAAAA	t
EN3	³¹² GGCATCAAGCATTTTAC	t
EN4	⁵⁰ TCAAATGCCTTAGTGGAATG	t

Notes: ^a Superscript number indicates base number in *nfsB*. A minus or plus sign refers to bases upstream and downstream, respectively, of the coding (i.e. translatable) region of *nfsB*. Lowercase letters indicate restriction enzyme recognition sites.

^b nt = non-transcribed strand (i.e. coding); t = transcribed strand (i.e. noncoding)

PCR System was used to amplify the genes from AB1157 followed by a 10 minute incubation at 72°C with 1 Unit of *Taq* polymerase (Canadian Life Technologies Inc.) to add a 3' A-overhang. The correct sequence of a successful clone, pTAB (Figure 2.2), was verified by sequencing with universal primers for pUC vectors as well as with specific internal primers for *nfsB*. The similarly constructed clone containing the *nfsA* gene, pTAA (Figure 2.2) was obtained from P. Koziarz. The *nfsB* PCR fragment was inserted into the *EcoRI* and *BamHI* sites of pUC118 to create pJAB (Figure 2.3a). The primers used for amplification of the 850bp *nfsB* fragment contained these 5' restriction endonuclease recognition sites to facilitate cloning. pJAC (Figure 2.3b) was constructed by subcloning the 469 bp *EcoRI*(filled)-*BglII* fragment of pJAB into the *SmaI* and *BamHI* sites of pKK232-8. The 3' termini of *EcoRI* were filled in by adding 2 µl each of dATP and dTTP 1 mM stocks, and 2 units of Klenow polymerase to the pJAB heat inactivated *EcoRI* digest.

2.2.10 Sequencing of clones and PCR products.

Sequencing of the PCR products from each mutant was achieved using the Sequenase™ v.2.0 T7 DNA Sequencing Kit and [α -³⁵S]dATP (Amersham Canada Ltd.). The double-stranded template (7µl, about 1-3µg) was denatured by boiling with 15 pmol (1µl) of primer and quick-chilled on ice. Chilled Sequenase reaction buffer (2µl), 0.1M dithiothreitol (1µl), 1:5 dilution of dGTP labeling mix (2µl) and 0.5µl (5µCi) of [α -³⁵S]dATP were added to the reaction. Immediately prior to the termination reaction, 2µl of diluted (1:7) Sequenase enzyme was added. For termination, 3.2µl of the reaction mixture was added to 2.5µl of each ddNTP, incubated at 37°C for 5 min., and stopped by

Figure 2.2 The linear pCR[®]2.1 vector (Invitrogen) is shown in the centre and the clones pTAA (Koziaz, 2000) and pTAB containing PCR amplified *nfsA* and *nfsB*, respectively, are indicated below. A circular vector (pTA) was derived from the linear form by digestion with *EcoRI* directly followed by ligation.

Figure 2.3 Plasmid clones of *nfsB*. (a) pJAB: PCR-amplified *nfsB* inserted in pUC118. (b) pJAC: Truncated *nfsB*, lacking the putative transcription terminator, inserted into the pKK232-2 vector containing the CAT gene. This construct was used to test for the presence of the *nfsB* promoter in the amplified sequence (approximately 140 bp upstream of the initiation codon).

the addition of 4 μ l STOP solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The reactions were preheated at 80°C for 2 minutes, and analyzed on an 8% acrylamide/bisacrylamide gel.

IS elements were identified by DNA sequencing the gene/IS element endpoints followed by a nonredundant similarity search of sequence databases and the *Escherichia coli* Databank (Nara Institute of Science and Technology, Japan) with BLASTX (Altschul et al., 1990).

2.2.11 Nitrofurazone sensitivity assay.

Overnight cultures (100 μ l) of each strain were added to 4 ml of LB top agar (LB medium solidified with 0.7% agar) to create a lawn of bacteria on which 10 μ l of various concentrations of nitrofurazone, in DMSO, were applied in triplicate to paper disks arranged on the lawn. Overnight incubation at 37°C yielded cleared zones in the bacterial lawn, the diameter of which was measured.

2.2.12 Nitrofurazone and furazolidone susceptibility assay.

LB (4 ml) was inoculated with a few colonies and grown at 37°C until $A_{600} \approx 0.3$. Two microtubes with 1 ml of the suspension were centrifuged at 14,000xg, 5 min., and the pellets were washed with 0.1M Tris-HCl (pH 7.6). One pellet was resuspended in 0.1 ml of 0.1M Tris-HCl (pH 7.6), the other in 0.1 ml of 0.1M Tris-HCl (pH 7.6) containing 0.2 mM EDTA. After incubation at 37°C for 2 min., 1 ml of Mueller-Hinton broth (Beef infusion, Casamino acids, starch) (Difco) was added. A sterile cotton swab was used to inoculate the entire surface of Mueller-Hinton Agar plates, and allowed to dry for no more than 15 minutes. Susceptibility Test Disks (Difco) infused with nitrofurazone (75

μg) or furazolidone (40 μg) were placed, in duplicate, on the plates. Overnight incubation at 37°C yielded cleared zones in the bacterial lawn, the diameter of which was measured.

2.2.13 Chloramphenicol acetyltransferase assay.

The specific activity of chloramphenicol acetyltransferase (CAT) was determined according to the previously described spectrophotometric assay (Rodriguez and Tait, 1983). This method measures free 5-thio-2-nitrobenzoate at $\lambda = 412\text{nm}$ which has a direct molar relationship to the acetylation of chloramphenicol in the presence of 5,5'-dithiobis-2-nitrobenzoic acid and acetyl CoA (Shaw, 1975). Cell extracts were prepared from 3 ml cultures grown to $A_{450} = 1.2$ in LB supplemented with 0.2% glucose. The harvested cells were washed with 1 ml of TDTT buffer (50 mM Tris-HCl pH7.8, 30 μM DL-dithiotheitol) and resuspended in 300 μl TDTT buffer. The suspensions were chilled on ice, sonically treated with a Kontes Micro-Ultrasonic Cell Disruptor (power setting 3.5) for two periods of 30 sec each, and centrifuged at 14,000×g, 4°C for 15 minutes. The reaction mixture contained 1 μM DTNB [5,5'-dithiobis-(2 nitrobenzoic acid) in 1M Tris-HCl pH 7.8], 0.1mM Acetyl-CoA and ddH₂O. The 0.588 ml reaction mixture was incubated at 37°C for 2 min. and the A_{412} was adjusted to 0.0. Crude extract (20 μl) was added, mixed well and equilibrated for 2 minutes. Background acetylation was measured for 2 min until the slope was constant. The reaction was initiated with the addition of 12 μl 5 mM chloramphenicol, mixed well, and A_{412} was recorded for 4 minutes. CAT activity was calculated using the molar extinction coefficient ($0.0136 \text{ nM}^{-1} \text{ cm}^{-1}$) and subtracting the background rate of acetylation.

2.2.14 Protein sequence analysis.

A similarity search of protein sequence databases was conducted using the BLAST server program (Altschul, 1990) with default parameters. Protein alignments were performed by the ClustalV program (Higgins and Sharp, 1989) using default parameters.

2.2.15 Induction of nitroreductase by paraquat and salicylate.

Overnight cultures of AB1157, SIL41, JVQ1 and JVQ2 were diluted 20-fold into fresh LB medium and incubated at 37°C for 1 hour. Paraquat (0.2 mM) and sodium salicylate (5 mM) were added to the appropriate cultures and incubation was continued for one hour. Clarified cellular extracts were prepared and assayed for protein concentration and nitroreductase activity. Two independent extracts were made and each was assayed in duplicate. The confidence interval (95%) of the mean nitroreductase activity in each strain was calculated in Microsoft Excel.

2.3 Results

2.3.1 Isolation of mutant strains.

Putative nitroreductase mutants derived from AB1157 were identified on the basis of their resistance to furazolidone after stepwise selection on gradient plates. Mutant resistance to nitrofurazone, as measured by growth on a gradient plate, was used to standardize the level of nitrofurazone resistance exhibited by these mutants, and allow comparison to mutants selected in previous studies (Bryant et al., 1981; McCalla et al., 1978). The first and second selection steps yielded mutants that were resistant to nitrofurazone concentrations of approximately 15 µg/ml and 35 µg/ml, respectively. Resistance occurred incrementally: there were no instances in which a single selection step yielded a mutant that exhibited resistance to the elevated concentrations of furazolidone characteristic of second step mutants. Subsequent selection steps did yield third step mutants that exhibited resistance to still higher concentrations of furazolidone (> 4 µg/ml) though they maintained characteristic second step nitrofurazone resistance. The focus of this study is primarily on the genetic analysis of first and second step mutants.

2.3.2 Genetic analysis.

DNA sequence analysis was performed on the PCR generated amplified products of *nfsA* (Koziaz, 2000) and *nfsB* obtained from all of the furazolidone resistant mutants isolated in this study, as well as from the nitrofurazone resistant mutants NFR402, NFR502 and SIL41 obtained previously by other workers (McCalla et al., 1978). Following sequence analysis, some mutants derived through a single experiment had

identical mutations. To ensure that all mutations were of independent origin, only one identical mutant from each experiment was considered in our analysis. The strain designations and mutations are shown in Table 2.2.

All eighteen of the first step nitrofurantoin resistant mutants isolated in this study and the previously isolated first step mutant NFR402 contain a mutation in the *nfsA* gene (Tables 2.2 and 2.3). No mutations in the *nfsB* gene were observed in any of the first step mutants, while all but three of the 53 second step mutants revealed a mutation in the *nfsB* gene (Tables 2.2 and 2.3). SIL41, derived from a genetic cross between an Hfr *nfsA* *nfsB* double mutant and AB1157, exhibits wild-type levels of resistance to nitrofurantoin. However, SIL41 can be mutated to high (two step) levels of nitrofurantoin resistance through a single mutational step. SIL41 has a wild-type *nfsA* gene, and contains an amber mutation, Gln-124 (CAG) to amber (UAG), at position C⁴²⁴ in *nfsB* (Figure 2.1).

The second step mutants isolated in this study were confirmed to have only the parental mutation in *nfsA*. Only single mutations were found in the *nfsB* gene of second step mutants (refer to Figure 2.1). Eighteen of the 53 second step mutants have a base substitution in *nfsB*: one results in an ochre mutation, 17 are missense mutations. Of the 17 missense mutations, nine effected changes in a 17 amino acid sequence, from Leu-33 to Ile-49, of the NfsB protein. One mutant, JVZ2, has a three base insert (TCT) at position T⁴⁷⁷ that results in the addition of a leucine residue between Leu-59 and Asp-60. The two mutants JVAE6 and JVI3 contain frameshift mutations in *nfsB* resulting from the insertion of a cytosine at position C³⁶⁹ and the deletion of A¹²¹, events causing premature termination of translation after two and 53 amino acids, respectively. In two

Table 2.2 Mutations in the *nfsA* and *nfsB* genes of first- and second-step nitrofurantoin resistant mutants, and the nitrofurantoin reductase activities of the mutant cellular extracts.

Strain	Parent	Mutation in <i>nfsA</i> ^a	amino acid change	Mutation in <i>nfsB</i>	amino acid change	NR activity ^b nmol/min/mg	SD	potential siblings ^c
AB1157		wild-type	wild type	wild-type	wild-type	55.2	8.9	
PREVIOUSLY CHARACTERIZED MUTANTS (NITROFURANTOIN RESISTANT)								
NFR402	AB1157	IS186(388)	insert	wild-type	wild-type			
NFR502	NFR402	IS186(388)	insert	C(134)→T	pro(45)→leu			NFR502I
SIL41		wild-type	wild type	C(424)→T	gln(142)→Amber	53.3	0.9	
FIRST STEP MUTANTS (FURAZOLIDONE RESISTANT)								
<i>NfsA</i> missense								
JVC1	AB1157	A(201)→C	gln(67)→pro	wild-type	wild-type	15.32	6.16	
JVJ1	AB1157	G(16)→T	glu(6)→ochre	wild-type	wild-type	7.162	0.06	
JVK1	AB1157	C(43)→T	arg(15)→cys	wild-type	wild-type	7.599	0.07	
JVO1	AB1157	T(266)→A	ile(89)→asn	wild-type	wild-type	8.05	0.1	
JVY1	AB1157	A(32)→T	his(11)→leu	wild-type	wild-type	7.995	1.01	
Average activity of all first-step mutants:						9.15^d	1.9	
NfsA other mutations								
JVA1	AB1157	IS1(38)	insert	wild-type	wild-type	10.669	0.14	
JVB1	AB1157	IS30(675)	insert	wild-type	wild-type	10.373	3.39	X1
JVD1	AB1157	IS186(388)	insert	wild-type	wild-type	8.532	2.09	E1
JVG1	AB1157	IS30(675)	insert	wild-type	wild-type	6.844	0.58	
JVH1	AB1157	IS1(119)	insert	wild-type	wild-type	7.671	0.27	
JVI1	AB1157	IS1(644)	insert	wild-type	wild-type	10.755	0.05	
JVL1	AB1157	IS186(388)	insert	wild-type	wild-type	11.012	1.95	AE1, AG1
JVN1	AB1158	IS1(-246)	insert	wild-type	wild-type	9.295	0.07	
JVP1	AB1157	IS1(560)	insert	wild-type	wild-type	8.838	1.54	
JVQ1	AB1157	(-637→813)	deletion	wild-type	wild-type	8.984	0.82	
JVR1	AB1157	(625)CGGGAT→CGGATA	+1 frameshift	wild-type	wild-type	8.967	0.5	
JVW1	AB1157	IS1(-235)	insert	wild-type	wild-type	9.2	0.74	
JVZ1	AB1157	IS1(-92)	insert	wild-type	wild-type	7.323	1.15	AA1
Average NfsA other:						9.11	1.3	

Table 2.2 (cont'd)

Strain	Parent	Mutation in <i>nfsA</i> ^a	amino acid change	Mutation in <i>nfsB</i>	amino acid change	NR activity ^b nmol/min/mg	SD	Potential siblings
SECOND-STEP MUTANTS								
NfsB missense								
JVA5	JVA1	IS1(38)	insert	A(118) → T	ser(40) → cys	2.326	0.28	
JVB4	JVB1	IS30(675)	insert	C(213) → A	asp(71) → lys	0.874	0.02	
JVE2	JVE1	IS186(388)	insert	C(116) → T	ser(39) → phe	1.845	0.4	E3
JVE4	JVE1	IS186(388)	insert	G(442) → A	gly(148) → ser	0.831	0.06	
JVG2	JVG1	IS30(675)	insert	G(313) → T	asp(105) → tyr	1.8	1.33	G4
JVG3	JVG1	IS30(675)	insert	G(523) → T	glu(175) → ochre	ND		
JVH2	JVH1	IS1(119)	insert	T(98) → C	leu(33) → pro	0.888	0.08	
JVI2	JVI1	IS1(644)	insert	T(248) → A	val(83) → asp	1.147	0.39	
JVL6	JVL1	IS186(388)	insert	T(560) → A	val(187) → glu	1.758	0.5	
JVN2	JVN1	IS1(-246)	insert	G(466) → C	ala(156) → pro	ND		
JVP2	JVP1	IS1(560)	insert	C(111) → A	ser(37) → arg	0.741	0.38	
JVQ4	JVQ1	-(637→813)	deletion	C(134) → A	pro(45) → gln	0.862	0.71	
JVR3	JVR1	(625)CGGGAT→CGGATA	+1 frameshift	A(131) → C	gln(44) → pro	0.754	0.56	
JVY2	JVY1	A(32) → T	his(11) → leu	G(776) → A	arg(207) → his	0.847	0.62	
JVAE2	JVAE1	IS186(388)	insert	T(146) → A	Ile(49) → Asn	ND		
JVAE5	JVAE1	IS186(388)	insert	C(134) → A	pro(45) → gln	0.665	0.42	
JVAG3	JVG1	IS186(388)	insert	T(547) → G	tyr(183) → asp	1.077	0.09	AG4, AG5
JVAG6	JVAG1	IS186(388)	insert	T(101) → G	leu(34) → arg	0.301	0.18	AG2
Average NfsB missense:						1.11	0.56	
NfsB other mutations								
JVA2	JVA1	IS1(38)	insert	IS1(165)	insert	1.261	0	
JVB3	JVB1	IS30(675)	insert	IS1(62)	insert	1.209	0.43	B2
JVD2	JVD1	IS186(388)	insert	IS5(43)	insert	0.831	0.13	
JVD3	JVD1	IS186(388)	insert	IS5(43)	insert	1.401	0.09	
JVE5	JVE1	IS186(388)	insert	IS5(43)	insert	0.886	0.06	
JVH3	JVH1	IS1(119)	insert	IS5(43)	insert	0.561	0.08	
JVH4	JVH1	IS1(119)	insert	IS1(3)	insert	1.438	0.19	

Average activity of all second-step mutants: 1.03^c 0.5

Table 2.2 (cont'd)

Strain	Parent	Mutation in <i>nfsA</i>	amino acid change	Mutation in <i>nfsB</i>	amino acid change	NR activity nmol/min/mg	SD	Potential siblings
JV14	JV11	IS1(644)	insert	IS5(43)	insert	0.913	0.04	
JV15	JV11	IS1(644)	insert	(118)AGCACCC→AGCCCCA	+1 frameshift	1.031	0.13	I3
JV16	JV11	IS1(644)	insert	IS1(110)	insert	1.456	0.07	
JV13	JVJ1	G(16)→T	glu→ochre	IS1(582)	insert	1.106	0.14	J2,J4-6,J8-9
JV17	JVJ1	G(16)→T	glu→ochre	IS5(43)	insert	1.118	0.72	
JV12	JVL1	IS186(388)	insert	IS5(43)	insert	ND		
JV13	JVL1	IS186(388)	insert	IS1(44)	insert	1.65	0.44	
JV14	JVL1	IS186(388)	insert	IS1(651)	insert	1.154	0.15	
JV17	JVL1	IS186(388)	insert	IS1(545)	insert	ND		
JVP3	JVP1	IS1(560)	insert	IS2(-3)	insert	1.177	0.26	
JVP4	JVP1	IS1(560)	insert	IS1(48)	insert	0.816	0	
JVP5	JVP1	IS1(560)	insert	IS1(49)	insert	1.187	0.3	
JVQ2	JVQ1	-(637→813)	deletion	IS5(43)	insert	0.725	0.04	
JVQ3	JVQ1	-(637→813)	deletion	-(257→342)	deletion	0.713	0	
JVR4	JVR1	(625)CGGGAT→CGGATA	+1 frameshift	-(267→343)	deletion	0.778	0.1	
JVW2	JVW1	IS1(-235)	insert	IS2(68)	insert	2.255	0.88	
JVW3	JVW1	IS1(-235)	insert	IS5(43)	insert	1.327	0.92	
JVX2	JVX1	IS30(675)	insert	IS1(158)	insert	0.502	0.03	
JVX3	JVX1	IS30(675)	insert	IS5(43)	insert	0.738	0.57	X4,X6
JVX5	JVX1	IS30(675)	insert	IS1(589)	insert	0.551	0.14	
JVX7	JVX1	IS30(675)	insert	IS1(620)	insert	0.531	0.1	
JVZ2	JVZ1	IS1(-92)	insert	+TCT(477)	asp(60)→ser	0.678	0.3	
JVAE3	JVAE1	IS186(388)	insert	IS2(610)	insert	0.458	0.03	
JVAE6	JVE1	IS186(388)	insert	(366)GTTCTT→GTTCT	-1 frameshift	0.239	0.18	
				Average NfsB other:				0.4
JVA6	JVA1	IS1(38)	insert	no mutation	no mutation	8.649	0.84	
JVN3	JVN1	IS1(-246)	insert	no mutation	no mutation	2.958	0.56	
JVO2	JVO1	T(266)→A	ile(89)→asn	no mutation	no mutation	1.759	0.3	
JVO3	JVO1	T(266)→A	ile(89)→asn	no mutation	no mutation	7.613 ^f		
JVAE4	JVAE1	IS186(388)	insert	C(678)→A	transcription terminator	1.656	0.4	

Table 2.2 (cont'd)

- ^a Data obtained by P. Koziarz.
 - ^b Average NR activity and standard deviations calculated from assays done at least twice on two independent extracts.
 - ^c Strains with identical mutations from a single mutant isolation step and thus were excluded from data analysis.
 - ^d Average for all 18 first step mutants.
 - ^e Average for all 44 second-step mutants for which a mutation has been characterized. The cultures of five second-step mutants were not viable after storage, and enzyme activity was not determined.
 - ^f NR assay only performed once.
- ND = Nitroreductase activity not determined as cultures were not viable after storage.

Table 2.3 Mutation distribution in *nfsA* and *nfsB* of nitrofurantoin resistant mutants.

	<i>nfsA</i> (of 1 st step mutants) ^a	<i>nfsB</i> (of 2 nd step mutants) ^b
Base substitutions		
Total	5	20^d
G:C → T:A	1	6
G:C → C:G	0	1
A:T → T:A	2	4
A:T → C:G	1	3
G:C → A:T	1	5 ^d
A:T → G:C	0	1
Frameshifts and insertions	1	3
Deletions	1 (177 bp)	2 (~80 bp)
Insertion elements	12	26
IS1	7	13
IS2	0	3
IS5	0	10
IS30	2	0
IS186	3 ^c	0
No mutation in gene	0	4
Total # of mutants	18 (19^c)	53 (55^d)

^aData obtained by P. Koziarz. The same mutations, and no others, are in *nfsA* of second step mutants derived from first step mutants.

^bThe *nfsB* gene of first step mutants is wild-type.

^cThe mutant NFR402 isolated by McCalla *et al.* (1978) is included.

^dThe mutants NFR502 and SIL41 isolated by McCalla *et al.* (1978) are included.

mutants, JVQ3 and JVR4, nearly identical deletions of approximately 80 bp are situated in the middle of the gene. The most frequent type of mutation identified in the *nfsB* gene of the furazolidone resistant mutants is IS element integration. Three IS elements, IS1, IS2, and IS5, account for 49% of the second step mutants isolated in this study. With the one exception of IS1 inserting near the Shine-Dalgarno sequence of JVP3, all IS elements integrated within the coding region of *nfsB*. IS elements demonstrated regional specificity for insertion in the regions proximate to either terminus of *nfsB*. This is particularly apparent in the first 165 bp of the gene's coding region where IS element integration occurred in 19 mutants. IS5 integrated at the identical sequence, after G⁴³, in all ten mutants with this element; IS5 inserted in the identical orientation in each mutant with the exception of JVD2 in which it was reversed. In four second step mutants, no mutations were observed within the coding region of *nfsB* although multiple sequencing reactions were carried out on both strands of the PCR products. Of these, one mutant, JVAE4, contained a transversion in the putative transcription terminator. The resistance of these mutants to a concentration of nitrofurazone (45 µg/ml) consistent with a second step mutant was confirmed. To ensure that there is not a mutation in or near the putative -35 region of *nfsB*, another primer, EBUP1 (see Figure 2.1), was used to amplify and sequence this region. No mutations were found.

2.3.3 Promoter activity of *nfsB*.

The wild-type sequence determined for the complete *nfsB* gene of the three second step mutants JVA3, JVN3 and JVO2 suggested the possibility that the promoter

region might be inactive. We confirmed the presence of a functional promoter in the PCR amplified product of *nfsB* by using the construct pJAC (Figure 2.3b). pJAC contains the promoterless chloramphenicol acetyltransferase (CAT) gene of plasmid pKK232-8 directly downstream from the truncated fragment of *nfsB*, containing the putative promoter region, so that any expression of CAT, and hence resistance to chloramphenicol, was due to the promoter activity of *nfsB*. Growth of transformants derived from pJAC on chloramphenicol medium confirmed the presence of a functional *nfsB* promoter. This construct enabled the measurement of promoter activity by determining the specific activity of CAT ($126 \text{ nmol min}^{-1} \text{ mg}^{-1}$). The specific activity of CAT in the negative control, containing only pKK232-8, was zero.

2.3.4 Nitroreductase activity.

Nitroreductase activities in cell extracts of AB1157 (wild-type), first step mutants and second step mutants were spectrophotometrically measured as the reduction of nitrofurazone using NADPH as a source of reducing equivalents. The average NADPH-dependent nitroreductase activities of AB1157, first step mutants, and second step mutants were 55.2 , 9.2 and $1.0 \text{ nmol min}^{-1} \text{ mg}^{-1}$, respectively (Table 2.2). Three second step mutants have significantly greater nitroreductase activity than the average: JVA6 and JVN3, two of the three second step mutants that do not have a mutation in *NfsB*; and JVA5, which has a missense mutation (Ser-40 $\rightarrow$ Cys) in *NfsB*. Of particular interest is JVA6, which has nitroreductase activity ($8.4 \pm 0.8 \text{ nmol min}^{-1} \text{ mg}^{-1}$) comparable to a first step mutant. JVN3 has intermediate nitroreductase activity ($3.0 \pm 0.56 \text{ nmol min}^{-1} \text{ mg}^{-1}$) that is approximately three fold less and greater than first- and second- step mutants,

respectively. The second step mutant, JVAE4, which has a mutation in the putative transcription terminator but not in the coding region of *nfsB*, has nitroreductase activity consistent with second step mutants. JVO2, the third mutant without a mutation in *nfsB*, has activity ($1.8 \pm 0.3 \text{ nmol min}^{-1} \text{ mg}^{-1}$) that is within the activity range for second step mutants. Although JVO3 is a potential sibling of JVO2, the NR activity of JV03 ($7.6 \text{ nmol min}^{-1} \text{ mg}^{-1}$) is similar to a first-step mutant. SIL41 has nitroreductase levels ($53.3 \pm 0.9 \text{ nmol min}^{-1} \text{ mg}^{-1}$) similar to the wild-type.

2.3.5 Restoration of nitrofurantoin sensitivity by cloned *NfsA* and *NfsB*.

The influence of plasmid encoded *NfsA* and *NfsB* *in vivo* was evaluated for the wild-type *E. coli* strain AB1157, a first step mutant containing a 177 bp deletion in *nfsA* (JVQ1), and a second step mutant derived from JVQ1 with IS5 integrated after position 43 of *nfsB* (JVQ2), by comparing their levels of nitrofurantoin sensitivity to those observed when the strains were transformed with pTAA (*nfsA*) or pTAB (*nfsB*) (Figure 2.2). Sensitivity was compared by measuring the diameters of cleared zones around a nitrofurantoin-infused paper disk on a lawn of bacteria (Table 2.4). The greatest differences in sensitivity were noted for concentrations between 5 mg/ml and 10 mg/ml of nitrofurantoin. Further increases in sensitivity were not observed at concentrations above 10 mg/ml, and concentrations of 1 mg/ml or lower did not yield cleared zones at all.

Cleared zone diameters obtained with each strain are shown in Table 2.4. From this experiment, four observations were made. (1) AB1157, JVQ1, and JVQ2 each had a significantly different sensitivity to nitrofurantoin; AB1157 demonstrated the greatest

Table 2.4 Diameters (mm) of cleared zones in a lawn of each strain with and without vector encoded NfsA (pTAA) and NfsB (pTAB) after the application of 5 mg/ml nitrofurazone.

Strain	vector alone (pTA)	+ pTAA	+ pTAB
AB1157	18.4 (0.4)	20.6 (0.5)	20.9 (0.5)
JVQ1	15.5 (0.5)	18.8 (1.3)	18.9 (0.7)
JVQ2	12.5 (0.5)	20 (1)	18.8 (0.8)

Note: Standard deviations calculated from five determinations are indicated in parentheses.

sensitivity, and JVQ2 the least. (2) Upon the introduction of either plasmid-encoded nitroreductase, an increase in sensitivity was observed over that exhibited by the strain transformed with vector alone. (3) Between strains, no significant difference between the sensitivity conferred by the *nfsA*- or the *nfsB*-containing clone was apparent. (4) Transformation with either *nfsA* or *nfsB* increases sensitivity of all strains to a similar level.

2.3.6 EDTA treatment and nitrofurantoin susceptibility

EDTA (0.2 mM) results in a nonspecific increase in permeability in *E. coli* (Leive, 1965). To test if the elevated resistance of JVA6 and two of the “third-step” mutants, JVE2 α and JVE2 β , was due to decreased cellular permeability, cells were treated with EDTA and a susceptibility disk assay was performed in duplicate. Related strains were tested as well, including wild-type AB1157 and the parental strains JVA1, JVE1 and JVE2. Susceptibility to nitrofurazone and furazolidone was not affected in any of the strains by the EDTA treatment (data not shown).

2.3.7 Induction of nitroreductase by paraquat and salicylate.

Figure 2.4 illustrates the NADH and NADPH-dependent nitroreductase activity of AB1157, SIL41 and JVQ1 before and after paraquat and salicylate induction. JVQ2 is not included as it has very little nitroreductase activity (< 2 nmol/min/mg) in both cases.

The NADPH:nitroreductase activity (uninduced) of AB1157 and SIL41 was similar (~ 32 nmol/min/mg) and significantly greater than JVQ1 (~ 7 nmol/min/mg). AB1157 had the highest level of NADH:nitroreductase activity (11.6 ± 2 nmol/min/mg), followed by JVQ1 (5.9 ± 3 nmol/min/mg). The NADH:nitroreductase activities of AB1157 and

Figure 2.4 The inducibility of NAD(P)H associated nitroreductase activity with paraquat and salicylate treatment in cellular extracts of *E. coli* AB1157 and its derived mutants. SIL41 lacks functional NfsB, and JVQ1 lacks functional NfsA. JVQ2, lacking both NfsA and NfsB, had too little nitroreductase activity to assay and thus is not included here. Error bars represent the confidence interval of nitroreductase activity at a significance level of 0.05 as derived from the standard deviation of four trials.

SIL41 were significantly less than those obtained with NADPH, about one-third and one-tenth respectively, while similar activities were observed in JVQ1 when either NADH or NADPH were used as cofactors.

Both paraquat and salicylate induction led to an increase in total oxygen-insensitive nitroreductase activity in AB1157, SIL41 and JVQ1. At the concentrations used, the inducers yielded similar levels of nitroreductase activity. For AB1157 and SIL41, induction of the NADPH associated activity was especially apparent relative to NADH:NR activity, while JVQ1 had nearly equal levels of NADH and NADPH associated nitroreductase activity.

A comparison of NADPH:nitroreductase activities demonstrates about a 4-fold induction in AB1157, and an approximate 3-fold induction in each SIL41 and JVQ1 following treatment with either paraquat or salicylate. While the uninduced nitroreductase activity of SIL41 and AB1157 is similar, about 32 nmol/min/mg, the induced level of SIL41 (~100 nmol/min/mg) is approximately one-third less than AB1157 (~140 nmol/min/mg). The induced NADPH:nitroreductase activity of JVQ1 (~20 nmol/min/mg) is significantly less than for both SIL41 and AB1157.

The induced NADH:nitroreductase activity in AB1157 (~20-35 nmol/min/mg) is approximately 2-3 fold greater than the uninduced activity (11.6 ± 2 nmol/min/mg). Induced NADH associated activity in JVQ1 is essentially the same as for NADPH activity, about 20 nmol/min/mg, which is similar to the NADH:nitroreductase levels of induced AB1157. The NADH:nitroreductase levels in SIL41 are very low even after

induction, <10 nmol/min/mg, and although some induction may exist, the low activity level precludes making reliable comparisons.

2.4 Discussion

2.4.1 Isolation of nitrofurantoin resistant strains

The stepwise manner in which furazolidone-resistant mutants were obtained in this study was comparable to that previously used to select mutants resistant to nitrofurazone (McCalla et al., 1970) and nitrofurantoin (Breeze and Obaseiki-Ebor, 1983; Sastry and Jayarman, 1984). Selection of *E. coli* K-12 nitrofurantoin-resistant strains on medium supplemented with furazolidone, nitrofurazone, and nitrofurantoin consistently resulted in two discrete steps of increased resistance. As compared to the parent strain AB1157 which is resistant to 5 µg/ml nitrofurazone, first and second step mutants selected on furazolidone were resistant to nitrofurazone concentrations of 15 µg/ml and 35 µg/ml, respectively; similar levels of resistance were observed in the mutants NFR402 and NFR502 that were initially selected on nitrofurazone (McCalla et al., 1970; 1978). Biochemical analysis of the nitrofurazone resistant mutants NFR402 and NFR502 suggested that the stepwise resistance of *E. coli* to nitrofurans is caused by the sequential inactivation of the proteins NfsA and NfsB: NfsA activity is absent from both the first step mutant, NFR402, and the second step mutant, NFR502, while NfsB activity is present only in NFR402 (Bryant et al., 1981). DNA sequence analysis of a large number of nitrofurantoin resistant mutants in this study strengthens this observed pattern and strongly suggests that two step resistance is a consequence of an obligatory first step event, mutation in *nfsA*, followed by mutational inactivation in *nfsB*. Further evidence that nitrofurantoin resistance occurs through an ordered inactivation of *nfs* genes can be found in the interesting resistance characteristics of SIL41. SIL41 was constructed by crossing an

Hfr *nfsA nfsB* double mutant and AB1157 (*nfsA*⁺ *nfsB*⁺) (Bryant et al., 1981). The resulting *nfsA*⁺ *nfsB* strain, in which the *nfsB* gene was inactivated by an amber mutation at C⁴²⁴, has a level of nitrofurazone resistance that is similar to the wild-type strain, suggesting that inactivation of *nfsB* is phenotypically silent in the presence of a wild-type *nfsA* gene. However, in contrast to wild-type bacteria, SIL41 can attain a high (second step) level of nitrofurazone resistance in a single step that presumably corresponds to inactivation of *nfsA*.

2.4.2 Characterization of the nitrofurant resistant mutants

The genetic characterization of these mutants corroborates previous biochemical studies suggesting that NfsA and NfsB nitroreductase activity is necessary for the majority of nitrofurant biological activity under aerobic conditions (Bryant et al., 1981; McCalla et al., 1978). Furthermore, by transforming strains with vector-encoded NfsA and NfsB, these proteins are shown to be sufficient for maximal nitroreductase activity. Evidence that the sensitivity of a cell to nitrofurants is dependent on the functional status of NfsA and NfsB was shown by introducing cloned *nfsA* and *nfsB* into AB1157 (wild-type) and its nitroreductase deficient derivatives. Significant increases in the cleared zone around the nitrofurazone impregnated filters were observed in the strains AB1157, JVQ1 and JVQ2 upon introduction of pTAA (*nfsA*) or pTAB (*nfsB*). The increases in AB1157 and JVQ1 upon the introduction of pTAB were presumably due to the multiple copies of the plasmid-encoded gene. The larger increase in sensitivity observed in the transformed first and second step mutants is attributable to a restoration of nitroreductase activity. While the percent increase in sensitivity was far greater in the mutants than in

the wild-type, the absolute sensitivity of the transformants, as indicated by the diameter of the cleared zone in the lawn, was similar to AB1157 transformants. This maximum diameter could represent a limit either for the chemical's diffusion, or for cellular sensitivity to nitrofurazone.

Of the mutants selected in this study, three second step mutants did not have a mutation in *nfsB* (Table 2.2) and one had a base substitution downstream of the coding region in the putative transcription terminator (Figure 2.1). Confirmation of the resistance of these mutants to elevated concentrations of nitrofurazone and furazolidone eliminates the possibility of mutant reversion. The complete sequence of *nfsB* in these mutants, including the -35 region, was determined to be wild-type. Confirmation that this *nfsB* fragment contained a functional promoter was achieved by demonstrating CAT activity in the plasmid pKK232-8, which contains a promoterless CAT gene, upon insertion of the putative promoter region of *nfsB*. NfsB is normally sufficient for biological activation of nitrofurans: sensitivity to nitrofurazone is increased upon the introduction of vector-encoded NfsB in both wild-type and mutant *E. coli* cells. Thus, the increased resistance demonstrated by these mutants suggests that additional factors may modulate nitrofurans resistance. Possible explanations include: (1) a nitroreductase enzyme distinct from NfsA or NfsB; (2) a factor mediating transport of the nitrofurans into the cell; or (3) a *trans* acting factor with which interaction is essential for full nitroreductase activity. In support of the first explanation, a third oxygen insensitive nitroreductase component, IB₂, was described by Bryant and coworkers (Bryant et al., 1981). It is conceivable that a minority of second step mutants could be due to

inactivation of this third minor component of nitroreductase. The last two possibilities may apply to JVA6; however, it is unlikely that transport of the nitrofurans into the cell is a factor, as treatment with 0.2 mM EDTA does not yield greater sensitivity of JVA6 to furazolidone or nitrofurazone. EDTA at noninhibitory levels causes a non-specific increase in the permeability of *E. coli* (Leive, 1965), including to nitrofurans (Obaseiki-Ebor, 1984).

Beyond the initial two levels of resistance, mutants isolated on nitrofurantoin and furazolidone appear to have nitrofurans tolerance properties not shared by nitrofurazone-resistant mutants. Third step furazolidone-resistant mutants displayed inconsistent and non-uniform resistance when plated on nitrofurazone medium. The third level of resistance observed in our studies is similar to that described in the higher order nitrofurantoin-resistant mutants observed by Breeze and Obaseiki-Ebor (1983). Those workers showed that putative third and fourth step mutants selected on nitrofurantoin did not display cross-resistance to nitrofurazone; rather, they were characterized by a level of nitrofurazone resistance that was comparable to second step mutants. It is possible that the principal mode of toxic action of the reactive metabolites derived from nitroreduction differs between nitrofurans derivatives. For example, nitrofurans have a variety of toxic actions including genotoxicity, metabolic effects, effects on nucleotide pools, and formation of active oxygen species (McCalla et al., 1975b; Tu and McCalla, 1975) although the bacteriostatic effect of nitrofurans appears to correlate primarily with the extent of chromosomal damage (Jenkins and Bennett, 1976; Lu and McCalla, 1978). Alternatively, the participation of other, as yet uncharacterized, nitroreductase activities

may contribute to the reduction of a subset of nitrofurans derivatives (e.g. furazolidone and nitrofurantoin, but not nitrofurazone) in the absence of NfsA and NfsB activity. A third possibility is that the high levels of unreduced nitrofurazone in higher order nitroreductase mutants may have an inhibitory effect on bacterial metabolism when mutants are plated on nitrofurazone medium (Tu and McCalla, 1975). Obaseiki-Ebor (1984) suggested that high-level nitrofurantoin resistant mutants were due to decreased permeability of the drug; however, mutants selected for nitrofurantoin resistance were not affected by EDTA treatment (Obaseiki-Ebor, 1984). Similarly, decreased permeability to furazolidone does not appear to be a factor for the higher level mutants tested in this study, as treatment with EDTA did not increase sensitivity of the mutants. Interestingly, Obaseiki-Ebor (1984) observed that EDTA treatment increased the nitrofurans susceptibility of aminoglycoside resistant mutants cross resistant to high levels of nitrofurantoin, suggesting another resistance mechanism for nitrofurans. A study by Morozov and colleagues (1994) also indicated that nitrofurans resistance is attributable to a different mechanism in these cross-resistant aminoglycoside mutants.

2.4.3 IS element hotspots are likely present in the nfsB gene

Among the 49 mutations in the *nfsB* gene of the furazolidone-resistant mutants isolated in this study, 26 (53%) were derived from the insertion of one of the insertion elements IS1, IS2 or IS5 (Table 2.3). Transposition is usually a rare event, occurring at frequencies of 10^{-7} to 10^{-5} per generation, with only 5-15% of all spontaneous mutations being attributed to IS elements (Galas and Chandler, 1989). Although there is a high degree of variability in the copy number and presence of IS elements in *E. coli* strains

(Boyd and Hartl, 1997), all IS element types have fixed locations on the chromosome of *E. coli* K-12 strains, as well as having additional strain specific locations (Birkenbihl and Vielmetter, 1989; Boyd and Hartl, 1997). All of the IS elements characterized in this study have been mapped in various *E. coli* strains (Birkenbihl and Vielmetter, 1989; Boyd and Hartl, 1997). Distribution of IS elements in a host genome is nonrandom, and though, in part, this must be due to positional constraints imposed by consequences on the host's fitness, it is also a result of insertion preference at certain sequences. Clustering of particular IS families to certain chromosomal locations is believed to result from local hopping, a process in which local transposition events give rise to daughter IS elements inserted in proximity (<10kb) to the parent element (Boyd and Hartl, 1997; Craig, 1997). The *nfsB* gene is located at 13 min. on the *E. coli* chromosome (Blattner, 1997). IS5, at 13.1 min., is the only element identified in this study to be located near *nfsB* in the three mapped *E. coli* K-12 strains (Birkenbihl and Vielmetter, 1989). Proximity of IS5 to *nfsB* on the chromosome, in addition to the probable IS5 hotspot in *nfsB* (discussed below), and the high transposition rate observed for IS5 (Naas et al., 1994) could account for the large number of *nfsB* mutants (19%) containing IS5 in this study. A hotspot for IS5 after G⁴³ is evident in *nfsB*: ten independent mutants that contained IS5 at the same site were isolated in this study. The target sequence for IS5 in *nfsB*, AAGG⁴³, does not conform to the previously described IS5 target site consensus sequence C(A/T)A(G/A) (Engler and van Bree, 1981). However, of the 12 sequences within the coding region of *nfsB* that did match this consensus sequence, two, CTAA⁴¹ and TTTG⁴⁹, flanked the insertion point of IS5.

All IS elements demonstrated specificity for insertion in the regions proximate to either terminus of *nfsB*, suggesting that despite the variable target specificity of IS elements there may be some common determinants in target site selection. In *nfsB*, the IS element most frequently observed was IS1. IS1 is often associated with regional preferences for segments of DNA, of approximately 100 bp, into which the IS elements randomly insert themselves (Galas et al., 1987; Meyer et al., 1980; Zerbib et al., 1985). Several studies have shown that IS1 has a preference for insertion into stretches of A+T rich sequences (Matsutani, 1997; Meyer et al., 1980; Zerbib et al., 1985). It has been suggested that IS1, like RNA polymerase, may require regions high in A+T for their low helix stability (Matsutani, 1997; Meyer et al., 1980); therefore, A+T rich promoter regions could be expected to be targets for IS1. Among the mutants described in the current study, one mutant had IS2 integrated upstream of the coding region of *nfsB*. The insertion of IS2 upstream from a gene can terminate transcription by the Rho-sensitive termination sites and transcription terminators present in these elements (Das et al., 1977).

Eight mutants with IS1 elements in *nfsB* had IS1 inserted within the first 165 bases of the coding region, possibly due to the proximity of the A+T rich promoter region (Matsutani, 1997). IHF induced DNA bending has also been suggested to be a signal for IS1 insertion (Galas and Chandler, 1989; Galas et al., 1987; Prentki et al., 1987). Although there is no obvious IHF binding site near this region, there is a high density of homopolymeric runs of adenine and thymine which, according to the wedge model of

DNA conformation, could promote bending of DNA (Goodrich et al., 1990; Hales et al., 1994; Koo et al., 1986; Ulanovsky and Trifonov, 1987).

Target site sequence specificity may sometimes be attributed to protein consensus sequences. In particular, *E. coli* integration host factor (IHF), a protein that induces DNA bending, has been implicated in some instances of *IS1* site recognition (Galas et al., 1987; Prentki et al., 1987). The *nfsB* gene contains one site at bases 640-652 that conforms to an IHF binding site consensus sequence WWWCAWNANNTTA (W is A or T) (Goodrich et al., 1990; Hagerman, 1990). Notably, all IS elements, five *IS1* and one *IS2*, that integrated near the 3' end of *nfsB* were inserted within 100 bp of this consensus sequence, and two integration sites were exactly nine bases on either side.

2.4.4 Implications of the mutations for the NfsB enzyme

The mutational distribution of the furazolidone and nitrofurazone resistant mutants in the *nfsB* gene (refer to Figure 2.1) implicates particular residues as having functional significance in the NfsB protein. NfsB shares 88% amino acid sequence identity with homologous nitroreductases isolated from *S. typhimurium* (Watanabe et al., 1989) and *E. cloacae* (Bryant et al., 1991), and 33% identity with the homologue FRaseI, the major flavin oxidoreductase of *Vibrio fischeri* (Zenno et al., 1994), compared with 16% identity with NfsA. The sequences of RdxA and FrxA from *Helicobacter pylori* (Goodwin et al., 1998), Nox from *T. thermophilus* (Hecht et al., 1995), three enzymes from *Bacillus subtilis* (Kunst et al., 1997), and DrgA from the cyanobacterium *Synechocystis* sp. PCC 6803 (Elanskaya et al., 1998) also share some amino acid sequence identity (>20%) with NfsB. These proteins have all been shown to have

nitroreductase activity, and inactivation of RdxA, FrxA and DrgA has been associated with resistance to nitroheterocyclic drugs. Moreover, the sequential inactivation of the *H. pylori* nitroreductase genes *rdxA* (HP0954) and *frxA* (HP0642) has recently been associated with the development of metronidazole resistance (Jeong et al., 2000).

Although the overall similarity of these sequences to NfsB is only about 20-30%, the identity between proteins in the 14 residue stretch corresponding to Ser-37 to Val-50 of NfsB (Figure 2.5) is high: it is identical for *E. coli*, *S. typhimurium* and *E. cloacae*; and *V. fischeri* shares 78% identity with NfsB. Most of the other putative NfsB-like homologues share protein identities over 50% within this region (NfsA is 28% similar), with the exceptions of FrxA (36%) and DrgA (43%) (Table 2.5). It is interesting that of the 17 *nfsB* mutants with missense mutations, 7 (41%) are located in this region. This region serves as a fingerprint for the identification of NfsB-like proteins, especially the invariant Trp-46. The other missense mutations mainly affected conserved residues: two such residues (Asn-71 and Arg-207) were conserved in 8 (73%) and 9 (82%), respectively, of the 11 probable homologues shown in Table 2.5, while the other amino acids altered by mutation in NfsB were conserved in at least four of the proteins. *E. coli* NfsB, the NfsB-like nitroreductases from *S. typhimurium* and *E. cloacae*, and FRaseI from *V. fischeri* constitute a protein family (Zenno et al., 1996) that can be extended to include the proteins from *H. pylori*, *B. subtilis*, *T. thermophilus* and *Synechocystis sp* that have nitroreductase activity and share identity with NfsB, especially within the 14 residue fingerprint. With the exception of RdxA, which exclusively uses NADPH, all of these enzymes, like NfsB, can use either NADH or NADPH as a source of reducing

Figure 2.5 ClustalV alignment of *E. coli* NfsB and the putative NfsB-like homologues. Shading = identity with NfsB.

NfsB	:	LVVGNFLLGVAAALGILDAVPLEGGDAAILDAEFGLKEKGYTSLVWVPVGHHSVEDFN	200	*	220	*	240	:	217
Cnr	:	LVVGNFLLGVAAALGILDAVPLEGGDAEVLDAAEFGLKEKGYTSLVWVPVGHHSVEDFN					ATLP-KSRLPONTITTEV		217
NfnB	:	LVVGNFLLGVAAALGILDAVPLEGGDAAILDEFGLEKKGFTSLVWVPVGHHSVEDFN					AGLP-KSRLPLETTLTEV		217
FraserI	:	LALGNAHTLRLNITSTTMEGILPELSEIFADELKGTECHVALALGYHPSEEDYN					ATLP-KSRLPLSTIVTEC		217
NOX	:	ILLGYLILLLEYGLCSVPMLEGGDPERVRILG					ASLP-KSRKAFEDVITIL		218
BnrB1	:	IVSMQLMTARAHGYDTNPICGYKKNIAETEG					SHRLEPLERVVLM-R		205
BnrB2	:	LAAMQLMTAAKCKYDTCAICGHNKEQFKQDD					EGYA-SYRLPIDTIAEWK		209
BnrB3	:	IALLGMMTAAAOICVDSCPLEGGHOYDHIHRILE					PAHQ-SNRLELSKVS		202
RdxA	:	IATGQICMGLSLMGDDSCIIGGFDPLKVGEVLE					KTFSAVEDVVKWV		221
FixA	:	IQMANMMMAALMGLIDSCPIEGYQEKVEYLE					KSRLKSKVDAILWL		210
DrgA	:	MAMQNLMAAKMGLIDSCPMIGFDLOKVAELVK					KTTHWKTVEIYEVIE		217
N.m.	:	IALAAMMTGALMAGIDSCPVTEGHNEMERILSG-QFGLFDAAEWGSVAATFGYRVQ-EIAT					GGQTLEELVWNSFA		201
H.i.	:	IALAAMTIGASALGIDSCPIEGHYDKMNECLA					KAPRPLEEVIW		221
P.sp.	:	IVSLQLMSAKNYGYETCPICGHNKKKINELK					KSRLKGLDEVVKWVG		220
C.j.	:	IALASITYSANALNIASCTIGGFDKEKDDSYLS					IEKKYLPILIVAIGKKVTKDEETKNFKMETTDTTHWL		198
D.r.	:	IATGFLMAARGCYDTVPMLEGGDQGVVRQILD					LDIQKERSLVALGYCND-KKNFQANRFSFDEVVKFI		201
	:						LPEHQQFAGILPLGKRAE-EGYP-HHHSVERVAKFY		215

Figure 2.5 (cont'd)

Table 2.5 Sequence identities of putative NfsB homologues and paralogues. Percent identity of the NR fingerprint region (Ser-37 to Val-50) is included.

	% identity	% identity in NR region
Homologues^a		
<i>S. typhimurium</i> Cnr ^b	88.5	100
<i>E. cloacae</i> NEC	88.4	100
<i>V. fischeri</i> FRaseI ^c	33	78
<i>T. thermophilus</i> Nox	24	50
<i>B. subtilis</i> BnrB1	24	64
<i>B. subtilis</i> BnrB2 ^c	22	57
<i>B. subtilis</i> BnrB3	28	57
<i>H. pylori</i> RdxA ^{b,d}	25	71
<i>H. pylori</i> FrxA ^b	26	36
<i>Synechocystis</i> sp. DrgA ^b	24	43
NfsB-like Homologues^e		
NEISSERIA MENINGITIDIS	28	71
<i>Haemophilus influenzae</i>	26	64
<i>Phytoplasma</i> sp.	22	64
<i>Campylobacter jejuni</i>	21	64
<i>Deinococcus radiodurans</i>	23	50
<u>Paralogues in <i>E. coli</i></u>		
NfsA ^d	16	28
YDJA ^f	9	50
P75894 ^f	15	29

^a Based on sequence, structure and biochemical evidence.

^b Inactivation implicated in resistance to nitro-substituted drugs.

^c Significant FMN reductase activity (greater than nitroreductase activity)

^d Can only use NADPH as a cofactor.

^e Based on sequence similarity.

^f Hypothetical proteins in *E. coli* identified as nitroreductases by the Pfam Database. Refer to Section 1.2.2 for more details.

equivalents, usually with a slight preference for NADH. It is more likely that RdxA is an orthologue of NfsB than NfsA as there is only 15% amino acid identity with NfsA (vs. 25% with NfsB), and its sequence is more characteristic of a NfsB homologue, particularly within the fingerprint region. As genome projects are completed, numerous hypothetical proteins that appear to fit a nitroreductase fingerprint arise. Five hypothetical proteins that are likely to be NfsB homologues based on sequence similarity are shown in Table 2.5, but many more exist (for details refer to Section 1.2.2).

2.4.5 NfsB: a putative member of the SoxRS/MarRAB stress response

With numerous genome projects underway, the newly defined NR family of proteins promises to extend indefinitely. However, the natural function and substrate(s) for nitroreductases remain unknown. The GenProtEC *E. coli* genome and proteome database (<http://genprotec.mbl.edu/start>) states that the physiological roles for NfsA and NfsB are anaerobic respiration and drug/analogue sensitivity, respectively. The accuracy of this information is questionable, however, as the cited literature (McCalla et al., 1978; Morozov et al., 1994) does not identify the physiological roles of these proteins. Both NfsA and NfsB certainly cause drug/analogue sensitivity, but a suicidal role is unlikely to be their primary function. Furthermore, the apparent redundancy of nitroreductase enzymes in *E. coli* (two putative oxygen-sensitive and three oxygen-insensitive enzymes) suggests a variety of *in vivo* roles. The inability to obtain mutants without oxygen-sensitive nitroreductase activity or even without the third putative oxygen-insensitive enzyme (Bryant et al., 1981; McCalla et al., 1975) suggests that these components are essential proteins. On the other hand, inactivation of the characterized nitroreductases in

E. coli (NfsA and NfsB) and *S. typhimurium* (SnrA and Cnr) has no apparent negative effect on cellular viability or function under laboratory conditions, and easily cultured clinical isolates of *H. pylori* metronidazole resistant mutants contain inactivated RdxA and FrxA enzymes.

It is interesting that there often appears to be more than one protein from the NR family within an organism. This leads to speculation on the evolutionary origin of the NR family. It could be that there was duplication of the hypothetical ancestral NR gene, after which each gene was adopted for a specific process, resulting in a paralogous relationship. This organism would thus represent the common ancestor for organisms containing the NRs. However, horizontal transfer could also be a contributing factor and a reasonable explanation for what is apparently an evolutionarily widespread distribution of NRs. A strong candidate for horizontal transfer is transposable element (TE) activity, a possibility that resonates with the large number of IS elements observed in the *nfsA* and *nfsB* genes of the nitrofurantoin resistant mutants described in this study.

It is possible that nitroreductases have physiological functions and substrates that are yet to be determined. For instance, NfsA uses NADPH exclusively as a cofactor, a characteristic often associated with biosynthetic processes, while NfsB is unusual in that it can use either NADH or NADPH. NADH is usually a cofactor in catabolic processes, so the ability to use both cofactors is suggestive of a broader physiological role for NfsB. The identification of NfsB and its homologues has largely been through studies of drug resistance (*E. coli*, *H. pylori*, *Synechocystis* sp.) or activation (*S. typhimurium*, *E. cloacae*). It is interesting that several other bacterial species with putative NfsB

homologues are also of clinical importance (ex. *N. meningitis*, *H. influenzae*, *C. jejuni*) or are associated with extreme environments (ex. *T. thermophilus*, *D. radiodurans*). The nitroreductase activity that bioactivates many clinically important synthetic antimicrobial agents and manmade (primarily) environmental contaminants may very well provide a protective response for the organism against other compounds that do not result in the cell's death. This catabolic role is consistent with NADH associated activity. That NfsB may play a protective role physiologically is consistent with its involvement in responses to environmental stresses, such as oxidative stress (see below) or starvation (A. Martin, personal communication).

Recently, *nfsA* and *nfsB* were shown to be putative members of the multiple-antibiotic-resistance (*marRAB*) regulon (Barbosa and Levy, 2000). The *nfsA* (Liochev et al., 1999; Paterson et al., 2002) and *nfsB* (this study) genes are positively regulated as part of the closely associated *soxRS* regulon. The *soxRS* and *marRAB* regulons respond to and provide protection against cellular oxidative stress and numerous antimicrobial agents, respectively. The activators of these regulons, SoxS and MarA, are homologous proteins (Martin et al., 1999). Indeed, the *soxRS* and *marRAB* regulons are often regarded as a single regulon. The binding sites for these promoters are generally referred to as the marbox, though the determinants of this element are not well understood (Martin et al., 1999). Indeed, genes activated by SoxS are commonly responsive to MarA and vice versa (Martin et al., 1999). There are some exceptions to this overlap, however, and MarA and SoxS are independently regulated, with neither protein affecting the expression of the other (Barbosa and Levy, 2000). As well, the individual regulons can be

selectively induced with certain chemicals, for instance *soxRS* by paraquat and *marRAB* by salicylate (Martin et al., 1999).

NfsA is a regulated member of the *soxRS* regulon (Liochev et al., 1999; Paterson et al., 2002). Nitroreductase activity associated with NfsA was shown to increase significantly when treated with paraquat, but while Liochev and coworkers suggested that NfsB might be slightly induced by paraquat, it wasn't within the scope of their study to resolve this possibility. Recently, increased expression of *nfsA* and *nfsB* was shown to result from the constitutive expression of MarA, and thus both are considered members of the *marRAB* regulon (Barbosa and Levy, 2000). In the current study, *E. coli* strains that varied with respect to their *nfsA* and *nfsB* genotypes were treated with paraquat and assayed for induced nitroreductase activity in order to discern whether *nfsB* is a member of the *soxRS* regulon. AB1157, the wild-type strain, obviously has oxygen insensitive nitroreductase activity attributable to both NfsA and NfsB (and to a much lesser degree the third nitroreductase component IB₂); SIL41 ($\Delta nfsB$) has an intact *nfsA* gene, but an inactive *nfsB* gene; JVQ1 ($\Delta nfsA$) has an intact *nfsB* gene, but an inactive *nfsA* gene; and JVQ2 ($\Delta nfsA nfsB$) is deficient in both genes and has negligible nitroreductase activity. Treatment with salicylate was included to confirm activation by MarA. The level of nitroreductase activity in JVQ2 was negligible and not noticeably induced with paraquat or salicylate, and thus only the three strains with measurable nitroreductase activity are discussed here. Paraquat induced nitroreductase activity in all three strains, although about one-fold less in SIL41 than in AB1157 (Figure 2.4). This suggests that *nfsB* is a regulated member of the *soxRS* regulon. Similar induction with salicylate confirmed *nfsB*

as a member of the *marRAB* regulon. As a member of the *soxRS* regulon, induction of *nfsB* would be expected to be present only in AB1157 and JVQ1, and induced nitroreductase activity in SIL41 would be solely attributable to *nfsA*. If *nfsB* was not SoxS responsive then induction would not have been evident for JVQ1, and AB1157 and SIL41 would have had similar levels of induced nitroreductase activity.

To further establish that increased activity upon induction with paraquat or salicylate was due to *nfsB* and not *nfsA*, the different cofactor dependencies of NfsA and NfsB were exploited. That is, while NfsA is NADPH dependent, NfsB can use either NADH or NADPH. Therefore NADPH:nitroreductase activity reflects total activity, while NADH:nitroreductase activity is virtually attributable to NfsB alone. This difference in cofactor dependency is reflected in the nitroreductase activities of the uninduced extracts: AB1157 has approximately two-thirds less NADH than NADPH associated nitroreductase activity; SIL41 has trivial NADH:nitroreductase activity despite NADPH activity comparable to AB1157; and JVQ1 has similar levels of NADH and NADPH associated nitroreductase activities (Figure 2.4). That the *nfsB* gene is a member of the *soxRS/marRAB* regulons is strongly suggested by the similar levels of NADH:nitroreductase activity that are observed for JVQ1 and AB1157, the lack of induction of NADH activity evident for SIL41, and the similar levels of NADH and NADPH nitroreductase activity that are apparent for JVQ1. The possibility that the third minor nitroreductase component, IB₂, is responsible for the induced NADH:nitroreductase activity is rejected because JVQ2, which contains IB₂ but not NfsA or NfsB, has negligible nitroreductase activity both before and after induction.

Although *nfsB* appears to be a member of the *marRAB* and *soxRS* regulons, no obvious binding site, or marbox, for MarA or SoxS is apparent in the promoter region of the *nfsB* gene based on the consensus sequence (AYnGCACnnWnnRYYAAAYn) (Martin et al., 1999). A gel shift assay and/or DNase footprinting could help to identify the putative MarA/SoxR binding site(s).

CHAPTER THREE: THE KINETIC CHARACTERIZATION OF *E. COLI* NFSB

Site-directed mutagenesis and determinants of cofactor specificity.

3.1 Introduction

The three dimensional structures of *E. coli* NfsB (Parkinson *et al.*, 2000) and NfsB complexed with nicotinic acid (Lovering *et al.*, 2001) were recently solved by X-ray crystallography. Nicotinic acid, a nicotinamide analogue, represents only a component of the NAD(P)H cofactor; therefore, not all of the residues involved in cofactor binding have been determined. Structural determination of the NAD(P)H binding site in NEC, the *Enterobacter cloacae* homologue of NfsB, could not be obtained by NMR (Koder and Miller, 1998a), and unstable crystals of NADH complexed with FRaseI, the *V. fischeri* homologue of NfsB, prevented resolution of the cofactor binding site in this enzyme as well (Koike *et al.*, 1998). Identification of residues in NfsB that are involved in the binding of cofactors could facilitate the engineering of a more specific enzyme, which would be an advantage for such applications as DEPT (see Section 1.3.1.2), or drug development (discussed in more detail in Chapter 4).

NfsB is unusual in that it does not display a strong preference for NADH or NADPH. NAD^+ and NADP^+ differ only by the presence of a phosphate group esterified to the 2'-hydroxyl group of ribose, and yet most enzymes have a strong preference for one of the coenzymes. Almost exclusively, NAD^+ is associated with degradations, and NADP^+ with reductive biosynthesis (Baker *et al.*, 1992; Carugo and Argos, 1997a; Kaufman, 1993); however, the mechanism(s) through which enzymes discriminate between the two coenzymes remains poorly understood. Identification of NAD(P)^+ binding domains is complicated by the lack of informative consensus sequences. The

most frequently occurring structural motif is the Rossman fold, which is comprised of two parallel β -strands linked by an α -helix (Carugo and Argos, 1997a); the most commonly cited consensus sequence for NAD(P)^+ binding is the GXGXXG/A motif (Wierenga et al., 1985), which overlaps the loop region between the β -strand and α -helix. Still, it is often only a three dimensional structure of cofactor bound enzyme that can absolutely reveal the binding site of NAD(P)^+ (Carugo and Argos, 1997a,b; Kinoshita et al., 1999).

In this study, an expression system was developed to purify *E. coli*-NfsB. The seven residue region from Gly-153 to Leu-159 of the putative active site was modified by PCR mutagenesis through the introduction of 16 independent missense mutations. Wild-type NfsB and selected mutants were kinetically characterized. Most of these mutations resulted in a dramatic reduction of nitroreductase activity. The exception was the G158S mutant: this single Gly $\rightarrow$ Ser mutation decreased the enzyme's catalytic specificity for NADPH but not NADH. Similar results were obtained for a mutant that contained two mutations, one of which, Val-154 $\rightarrow$ Ala, is within this target region. These results suggest that the Gly-158 residue, and possibly other residues in this region, is important for the utilization of NADPH. A serendipitous Phe $\rightarrow$ Ser mutation at residue 70 is also interesting. F70S did not influence cofactor use; but this mutant did display an increased turnover number, k_{cat} , for nitrofurazone, which may be indicative of a decrease in transition state energy. Here, the kinetic data obtained for the mutant enzymes are interpreted with consideration for possible perturbations in their secondary and tertiary structures, as revealed by fluorescence spectrophotometry and computer simulation,

respectively. The structural and functional implications of these mutations are more thoroughly explored in Chapter 4, particularly with regard to the active site of NfsB.

3.2 Methods and Materials

3.2.1 Bacterial strains, plasmids and growth conditions.

E. coli K-12 strain DH5 α [*SupE44 lacU169* (ϕ 80/*lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Jessee, 1986) is a standard laboratory strain used for most plasmid manipulations. *E. coli* K-12 strain AB1157 [*F⁻ thr-1 leu-6 thi-1 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 λ^- sup-37*] (Bachmann, 1972) was provided by Dr. D. Bryant, McMaster University, Hamilton, Canada. *E. coli* B strain GJ1158 (Bhandari and Gowrishankar, 1997) is a derivative of BL21 (*F⁻ompTr_{BM}⁻ hsdS gal*) (Studier and Moffat, 1986) with a *proUp*-T7 RNA polymerase fusion integrated at the *malTPQ* locus, allowing NaCl-induced overexpression of cloned gene products.

The vectors used in this study were pCR[®]2.1 from the Original TA Cloning[®] Kit (Invitrogen), pCYB3 from the IMPACT[™] I Kit (New England Biolabs), pET23d (Novagen), pNEB193 (New England Biolabs), and pUC128 (Keen et al., 1988). The primers in this study are listed in Table 3.1.

Bacterial strains DH5 α and AB1157 were grown at 37°C in Luria-Bertani (LB) medium or on LBA (LB medium with 1.5% agar), obtained as a premixed powder from Canadian Life Technologies Inc. (Burlington, Canada). The NaCl-inducible strain GJ1158 was grown in LB broth with NaCl omitted (LB-N: 10g tryptone, 5g yeast extract per litre). Unless otherwise stated, GJ1158 was grown at 37°C. When appropriate, medium was supplemented with 100 μ g ampicillin per ml and 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal) per ml. All chemicals and reagents were

Table 3.1 The primers used in this study.

Primer	Primary Application	Sequence 5'→3' ^b	Restriction site	Strand ^c	Location
ET2	S	²¹⁶ GCGTAAAAATGCTTGATG	N/A	nt	<i>nfsB</i> coding
ET3	S	⁴⁹⁹ TTTGACGCCGCCATCCT	N/A	nt	<i>nfsB</i> coding
EN2	S	⁵⁹² GGATGGCGCGTGCAAAA	N/A	t	<i>nfsB</i> coding
EN3	S	³¹² GGCATCAAGCATTTTAC	N/A	t	<i>nfsB</i> coding
EN4	S	⁵⁰ TCAAATGCCTTAGTGGAATG	N/A	t	<i>nfsB</i> coding
IMPBU	P	GAGTCTcc ¹ atggATATCATTTCTGTC	<i>Nco</i> I	nt	Beginning of <i>nfsB</i> coding
IMPBL	P	CCGGCAgctcttcCGCA ⁶⁵¹ CAC TTCGGTTAAGGTGATG	<i>Sac</i> I	t	End of <i>nfsB</i> coding
MNU	M	⁴⁵¹ CTGCTC(GX)GCG(TX)G(GX)CG(GX)CTC(TX)G(GX)GTC(TX)GGACGC	N/A	nt	Mutagenesis target in <i>nfsB</i>
MNL	M	⁴⁸² GCGTCCAGACCCAGAGCCGCCACGCCGAGCAG	N/A	t	Mutagenesis target in <i>nfsB</i>
DMNL	M	⁴⁸² GCGTCC(AX)GAC(CX)C(AX)GAG(CX)CG(CX)C(AX)CGC(CX)GAGCAG	N/A	t	Mutagenesis target in <i>nfsB</i>
ILH	P	CAAAA TaagcttTATTTGAAGCC	<i>Hind</i> III	t	Intein

Notes: ^aS = sequencing, P = PCR amplification and cloning, M = mutagenesis

^bLowercase letters indicate restriction enzyme recognition sites. Superscript number indicates base number in the nontranscribed strand of *nfsB*.

^cnt = non-transcribed strand (i.e. coding); t = transcribed strand (i.e. noncoding)

molecular biology grade or equivalent. Restriction endonucleases and modification enzymes were purchased from New England Biolabs or Gibco BRL.

3.2.2 Manipulation of DNA.

AB1157 genomic DNA was isolated from a 5 ml overnight culture. The cells were harvested by centrifugation and the pellet was resuspended in 1.9 ml TE (0.1 mM Tris, 0.01 mM EDTA, pH 8.0). The cells were lysed with 0.5% SDS and 30 µg/ml proteinase K during a one hour incubation at 37°C. NaCl (0.7M) was added and mixed vigorously prior to the addition of 1% hexadecyltrimethyl ammonium bromide (CTAB)/0.07M NaCl (from a 10% CTAB, 0.7M NaCl stock solution) and the mixture was incubated for 10 min at 65°C. The proteins and cellular debris were extracted with 5 ml of chloroform/isoamyl alcohol (24:1). RNase A (20 µl of 10 mg/ml) was added to the aqueous phase and incubated for 30 min at 37°C. The remaining proteins and RNase A were extracted with 5 ml phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated from the aqueous fraction with 0.6 volumes of isopropanol, washed in 1 ml of 70% ethanol, dried and resuspended in 50 µl of TE.

Plasmid DNA was isolated either by using a Wizard™ miniprep kit (Promega), or by the boiling miniprep method (Holmes and Quigley, 1981) described in Section 2.2.7. If further purification was necessary it was done with a GeneClean™ kit (Bio 101 Inc.). Standard procedures were used for molecular cloning. Amplification product and restriction endonuclease analyses were done by agarose gel electrophoresis (1%) in the presence of ethidium bromide. Transformation of DH5α cells by ligation mixtures was accomplished by electroporation. The ligation mixes were purified with a GeneClean™

kit, and half was used to transform 40 µl of host cells. Competent cells were prepared for electroporation by four successive washes of sterile distilled water and stored at -80°C in 10% glycerol. Transformation of GJ1158 by constructs was achieved by calcium chloride transformation. Competent cells were prepared by a wash with one-quarter volume of 100mM MgCl₂, brought up in 1/20 volume of 100mM CaCl₂, and stored at -80°C with 10% glycerol. Transformation was achieved by mixing 1-5 µl of plasmid (about 0.5-2 µg) with 100 µl of competent cells, incubation on ice for one hour, followed by heat-shock at 37°C for 5 min, and recovery with gentle shaking at 37°C for up to one hour after the addition of 1ml LB-N broth.

3.2.3 Amplification of the *nfsB* gene.

Amplification of a 656 bp fragment containing the coding region of the *nfsB* gene was accomplished by PCR with the Expand™ High Fidelity PCR System (Boehringer Mannheim Canada). The upper (IMPBU) and lower (IMPBL) primers (Table 3.1) contained 5' *NcoI* and *SapI* restriction sites, respectively, to facilitate cloning. The reaction mixture (100 µl) included the Expand High Fidelity buffer with 15 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 300 nM of each primer, 1.7 U of Expand High Fidelity enzyme mix, and approximately 0.5 µg AB1157 genomic DNA. The amplification consisted of a 3 min preincubation at 94°C, a 7 min postincubation at 72°C, and 30 repetitions of the following cycle: 15 s at 94°C, 30 s at 55°C, 1 min at 72°C. The PCR product was purified using a High Pure PCR Product Purification Kit (Boehringer Mannheim Canada).

3.2.4 Cloning of *nfsB*.

The P_{tac} promoter of the IMPACT™ I kit supplied vector pCYB3 was replaced with the promoter from gene 10 of T7 bacteriophage. The T7 promoter was removed from pET23d on a *PvuII*/*NcoI* fragment, and inserted into the *MscI*/*NcoI* sites of pCYB3 (which also removed the *lacI* gene) to make the expression vector pIT. The amplified *nfsB*-containing fragment and pIT were digested overnight with *NcoI* and *SapI* prior to ligation. The desired clone, pITB (Figure 3.1), was verified by restriction endonuclease analysis and DNA sequencing as described in Section 2.2.10.

3.2.5 Expression and purification of *NfsB*.

NfsB was purified with the IMPACT™ I kit from New England Biolabs. This system uses the intein, a self-cleavable protein splicing element, from the *Saccharomyces cerevisiae* *VMA1* gene, for the affinity purification of native proteins free of any affinity tag. In the presence of thiols (e.g. dithiothreitol) the modified intein undergoes self-cleavage at its N-terminus. As Figure 3.2 illustrates, the chitin binding domain (CBD) from the chitinase A1 protein of *Bacillus circulans* WL-12 is added to the C-terminus of the intein to allow for affinity purification on a chitin bead column. Thus, the triple fusion (NfsB, intein and CBD) is bound to a chitin bead column, and native NfsB is cleaved from the fusion after treatment with DTT as follows (refer to Figure 3.2).

An overnight culture (5 ml) of GJ1158, freshly transformed with pITB, was used to inoculate 0.5 L LB-N medium supplemented with ampicillin. The cells were grown to $A_{600} \approx 0.7-0.8$ at 37°C with shaking (about 2.5-3 hours). For induction, 5M NaCl was

Figure 3.1 (a) NfsB expression vector pITB. See text for details of construction.

Approximate location of mutagenesis target region is indicated. (b) Target region and potential mutations after PCR mutagenesis. Bold font denotes wild-type bases and residues and their respective numbers. Bases that were degenerate in the PCR primers (i.e. targets) are underlined, and the base numbers are included below. The inner table indicates the residue change for each possible nucleotide substitution. Bold italics = mutants that retained some nitroreductase activity. Square brackets = mutations that were obtained, but the proteins were not expressed. Parentheses = mutations that were not obtained.

b.

Nucleotide substitution	Target						
	ggc 457	gtg 461	gcg 463	gct 466	ctg 470	ggt 472	ctg 476
	Gly- 153	Val- 154	Ala- 155	Ala- 156	Leu- 157	Gly- 158	Leu- 159
A	<i>Ser</i>	Glu	[Thr]	(Thr)	Gln	<i>Ser</i>	Gln
T	Cys	X	Ser	(Ser)	X	[Cys]	X
G	X	Gly	X	X	Arg	X	(Arg)
C	Arg	Ala	Pro	Pro	Pro	(Arg)	(Pro)

Figure 3.2 (a) Illustration of the IMPACT I Purification System, adapted from the manufacturer's manual (New England Biolabs). The purification procedure was done at 4°C. MCS = multiple cloning site; CBD = chitin binding domain. *Italics* refer to the genetic elements encoding the intein and CBD. (b) Regeneration of the chitin column at room temperature.

a.

b.

At room temperature:

added to a final concentration of 0.3 M and the cells were incubated at 15-20°C with gentle shaking for between 6 and 12 hours. The cells were harvested by centrifugation at 5000 x g and washed with 0.15 M NaCl (at this stage the pellet could be stored at -20°C, if necessary).

The pellet was resuspended in 10 ml column buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1 mM EDTA) with one Complete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablet (Roche). The cells were chilled on ice and sonicated with a Virtis VirSonic 60, power 15, for 1x 30 s and 2-4x 15 s bursts to yield the clarified extract which was immediately centrifuged at 12 000x g for 30 min at 4°C. The clarified extract was promptly loaded onto the chitin bead column.

The following purification procedure was done at 4°C (Figure 3.2a). Approximately 10 ml of chitin beads were loaded onto a column (Bio-Rad) and equilibrated with 100 ml column buffer. The clarified extract was loaded onto the column and an elution rate of no greater than 0.5 ml/min was set. The loaded chitin beads were washed with 100 ml column buffer, followed by 50 ml of a high salt buffer (50 mM Tris pH 8.0, 1 M NaCl, 1 mM EDTA), and finally equilibrated with 50 ml cleavage buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1 mM EDTA). Cleavage of the fusion protein by the intein was induced by flushing the column with 30 ml cleavage buffer containing 30 mM dithiothreitol (DTT) followed by incubation for 36-48 hours at 4°C. NfsB was eluted with 5 ml 50 mM Tris-HCl pH 7.0, and stored at -20°C in 40% glycerol.

After elution of the protein, 3 column volumes (30 ml) of stripping buffer (20 mM Na-Hepes pH 8.0, 500 mM NaCl, 1% SDS) were applied (Figure 3.2b). The column was regenerated by washing with 5 column volumes (50 ml) ddH₂O followed by 50 ml column buffer. Sufficient column buffer to cover the resin was left in the column, which was then stored at 4°C. The chitin bead column could be used up to five times following stripping and regeneration of the resin.

3.2.6 Protein analysis.

Protein concentrations were determined as described by the manufacturer for the Bio-Rad protein assay with a bovine serum albumin standard curve. Protein products were run on 10% or 12.5% SDS-PAGE gels according to standard protocol. The gels were stained for 30 min in Coomassie Blue (2.5 g Coomassie Brilliant Blue, 450 ml methanol, 450 ml ddH₂O, 100 ml acetic acid) and destained with 10% acetic acid/12.5% isopropanol.

3.2.7 PCR mutagenesis.

The mutational target in NfsB consists of 7 amino acids, G153-L159. The MNU and DMNL primers were degenerate at 7 bases, designed to introduce missense mutations in this region of *nfsB* (refer to Figure 3.1b for all possible missense mutations). Introduction of a mix of nucleotides at the target positions of the oligonucleotides during synthesis allowed a pool of primers with varying numbers of mutations to be created during primer synthesis. At each target position during synthesis the bases were drawn from two sources in equal proportions such that 50% were from the correct base, and 50% were from an equal mix of all four bases. Therefore, at each position there was a

5/8 chance of having the wild-type base inserted. The fraction of oligonucleotides expected to contain a single mutation was calculated using the formula: $n!P^{n-r}(1-P)^r/(n-r)!r!$ where n is the number of bases in the target sequence to be mutated (in this case 7), P is the probability of any target nucleotide being unchanged (here, 5/8 or 0.625), and r is the number of mutations per oligonucleotide (ideally $r=1$) (Sambrook et al., 1989).

Figure 3.3 illustrates the PCR mutagenesis approach, which involved two sequential reactions. The first round of PCR consisted of one reaction for each of the two primer pairs: MNU + ILH, and IMPBU + DMNL. Each reaction (100 μ l) contained Taq reaction buffer (Gibco BRL), 2 mM MgCl₂, 300 nM of the appropriate primers, 0.2 mM of each deoxynucleoside triphosphate, 2 U Taq DNA polymerase (Gibco BRL) and approximately 50 ng pITB as template. Amplification (program #1) was achieved through 30 repetitions of a cycle of denaturation for 20 s at 94°C, annealing for 30 s at 50°C, and elongation for 45 s at 72°C. A preincubation at 94°C for 2 min and a postincubation at 72°C for 5 min were included. The second round of PCR used the amplification products from the first round as template. This reaction mixture (100 μ l) included 10 μ l of amplification product from each first round reaction, 200 nM each of the primers ILH and IMPBU, Taq reaction buffer, 2 mM MgCl₂, 0.2 mM each dNTP and 2 U Taq DNA polymerase. The amplification cycle (program #2) was the same as for the first round, except that the elongation time was extended to 1.5 min, and it was repeated only 15 times. The PCR product (12 μ l) was run on a 0.7% agarose gel in the presence of ethidium bromide and the ~1.2 kb fragment was excised and purified using a GeneClean kit. This product was freshly amplified immediately prior to directly cloning it into

Figure 3.3 Targeted PCR mutagenesis using degenerate primers. Lowercase italics indicate restriction enzyme recognition sites; bold capitals refer to the primers (see Table 3.1) used for amplification at these steps. The '●' represents the mutation(s).

pCR[®]2.1 (explained below). The reaction mixture (100 µl) contained Taq reaction buffer, 2 mM MgCl₂, 0.2 mM each dNTP, 2 U Taq DNA polymerase, 600 nM each of the primers IMPBU and ILH, and 1/100 of the PCR product. Amplification was achieved by program #2 described above.

Direct cloning of PCR amplification products was facilitated by the Original TA Cloning[®] Kit (Invitrogen). The kit supplied vector, pCR[®]2.1, is linearized and contains single 3' thymidine residues. *Taq* polymerase adds a single deoxyadenosine to the 3' ends of amplification products, so immediate ligation of PCR products into pCR[®]2.1 can occur without any restriction enzyme digestion step(s). pCR[®]2.1 is a high copy number vector and inserts can be detected using blue/white screening. Here, the freshly amplified PCR product (1 µl) was ligated with pCR[®]2.1 (8 ng) according to the manufacturer's directions. Transformed DH5α was plated on LBA+Ap+Xgal and plasmids extracted from white colonies were screened for the presence of the ~1.2 kb insert by digestion with *Eco*RI. The target region of each plasmid with an insert was sequenced with primer ET2. If the target contained a single mutation, the whole *nfsB* gene was sequenced to verify that no other mutation(s) had been introduced.

3.2.8 Subcloning of selected mutations.

Plasmids containing unique mutations within the target site (including some with two and three mutations) were subcloned into the *Nco*I/*Hind*III sites of pIT, and the constructs were used to transform the host strain GJ1158. One mutant, VAFS, has two mutations, T209→C and T461→C, of which the former is located beyond the target

region. After insertion into pIT, each mutation of VAFS was subcloned as described below (refer to Figure 3.1a for restriction site locations).

3.2.8.1 V154A

The T461→C mutation of VAFS translates to a Val154→Ala substitution in NfsB. To subclone this mutation, the *XbaI/HindIII* fragment of pITB, which contains the complete *nfsB* gene, was inserted into pUC128 to create pUCIB. The *BglII/HindIII* fragment of pUCIB was then replaced by that of pITVAFS to make the intermediate construct pUCV154A, which contained only the T461→C mutation. The V154A expression construct, pITV154A, was obtained by inserting the *NcoI/HindIII* fragment of pUCV154A into pIT.

3.2.8.2 F70S

The T209→C substitution of VAFS yields a Phe70→Ser mutation in NfsB. Each attempt to make an expression construct of F70S according to the cloning scheme described for V154A (Section 3.2.8.1) failed, resulting in a truncated or mutated *nfsB* gene. An expression construct of F70S was finally obtained by making an intermediate clone that contained the *nfsB* gene in the opposite orientation. First, the *XbaI/HindIII* fragment of pITVAFS was inserted into pUC128 to make pUCVAFS. Ligating the *XbaI/BglII* fragment of pUCVAFS and the *BglII/HindIII* fragment of pITB in a single reaction with the vector pNEB193 made the intermediate clone pNEBF70S. The pNEB193 vector contains the identical multiple cloning site as pUC128 but in the opposite orientation. Finally, the *NcoI/HindIII* fragment of pNEBF70S was subcloned into pIT to create the expression construct pITF70S.

3.2.9 Confirmation of protein expression and nitroreductase activity

A small scale culture of each mutant was grown and the cells were run on a 10% SDS-PAGE gel to ensure that the triple fusion protein was expressed. LB-N (10 ml) with ampicillin was inoculated with 100 μ l of an overnight culture and grown at 37°C for 2.5 hours. The culture was divided into two tubes and one was induced with 0.3 M NaCl; both tubes were gently shaken at 20°C for 5 hours. A 0.5 ml sample was removed from each tube, centrifuged, and the pellets were resuspended in 25 μ l SDS-PAGE gel loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.01% bromophenol blue, 10% glycerol) of which 5-10 μ l was loaded on a 10% SDS-PAGE gel. The remaining culture was centrifuged and the pellet was used to make cell extracts according to the procedure described in Section 2.2.4. Nitroreductase activity was assayed as nitrofurazone reductase activity (Section 2.2.6).

3.2.10 Intein activity

Cleavage of fusion protein was induced in cell extracts of the mutants to ensure that the intein was functional. DTT (50 mM) was added to the 100 μ l cell extract, which was then incubated at 4°C for 36 hours; cell extract/DTT mixture (10 μ l) was run on a 10% SDS-PAGE gel to confirm the presence of a band corresponding to NfsB (approximately 27 kD) in the induced fraction.

3.2.11 Fluorescence spectroscopy

Fluorescence spectra of wild-type NfsB and the mutants VAFS, F70S, V154A, G158S, G153S and A156P were acquired at room temperature on a Hitachi F-2000 fluorescence spectrophotometer. The purified protein samples were adjusted to 1 μ M in

50 mM Tris-HCl pH 7.0 buffer, except for mutant A156P for which only a 0.5 μ M sample was used due to low yields of purified protein. Tryptophan residues were specifically excited at 290 nm and emission spectra were recorded from 300 to 450 nm. FMN was excited at 464 nm, an optimal wavelength for excitation as determined from an excitation scan (emission 525 nm) of 300-800 nm, and emission spectra were recorded from 500-600 nm. The fluorescence emitted by the buffer was subtracted from all spectra.

3.2.12 Ultraviolet spectroscopy

Samples (0.1 μ M) from the same protein preparations analyzed for fluorescence were used to measure tryptophan and tyrosine absorption (280 nm) on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer.

3.2.13 Enzyme kinetics

Nitroreductase activity was measured as nitrofurazone reductase on a Dynatech Model MR5000 microplate reader. The reduction of nitrofurazone was measured as a decrease in absorption at 405 nm (molar extinction coefficient 11,100 $\text{M}^{-1}\text{cm}^{-1}$) at 23°C. The reaction mixture (195 μ l) contained 50 mM Tris-HCl (pH 7.0), 0.2 μ g purified enzyme, and appropriate concentrations of nitrofurazone and NADPH or NADH. Nitrofurazone and NAD(P)H concentrations were determined spectrophotometrically (NAD(P)H molar extinction coefficient 6,220 $\text{M}^{-1}\text{cm}^{-1}$ at 340 nm). For each set of reactions, the kinetic parameters were measured for 5 concentrations of nitrofurazone each at 6-8 concentrations of NAD(P)H. Each strain was analyzed at least four times for each cofactor, with at least two independent enzyme preparations, and on separate days.

The reaction velocities were measured at 10 s intervals for 45 readings, and the raw data was stored as an ASCII file by the Biolinx 2.0 program (Dynatech Laboratories, Inc.). The reaction rate data were calculated by linear regression using the Microplate Analysis (MPA) Program (Brooks, 1994). This program uses an absorbance factor to determine the enzyme velocity of each reaction. The absorbance factor is calculated as follows:

$$\text{absorbance factor} = 1000 \times \text{ABS}_{\text{STD}} \times 60 / (A \times \epsilon \times l_{\text{STD}}) = \text{nmol/min (mU)} \quad (1)$$

The absorbance of the standard, ABS_{STD} , was determined from six independent absorbance measurements on an independently calibrated Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer (not a microplate reader).

$$\text{ABS}_{\text{STD}} = \underline{0.745} = \text{absorbance of the standard (67.1 } \mu\text{M nitrofurazone)} \quad (2)$$

The machine (microplate reader) slope, A , was determined by plotting the machine absorbance versus microplate well volume (μl) (50, 100, 150, 200, 250, 300 μl well volumes were used), and calculating the slope.

$$A = \underline{0.0026} \text{ ABS}/\mu\text{l} = \text{machine slope} \quad (3)$$

The molar extinction coefficient of nitrofurazone at 405 nm was determined on the independently calibrated spectrophotometer.

$$\epsilon = \underline{11\ 100} \text{ M}^{-1}\text{cm}^{-1} = \text{molar extinction coefficient of nitrofurazone (405nm)} \quad (4)$$

The pathlength, l_{STD} , is 1.0 cm, and the values of 60 and 1000 are present to convert the result into minutes and nmol, respectively. Therefore, substitution of the values

calculated in equations 2-4, along with I_{STD} and the conversion factors into equation (1) yields an absorbance factor of 1548.86 nmol/min.

$$\text{absorbance factor} = 1000 \times 0.745 \times 60 / (0.0026 \times 11\,100 \times 1.0) = \underline{\underline{1548.86}}$$

nmol/min (5)

The enzyme velocity data were saved as a Kinetics file, imported into the Kinetics Program (Brooks, 1992) and used to calculate the kinetic parameters K_m^{app} and V_{max}^{app} for NAD(P)H. Calculation of the kinetic parameters for nitrofurazone was achieved by reentering the same data sets with nitrofurazone concentrations as the variable substrate. The enzyme activity curves and Lineweaver-Burk plots were viewed in the Kinetics Program, but in order to print the curves the data was reentered into Microsoft® Excel.

Double reciprocal plots of substrate (nitrofurazone, NADH or NADPH) against enzyme velocity were plotted, but as the reactions were not conducted under saturating conditions, replots of the apparent kinetic parameters (K_m^{app} , V_{max}^{app}) derived from these primary double reciprocal plots were used to determine the actual K_m and V_{max} values. Diagrams of the primary double reciprocal plots (Figure 3.4a) and of the secondary replots (Figure 3.4b,c) illustrate this method for the graphical determination of kinetic parameters as described in Copeland (2000).

The K_m values for nitrofurazone and NAD(P)H were determined from their respective V_{max}^{app} reciprocal plots. The actual V_{max} value was taken as an average of the two plots (i.e. nitrofurazone and NAD(P)H V_{max} values). The turnover number, k_{cat} , was calculated as: $V_{max}/E(t)$ where $E(t)$ is the total amount of enzyme in the reaction (in moles). The errors were calculated based on regression analysis (R^2 value) of the

Figure 3.4 (a) Double reciprocal plot for a ping-pong Bi Bi reaction (adapted from Copeland, 2000). Replots as (b) $1/V_{\max}^{\text{app}}$ versus $1/[B]$ and (c) $1/K_m^{\text{AX app}}$ versus $1/[B]$, illustrating the graphical determination of K_m^{AX} , K_m^{B} , and $V_{\max}$ for a ping-pong Bi Bi reaction (adapted from Copeland, 2000).

secondary plots. Standard error analysis rules were used when combining these errors in further calculations (ex. k_{cat}/K_m values).

3.2.14 Structural analysis

Three dimensional protein structures were downloaded as PDB files from the Protein Databank (www.rcsb.org) and viewed with Swiss-PdbViewer v.3.7b2, obtained from <http://www.expasy.ch/spdbv> (Guex and Peitsch, 1997). Distances between atoms, bond lengths and structural alterations after residue changes were obtained with this program. A more comprehensive analysis of these structures is the focus of Chapter 4.

3.3 Results

3.3.1 Expression and purification of NfsB.

The key challenge in developing a purification scheme for NfsB is its tendency to aggregate and stick to surfaces. Expression at over 25°C leads to the formation of inclusion bodies, affinity purification is frustrated by on-column aggregation, and storage of purified NfsB in glass containers with at least 40% glycerol is necessary otherwise the protein disappears, presumably as it sticks to the container walls. Aggregation of NfsB was particularly evident during initial attempts to purify it with an N-terminus His-tag (data not shown). The importance of the N-terminus residues for NfsB function, and the possibility that the His-tag was contributing to protein aggregation, motivated the search for a different purification strategy. The appeal of the IMPACT™ I kit was the one step purification of native enzyme. Purification of NfsB with the IMPACT™ I system is not work intensive, but it is time consuming. For instance, cleavage of the fusion protein by the intein is achieved with incubation at 4°C for two days. However, pure NfsB without any affinity tags is obtained directly.

The first attempts to purify NfsB with the IMPACT™ I kit were not successful as the presence of the NfsB fusion protein was not obvious after expression with the p_{tac} regulated *nfsB* construct. To reduce the background of host proteins and increase the expression of NfsB a new construct, pITB (Figure 3.1a), was created such that the triple fusion protein was under the control of the strong gene 10 promoter of bacteriophage T7. Expression of the protein thus relies upon the presence of T7 RNA polymerase (RNAP). Proteins under the control of a T7 promoter are usually expressed in the *E. coli* strain

BL21(DE3) which encodes T7 RNAP on a lambda prophage under the control of the *lacUV5* promoter. Induction of T7 RNAP by IPTG results in the overexpression of the target protein. An alternative to induction by IPTG is possible with GJ1158, a modified strain of BL21(DE3) (Bhandri and Gowrishankar, 1997). In this strain, T7 RNAP is under the transcriptional control of the *cis*-regulatory elements of the osmoresponsive *proU* operon, and may be induced by NaCl. The lower cost and toxicity of NaCl make it an appealing alternative to IPTG. Above 25°C, most of the fusion protein is localized in the insoluble fraction (see Figure 3.5), presumably due to the formation of inclusion bodies. Therefore, cultures were incubated at 15-20°C with gentle shaking for six to twelve hours after the addition of NaCl. Under these conditions, most of the fusion protein was present in the soluble fraction. Reduction of inclusion bodies has been noted previously when GJ1158 is used as an expression host, perhaps due to the intracellular accumulation of glycine betaine, thought to reduce inclusion bodies, that occurs upon osmotic upshock (Bhandri and Gowrishankar, 1997; Blackwell and Horgan, 1991). However, compared to the expression of fusion protein in BL21 induced with IPTG, any increase in solubility provided by GJ1158 appeared negligible for NfsB (data not shown).

Initial purification attempts were not successful in obtaining native NfsB protein, as the protein would mostly elute from the column following denaturation with SDS (i.e. when the column was stripped) (Figure 3.6). This was likely due to on-column aggregation of the protein, which can occur when too much protein is loaded onto an inadequate amount of resin. Therefore, less cellular extract was loaded, and greater column bed volumes were used in subsequent purification attempts. Purification of NfsB

Figure 3.5 Expression of the NfsB triple fusion protein at a range of temperatures. At elevated temperatures the protein is increasingly localized within the insoluble fraction of the induced crude extracts. Arrows indicate the triple fusion protein with NfsB. UI = uninduced; I = induced.

Figure 3.6 Initial NfsB purification attempt. Lanes: E = Eluted (purified) fraction; M = marker (BioRad Broad Range); S= stripped fraction. Arrows point to (from the top) the triple fusion, double fusion and NfsB proteins.

was further frustrated by the presence of contaminating bands in the elution fractions (Figure 3.6). Elimination of these contaminants was achieved with an additional wash with a high salt (1 M NaCl) buffer during the purification process, and by excluding the detergent component (Triton-X 100) of the column buffer. NfsB of high purity (about 25 mg/L) was subsequently obtained (Figure 3.7), and stored in glass tubes with 40% glycerol at -20°C. However, purified NfsB represented less than 1% of the protein in the soluble fraction of the cellular extract, which was mostly a consequence of the low levels of expression necessary to minimize protein aggregation.

3.3.2 Characterization of wild-type NfsB

3.3.2.1 NR Activity

In crude cellular extract, the host strain, GJ1158, exhibited about two-fold more NADPH-associated NR activity (42.3 nmol/min/mg) than NADH-associated NR activity (17.9 nmol/min/mg) (Table 3.2). The greater NR activity associated with NADPH is attributable to the existence of NfsA, the major, and NADPH-linked, NR component of *E. coli*. The presence of vector encoded wild-type NfsB did not yield an increase in NR activity without induction of the NfsB protein. However, following induction the increase in NADPH and NADH associated NR activities were approximately 7- and 18-fold, respectively.

3.3.2.2 Structural integrity

Fluorescence spectra are sensitive to changes in the solvent polarity near the fluorophore, which makes this technique a useful tool to evaluate the structural integrity of proteins. In NfsB there are two primary sources of fluorescence: the indole and phenyl

Figure 3.7 Purified NfsB. Lanes 1-4: eluted fractions; Lane M is the standard marker (BioRad Narrow range); S1, S2 are the stripped fractions (SDS eluent).

Table 3.2 Nitrofurazone reductase specific activities (nmol/min/mg) of cell extracts containing vector encoded wild-type and mutated NfsB.

Strain	Amino acid mutation	NADH	NADPH
Wild-type	None	320.1 (48)	290.8 (51)
GJ1158 (no vector)	None	17.9 (3.3)	42.3 (5.1)
G158S ^a	Gly158→Ser	232.8 (71)	298.9 (65)
VAFS ^{a,d}	Val154→Ala	334.0 (108)	358.3 (121)
	Phe70→Ser		
G153S ^b	Gly153→Ser	96.7 (25)	142.6 (32)
A156P ^c	Ala156→Pro	17.6	44.0

Note: Values averaged from at least 3 trials with the exception of A156P. Average deviations are shown in parentheses.

^aMutants with activity that were further characterized.

^bMutant had activity but was eventually discarded as enzyme velocity was too low to assay accurately.

^cNo activity greater than the host strain. Similar values obtained for all mutants with the exceptions listed here.

^dThe Phe70→Ser mutation is responsible for this mutant's activity.

fluorophores of the aromatic amino acids (tryptophan and tyrosine residues), and FMN. Excitation at 290 nm or greater minimizes the contribution of tyrosine residues such that fluorescence emissions from tryptophan can be isolated (Yelamos et al., 1999). Fluorescence spectra of 1 μ M wild-type NfsB were obtained for both tryptophan and FMN.

NfsB contains three tryptophan residues (W46, W94, W138) that are within approximately 10 Å of one another. W138 is less than 5 Å from bound nicotinic acid, and within 8 Å of FMN and Phe-124, a residue of importance in substrate recognition (Zenno et al., 1996c). Excitation of NfsB at 290 nm yields an emission peak at 345 nm (Table 3.3), which is within the normal range of tryptophan emission peaks (Creighton, 1993). Tryptophan absorbance was measured by UV spectroscopy as well. At 280 nm, 0.1 μ M of NfsB had an absorbance value of 0.213 (Table 3.3).

An excitation scan of NfsB revealed a peak at 464 nm (Figure 3.8a) that is within the characteristic range of FMN-containing proteins (Munro and Noble, 1999). Emission scans between 500-600 nm revealed two peaks ascribed to bound FMN in NfsB at 525 nm (peak I) and 550 nm (peak II), also within the expected range, while FMN alone had a single peak at 525 nm (Figure 3.8b). Peak I exhibits greater intensity than peak II (Figure 3.8b,c), with a peak I:peak II intensity ratio of 1.3 (Table 3.3).

3.3.2.3 Enzyme kinetics

Kinetic analysis can reveal two important aspects of an enzyme's function: kinetic parameters, and evidence concerning the reaction mechanism. However, the determination of initial velocities in two substrate systems is problematic, particularly if

(a)

(b)

(c)

Figure 3.8 Fluorescence spectra. (a) Wild-type excitation scan, emission at 525 nm. (b) Emission scans of wild-type NfsB and the mutant enzymes, excitation at 464 nm. Peak I and peak II are identified (see text for details). (c) A closer view of the wild-type, F70S and V154A emission scans from (b).

Table 3.3 Fluorescence emission peaks (excitation at 290 nm and 464 nm) and UV absorbance values (280 nm) for wild-type and mutant NfsB proteins.

Strain	NR ^a activity	UV ^b 280 nm	Trp ^c Peak (nm)	FMN ^d ex. 464 nm		
				Peak (nm)	Intensity	ratio ^e
FMN	N/A	N/A	N/A	525	50.0	N/A
Wild-type	Full	0.213	345	525 (I) 550 (II)	4.4 3.5	1.3
F70S	Full	0.220	345	520	4.0	1.3
VAFS	Full	0.202	345	520	11.2	N/A
G158S	Partial	0.386	375	525 550	3.3 2.8	1.2
G153S	Partial	0.504	375	525 550	6.9 5.6	1.2
V154A	None	0.604	375	530 555	3.8 3.5	1.1
A156P	None	0.508	375	525 555	1.1 1.2	0.9

^a NR = nitroreductase activity relative to wild-type. Refer to Tables 3.2 & 3.4 for actual NR activities.

^b Absorbance ($\lambda = 280$ nm) of 0.1 μ M protein as determined by Bradford assay.

^c Tryptophan specifically excited at 290 nm, emission recorded 300-450 nm. 1 μ M protein used for measurements except for A156P (0.5 μ M).

^d FMN specifically excited at 464 nm, emission recorded 500-600 nm. Fluorescence due to buffer components is subtracted.

^e ratio of the intensities of Peak I (~525 nm):Peak II (~550 nm)

the K_m of the measured substrate is so high that saturation cannot be achieved and Michaelis-Menten single substrate kinetics is not approximated. The NR assay used in this study measures the reduction of nitrofurazone at 405 nm, which introduces a limitation to the maximum concentration of nitrofurazone ($\sim 132 \mu\text{M}$) that can be used in a reaction due to the spectrophotometer's sensitivity limit (absorbance of approximately 1). As the K_m of nitrofurazone is greater than $132 \mu\text{M}$, the saturating conditions necessary to mimic single substrate Michaelis-Menten enzyme kinetics could not be met. The solution to this limitation is to use a matrix of substrate concentrations such that at least 25 velocity measurements are made in a single experimental trial (Allison and Purich, 1979). A double reciprocal plot of initial velocity versus the variable substrate concentration (here, NADH or NADPH) is made for at least 4 fixed concentrations of the other, fixed variable substrate (here, nitrofurazone). This primary double reciprocal plot reflects the putative reaction mechanism of the enzyme, as described below in Section 3.3.2.3.1. As well, the values of $-1/K_m^{\text{app}}$ and $1/V_{\text{max}}^{\text{app}}$ are derived from the x and y intercepts, respectively, of this plot (see Figure 3.4a). A secondary plot of the intercepts yields estimates for the actual $1/V_{\text{max}}$ and $1/K_m$ values of the substrates (Figure 3.4b,c) (Copeland, 2000). Using this approach, the kinetic parameters of NfsB for nitrofurazone, NADH and NADPH were determined. Enzyme assays were conducted for 6-8 NADH and NADPH concentrations, each at 4-5 nitrofurazone concentrations and the results were analyzed by least squares analysis (Cleland, 1979).

3.3.2.3.1 Mechanism

The double reciprocal plots described previously in section 3.3.2.3 can test the postulated reaction mechanism of an enzyme. Parallel lines of these plots for the fixed variable substrate often correspond to a ping-pong Bi-Bi kinetic mechanism, while converging lines indicate an ordered (also known as a sequential) reaction mechanism (Segel, 1975). Figures 3.9-3.12 illustrate representative primary double reciprocal plots obtained for wild-type NfsB.

The primary double reciprocal plots, $1/V_i$ versus $1/[\text{substrate}]$, exhibit parallel lines for both NADH (Figure 3.9a) and NADPH (Figure 3.10a) with nitrofurazone as the fixed variable. Likewise, parallel lines are apparent for nitrofurazone with each of NADH (Figure 3.11a) and NADPH (Figure 3.12a) as fixed variables. This suggests that a ping-pong Bi-Bi reaction mechanism is consistent with NfsB kinetics.

3.3.2.3.2 Kinetic parameters

The kinetic parameters, K_m and k_{cat} , of NfsB for the cofactors and nitrofurazone are shown in Table 3.4. These were derived from the secondary double reciprocal plots shown in Figures 3.13-3.16 as described in Section 3.3.2.3. The reduction of nitro-compounds by nitroreductase follows a double-displacement, or ping-pong, reaction mechanism (Figure 3.17). As such there are two activities of the enzyme that must be considered during kinetic analysis. First, the transfer of reducing equivalents, in the form of hydrogen atoms, from the NAD(P)H cofactor to the bound FMN; and second, the transfer of electrons from FMN to the nitro-substituted substrate. Thus, analysis of the reaction mechanism requires the determination of two K_m values (Table 3.4). The first is

Figure 3.9 Primary double reciprocal plots of initial velocity versus NADH concentration for wild-type NfsB and the mutant enzymes. The numbers in the legend specify the five concentrations (μM) of the fixed variable, nitrofurazone, at which each set of assays was performed. There is no symbol for the line representing 93.2 μM nitrofurazone.

Figure 3.10 Primary double reciprocal plots of initial velocity versus NADPH concentration for wild-type NfsB and the mutant enzymes. The four numbers in the legend signify the concentrations of the fixed variable, nitrofurazone, that were used for each plot. There is no symbol for the line representing 80.1 μM .

Figure 3.11 Primary double reciprocal plots of initial velocity versus nitrofurazone (NF) concentration for wild-type NfsB and the mutant enzymes. The legend shows the 7 concentrations (μM) of the fixed variable NADH at which each set of assays was done.

Figure 3.12 Primary double reciprocal plots of initial velocity versus nitrofurazone concentration for wild-type NfsB and the mutant enzymes. The legends show the eight concentrations (μM) of the fixed variable NADPH at which each set of assays was done.

Figure 3.13 Secondary double reciprocal plots of V_{max}^{app} versus NADH concentration. The data for VAFS were associated with such large errors that a plot was meaningless, and thus was not included.

Figure 3.14 Secondary double reciprocal plots of $V_{\max}^{\text{app}}$ against NADPH concentration.

Figure 3.15 Secondary double reciprocal plots of V_{max}^{app} versus nitrofurazone concentration with NADH as the cofactor.

Figure 3.16 Secondary double reciprocal plots of V_{max}^{app} versus nitrofurazone concentration with NADPH as the cofactor.

Figure 3.17 Schematic diagram of a ping-pong reaction mechanism (adapted from Zenno et al., 1996b). E = Native NfsB. E-FMN and E-FMNH₂ represent the complexes between NfsB and FMN^{ox} and FMN^{red}, respectively. Between each form of enzyme, (quaternary) transitory complexes are formed which cannot participate in reactions.

- (1) Either 2- or 4-electron transfer to bound FMN. Zenno et al. (1996b) suggest that it is a 2-electron transfer step.
- (2) Transfer of the electrons to the substrate nitrofurazone. This step either yields the intermediate nitroso derivative, or the fully reduced amino end product. According to a hexa uni ping-pong terreactant reaction mechanism, the substrate (NF) would remain bound here until fully reduced by four electrons.

for the cofactor itself, $K_{m(NAD(P)H)}$, which approximates the affinity of the enzyme for the coenzyme. Here, the K_{mNADH} of NfsB, 25.1 μM , is slightly lower than the K_{mNADPH} , which is 31.8 μM . The second K_m value is for nitrofurazone in order to approximate the affinity of NfsB for the electron acceptor. In this study the K_m value for nitrofurazone was calculated with each cofactor to yield two values, $K_{mNF(NADPH)}$ and $K_{mNF(NADH)}$. The K_m values for nitrofurazone were larger than for the cofactors, and the K_m of nitrofurazone with NADPH (275 μM) is greater than that with NADH (174 μM).

Two secondary double reciprocal plots were made, one for the cofactor (Figures 3.13 & 3.14) and one for nitrofurazone (Figures 3.15 & 3.16), and two V_{max} values were obtained for each cofactor. The turnover number, or k_{cat} , was calculated from an average of these two V_{max} values. The turnover number of nitrofurazone was slightly higher with NADPH as the coenzyme, though close to 100 sec^{-1} with each cofactor (Table 3.4).

Catalytic specificity, calculated as k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$), is the most important measure when assessing changes in an enzyme, particularly changes with regards to specific substrates (Copeland, 2000; Plesner, 1986). The catalytic specificity of NfsB for nitrofurazone with each cofactor, $k_{cat}/K_{mNF(NAD(P)H)}$ (Figure 3.18a), and with each cofactor, $k_{cat}/K_{mNAD(P)H}$ (Figure 3.18b), was also calculated independently. A comparison of catalytic specificities for the cofactors and nitrofurazone was accomplished by determining the absolute ratio of NADH:NADPH k_{cat}/K_m values (Figure 3.18c). Thus, an absolute ratio of greater than one indicates a preference for NADH. Again, both sides of the reaction must be considered, and hence a comparison of cofactor specificity (cofactors on Figure 3.18) and nitrofurazone specificity calculated

Figure 3.18 Catalytic specificity of wild-type NfsB and the mutant enzymes G158S, VAFS and F70S measured as k_{cat}/K_m . a) The catalytic specificity of each enzyme for nitrofurazone (NF), separately with each cofactor (NADH, NADPH). b) The catalytic specificity for NADH and NADPH. c) Absolute ratio of NADH to NADPH catalytic specificity for nitrofurazone with each cofactor (NF) and for the cofactors. d) Relative ratio of specificity as compared to the wild-type. See text for details.

with each cofactor (NF on Figure 3.18) is necessary. The NF and cofactor absolute ratios for wild-type NfsB of 1.3 and 1.1, respectively, indicate a preference for NADH (Figure 3.18c).

3.3.3 PCR mutagenesis

The stretch of amino acids from G153 to L159 in NfsB was selected as a mutagenesis target (Figure 3.1b) to determine the relevance, if any, of these residues on NAD(P)H usage. Rather than introduce single mutations by means of numerous individual site-directed mutagenesis reactions, a two-step PCR mutagenesis approach, using primers degenerate at 7 positions, allowed numerous distinct missense mutations to be obtained simultaneously within the target region (Figure 3.3).

In initial attempts to create mutations within the *nfsB* sequence encoding Gly-153 to Leu-159, direct insertion of the amplified mutated DNA fragment into the expression vector pIT was attempted by using the lower PCR primer, IMPBL, during the second round of PCR. This approach was arduous and ineffective as the low copy number of pIT was not amenable to manipulation and the *SapI* digestion was often incomplete, which usually led to aberrant clones (i.e. containing truncated or mutated DNA, or inserts of unknown origin). These problems were circumvented by: (1) use of a different primer, ILH, that is within the intein coding region and contains a 5' *HindIII* site, and (2) insertion of the amplified fragment into pCR[®]2.1 with subsequent subcloning into pIT. The direct insertion of PCR amplified fragments into the high copy number vector pCR[®]2.1 (as explained in Section 3.2.7) facilitated sequencing and subcloning, especially since blue/white screening of inserts on medium containing Xgal was possible. Indeed,

Table 3.4 K_m (μM) and k_{cat} (sec^{-1}) values of wild-type and mutant NfsB proteins with NADH and NADPH as cofactors and nitrofurazone (NF) as the electron acceptor. The K_m for NF was independently determined with each cofactor (i.e. $K_{m_{NF(NAD(P)H)}}$). The standard deviation for each value is indicated in parentheses.

	NADH		NADPH	
	k_{cat} (sec^{-1})	$K_{m_{NF(NADH)}}$ (μM)	k_{cat} (sec^{-1})	$K_{m_{NF(NADPH)}}$ (μM)
Wild-type	88.9 (9.9)	174 (28)	105 (52)	275 (14)
G158S	45.6 (10)	179 (29)	43.8 (8.9)	206 (27)
F70S	203 (53)	293 (25)	149 (32)	302 (107)
VAFS	119 (36)	241 (73)	104 (9.0)	174.4 (85)
				31.8 (1.6)
				64.8 (2.7)
				53.7 (12)
				124.4 (91)

104/167 (62%) of the white pCR[®]2.1 transformants contained the correct insert compared to only 40/153 (26%) of plasmids with inserts in the original pIT cloning approach. Moreover, where the pIT clones were usually aberrant, the pCR[®]2.1 clones usually contained the entire and correct insert.

The target region of the 104 transformants with the correct insert was sequenced. Table 3.5 lists the mutations of the screened transformants. In six mutants the sequence was illegible and analysis was not continued. Table 3.6 summarizes the frequency of the mutation number (single, double, etc.) obtained in the target region of *nfsB* in the 98 transformants for which unambiguous sequence was ascertained. A single mutation within the target region was desired, but with 7 degenerate positions multiple mutations were expected in the majority of cases. The ratio of correct to contaminating base at each target site was 0.625, which was predicted to yield 15.6% of PCR generated oligonucleotides with a single mutation (see Section 3.2.7). In actual fact, 30, almost one-third, of the correctly cloned fragments had a single mutation, of which 16 (out of a possible 21) were unique. Mutation frequency at the target sites was not equal with half (15) of all single mutations obtained at two positions: 461 (7 mutants) and 470 (8 mutants).

Figure 3.1b and Table 3.5 illustrate all of the potential missense mutations and indicate the 16 (of 21 possible) unique missense mutations that were obtained. Mutants that contained inserts with a single mutation were further characterized by sequencing the entire *nfsB* gene. With the one exception of mutant #96, no additional mutations were found within the *nfsB* coding region. The extra mutation in mutant #96 was T²⁰⁹→C,

Table 3.5 Mutations introduced by targeted PCR mutagenesis. The target region includes residues Gly-153 to Leu-159 in NfsB. The targeted nucleotides are indicated above their encoded residues. Figure 3.1b shows all of the possible mutations in the target region.

SINGLE MUTATIONS							Mutant numbers
G 457	T 461	G 463	G 466	T 470	G 472	T 476	
Gly-153	Val-154	Ala-155	Ala-156	Leu-157	Gly-158	Leu-159	
Cys							137
Ser ¹							242 ¹
Arg							267
	Gly						21,111,212
	Ala						96 ¹ ,131
	Glu						110,263
		Pro					50,79
		Ser					53,63
		(Thr ²)					177 ²
			Pro				75,207
			(Thr)				None
			(Ser)				None
				Gln			210,245,278
				Arg			271,272
				Pro			9 ² ,240,266
					Ser ¹		94 ¹ ,113,221
					(Cys ²)		174 ²
					(Arg)		None
						Gln	238
						(Arg)	None
						(Pro)	None
DOUBLE MUTATIONS							
Cys	Glu						22
Cys					Ser		243
Ser		Pro					178,195
Ser			Thr				164,217
Ser				Pro			18
Arg		Ser					24,180
	Glu	Ser					125
	Glu		Ser				253
	Ala			Pro			16
	Ala					Arg	65
	Gly		Thr				146
	Gly			Gln			229
	Gly	Pro					244
	Gly		Pro				269
	Gly				Cys		281
		Ser		Arg			30
			Pro	Arg			256
			Thr			Arg	282
				Gln	Ser		106
				Gln	Arg		134
				Pro	Ser		128
					Cys	Pro	47
					Ser	Gln	168

Table 3.5 cont'd.

TRIPLE MUTATIONS							<i>Mutant numbers</i>
Gly-153	Val-154	Ala-155	Ala-156	Leu-157	Gly-158	Leu-159	
Arg		Pro	Thr				112
Cys		Thr			Ser		120,138
Ser	Gly	Ser					167
	Glu	Ser				Gln	74
	Glu	Ser	Pro				124
	Gly				Cys	Arg	218
		Thr			Arg	Arg	17
		Thr			Ser	Pro	39
		Thr	Thr			Pro	114
		Thr	Ser	Pro			119
		Thr		Pro		Pro	283
		Ser		Pro	Arg		220
		Ser			Cys	Gln	235
QUADRUPLE MUTATIONS							<i>Mutant numbers</i>
Gly-153	Val-154	Ala-155	Ala-156	Leu-157	Gly-158	Leu-159	
Cys	Ala	Pro	Ser				66
Ser	Gly	Pro		Gln			157
		Pro	Thr		Ser	Gln	14
OTHER MUTATIONS							<i>Mutant numbers</i>
Gly-153	Val-154	Ala-155	Ala-156	Leu-157	Gly-158	Leu-159	
Arg			-G frameshift				215

Note: Parentheses indicate that the mutation could not be tested, as either the mutation was not obtained, or the protein could not be expressed.

¹ Mutants had some NR activity and the proteins were analyzed further.

² The protein was not induced so analysis was not possible. In the cases of #174 and #177 where the mutation was not otherwise obtained these mutations could not be studied.

Table 3.6 Predicted frequency of mutation number in the target region of NfsB, and the actual frequency in 98 mutants.

No. of mutations	Frequency (%)	Predicted Frequency (%)
0 (wild-type)	24.5 (24)	3.7
1	30.6 (30)	15.6
2	26.5 (26)	28.2
3	14.3 (14)	28.2
4	3.1 (3)	16.9
?	1 (1)	--
other	--	7.4

Note: Numbers in parentheses are the actual number of mutants

which translates to Phe-70→Ser in NfsB. All products with unique single mutations, plus that of mutant #96, were subcloned into pIT. As well, 4 double mutation and 3 triple mutation inserts were subcloned (refer to Table 3.5). The two mutations in isolate #96 were subsequently separated. The T⁴⁶¹→C (Val-154→Ala) mutation was easily subcloned, but the T²⁰⁹→C (Phe-70→Ser) mutation was more difficult. A construct was finally obtained by reversing the gene's orientation during the intermediate cloning step (insertion into a pUC vector) so that it would not be expressed during manipulations (see Section 3.2.8.2).

3.3.4 Purification and characterization of mutants

3.3.4.1 NR activity

Mutants were selected for kinetic analysis by screening for enzyme activity. Two of the mutants, #174 and #177, did not express the triple fusion protein and further analysis were not possible. The nitroreductase (NR) activity of the remaining 14 mutant proteins was measured both in a crude extract and after purification. In cell extracts, most of the mutants had nitroreductase activity similar to that of the host strain GJ1158 alone (~18 μmol/min/mg); the only mutants with NR activity significantly greater, that is with a difference of at least two standard deviations, than GJ1158 were #94 (G158S), #96 (V154A, F70S) and #242 (G153S) (Table 3.2). Herein each mutant is referred to according to its amino acid mutation, shown in parentheses above, except for isolate #96, which has two mutations and is designated VAFS. Each of these mutants also displayed activity when assayed with NADPH as a cofactor. In cell extracts, the mutants generally exhibited less NR activity than vector-encoded wild-type NfsB. The exception is VAFS,

which often demonstrated greater overall NR activity than wild-type NfsB, although there were large fluctuations in NR activity between different preparations of VAFS. This fluctuation in NR activity for VAFS was also apparent in purified preparations of the enzyme and is probably due to variations in the stability of the enzyme as a consequence of the two mutations.

NfsB from all of the mutants, even those without discernable cell extract activity, was purified and the NR specific activity was measured. Sufficient enzyme was obtained to determine the specific activity of the 14 mutant enzymes even though expression levels of the mutants were usually lower than for the wild-type protein, and aggregation was often a hindrance during purification (data not shown). Mutants with low specific activity in the cellular extract had correspondingly low activity after purification. The mutant with the lowest assayable activity, G153S, had such low overall activity that kinetic analysis was not possible. The Val-154→Ala substitution subcloned from VAFS resulted in the elimination of NR activity, while F70S retained full NR activity.

3.3.4.2 Structural integrity

After the nucleotide sequences of the mutant genes were confirmed, and the molecular weight of the proteins was established by SDS-PAGE, the structural integrity of the enzymes was examined using fluorescence spectroscopy. Fluorescence spectra of 1 μ M G158S, VAFS, V154A, F70S, G153S and 0.5 μ M of the null mutant A156P were obtained for both tryptophan and FMN, and compared to the wild-type spectrum (Section 3.3.2.2). Spectra of the other mutants could not be obtained, as concentrations of the enzyme preparations were too low.

Changes in the Trp fluorescence spectra of the NfsB mutant proteins

contribute information on the overall structural integrity of NfsB, but could also indicate perturbation near the putative substrate binding site. The three tryptophan residues (W46, W94, W138) in NfsB are within approximately 10 Å of one another. W138 is in the FMN binding pocket of NfsB, and W46 is a highly conserved residue among the NfsB homologues that may be involved in substrate binding (see Chapter 4, Section 4.4.2.3). NfsB proteins that exhibit considerable NR activity, such as the wild-type, VAFS and F70S, had an emission peak at 345nm. A peak of significantly less intensity was evident at 375nm for the mutants that exhibit less NR activity (Table 3.3). This shift to a longer wavelength suggests that at least one of the Trp residues is more exposed to the solvent in the mutant enzyme as compared to the wild-type; completely buried tryptophan residues generally exhibit emission maxima near 320 nm (Creighton, 1993). The 375 nm peak is evident only in the target region mutants, but VAFS also has a mutation within this region (V154A) and does not have a peak at this wavelength. Similarly, the FMN spectrum of VAFS resembles that of F70S, rather than V154A and the other mutants with less NR activity (discussed below). Thus, spectral variations appear to reflect differences in NR activity, suggesting that the diminished NR activity of the mutant enzymes is due at least in part to structural changes.

The UV absorbance at 280 nm was obtained for 0.1 µM of the same protein preparations used for the fluorescence spectra (Table 3.3). F70S and VAFS had absorbance values similar to the wild-type protein, about 0.2, while the other mutants had absorbance values greater than 0.35. Indeed, the least active mutants G153S, V154A and

A156P had the highest absorbance values (0.5-0.6). This correlation with the fluorescence spectra peaks emitted after Trp excitation may also indicate that the aromatic residues are more exposed to the solvent in the less active mutants.

Emission scans of the mutants after excitation of the FMN molecule at 464 nm exhibited some differences from the wild-type scan (Figure 3.8b). Although an equal concentration of each enzyme was used (1 μ M except for 0.5 μ M A156P), the intensities of some spectra, particularly those of VAFS and G153S, were different, which suggests that the environment of the FMN molecule has changed. Unlike absorbance, fluorescence usually does not follow Beer's law and greater concentrations of enzyme can actually yield less fluorescence due to the inner filter effect (Munro and Noble, 1999), while buried residues often produce peaks of greater intensity than exposed residues (Copeland, 2000).

The shapes of the spectra are also indicative of structural alterations around residues that influence FMN binding. As described in Section 3.3.2.2, peak I (525 nm) is more defined and is of greater intensity than peak II (550 nm) in wild-type NfsB. Likewise, ratios of peak I:peak II in the mutant enzymes with NR activity are greater than one (Table 3.3). Peak II is practically absent for F70S and VAFS, such that their spectra resemble the single peak of free FMN (Figure 3.8b). In addition, there is a slight blue shift, to 520nm, of peak I in the F70S and VAFS mutants. In V154A peak I underwent a slight red shift, to ~528nm. Peak II is most pronounced for V154A and A156P, the mutants with no detectable NR activity (peak I:peak II ratios of 1.1 and 0.9 respectively). Furthermore, peak II undergoes a slight red shift, to 555nm, in these mutants. The

mutants with intermediate NR activity, G153S and G158S, exhibit an intermediate ratio (1.2) of peakI:peakII.

The spectral perturbations observed for the A156P, V154A and G153S mutants are probably due to disruption of the α G helix (Figure 3.19). Simulation of the mutated residues in the three-dimensional structure of NfsB with the program SWISS-PDBviewer (Guex and Peitsch, 1997) demonstrated that many new bonds are probably formed in these mutant proteins, whereas new bonds are not predicted to form with the F70S mutation. It is unclear why the spectra of VAFS resemble F70S despite the V154A mutation.

3.3.4.3 Enzyme kinetics

The reaction mechanism and kinetic parameters of the mutant enzymes VAFS, F70S and G158S were analyzed and compared to wild-type NfsB. Kinetic analysis of G153S was not possible as this mutant enzyme has too little activity to obtain a sufficient range of initial velocities. The difference in initial velocities was negligible for the range of substrate concentrations necessarily used here due to equipment limitations (refer to Section 3.3.2.3). Kinetic analysis of the bisubstrate NfsB enzyme involves the determination of at least four reaction curves (refer to Section 3.3.2.3). If much higher concentrations of nitrofurazone could be used, then it would be possible to obtain a few enzyme reaction curves with significantly variable initial velocities such that the actual V_{max} and K_m values of G153S could be determined from the primary plots.

Figure 3.19 Nucleotide sequence of the *nfsB* gene with the translated sequence below it. Mutagenesis target region is shown in the box. Rectangles above the sequence indicate the alpha helices (α) and beta sheets (β) of the secondary structure derived from the 3D structure of NfsB (Parkinson et al., 2000). Restriction endonuclease sites are underlined in the nucleotide sequence and identified above the sequence. The primers used for amplification and cloning of the mutated fragment are shown as arrows above the nucleotide sequence.

3.3.4.3.1 Mechanism

At least three primary double reciprocal plots from independently determined data were obtained for the mutant enzymes. Figures 3.9-3.12 illustrate the representative plots. In most cases a ping-pong Bi-Bi mechanism is implicated for the mutant enzymes. Parallel lines were obtained for the primary double reciprocal plots with NADH (Figure 9b-d), and G158S and F70S exhibit parallel lines with NADPH (Figure 10b,d). One possible exception is revealed by the slight convergence of lines for VAFS with NADPH (Figure 10b). However, large errors associated with VAFS preclude any conclusions regarding its reaction mechanism.

Another possible deviation from a ping-pong Bi-Bi mechanism is detected in the plots of G158S (Figures 3.9b & 3.10b). At increased concentrations of nitrofurazone there is an increase in the slope values derived from the $1/V_i$ versus $1/[NADPH]$ plot. This trend does not conform to a recognized pattern of reaction mechanism (e.g. ordered or random) (Segel, 1975). The lines may essentially be parallel as the deviation is slight and within the limits of error, however, it could also be an indication of substrate inhibition. A slight increase in slope values is also apparent for the $1/V_i$ versus $1/[NADH]$ plot, but if substrate inhibition is present for this cofactor it is less pronounced than for NADPH.

3.3.4.3.1.2 Substrate inhibition

Competitive substrate inhibition is distinguished by a linear replot of slopes from the primary double reciprocal plot against the inhibitory substrate (Segel, 1975). Here, the slopes of the $1/V_i$ vs $1/[NADPH]$ plot for G158S (Figure 3.10b) versus nitrofurazone

concentration yield a line with a slope of 0.68 (Figure 3.20). A similar replot for wild-type gives a lesser slope of 0.29 and F70S yields no positive slope at all. The large sampling fluctuation associated with VAFS prevents a replot for this mutant. This observed linearity may indicate that G158S experiences substrate inhibition by nitrofurazone. If so, then the inhibition constant (K_i) of nitrofurazone can be extrapolated from Figure 3.19 to give approximately 123 μM (Segel, 1975). Likewise, the K_i of nitrofurazone for the wild-type enzyme would be 194 μM . Table 3.7 lists the possible K_i values derived from the primary NAD(P)H double reciprocal plots. Of course, experimental error or an inadequate range of substrate concentrations may account for these linear slope plots. However, linear plots are not obtained from the $1/V_i$ vs. $1/\text{nitrofurazone}$ graphs, which indicates that NADH and NADPH are probably not inhibitory. It should be noted that if the K_i for a substrate were much greater than its K_m value, then parallel lines would be apparent on the primary double reciprocal plot (Segel, 1975); therefore, it might be that the cofactors are inhibitory but that their K_i values are much greater than their K_m values.

3.3.4.3.2 Kinetic Parameters

The kinetic parameters, K_m and k_{cat} , of the mutant enzymes for the cofactors and nitrofurazone are shown in Table 3.4. As described in Section 3.3.2.3.2, the catalytic specificities for the cofactors and nitrofurazone are compared by an absolute ratio of NADH:NADPH k_{cat}/K_m values (Figure 3.18c). Like wild-type NfsB, all of the enzymes exhibit a preference for NADH: the absolute NADH:NADPH k_{cat}/K_m ratios are close to or greater than one for nitrofurazone and the cofactors (Figure 3.18c).

a.

b.

Figure 3.20 Linear replots of slopes at different nitrofurazone concentrations from the double reciprocal plots of $1/v$ vs (a) $1/\text{NADPH}$ and (b) $1/\text{NADH}$. X-intercepts correspond to $-K_i$ value if substrate inhibition is present.

Table 3.7 K_i values (μM) for nitrofurazone with each cofactor in wild-type NfsB and two of the mutant enzymes. Refer to Figure 3.20 for the plots from which these values were derived, and Section 3.3.4.1.3.2 in the text for details.

Protein	Nitrofurazone K_i value (μM)	
	NADPH	NADH
Wild-type NfsB	194	123
mutant G158S	123	46.4
mutant F70S	N/A	146

The catalytic specificities of the mutant enzymes for each substrate are compared to those of the wild-type enzyme by a relative mutant:wild-type ratio (Figure 3.18d). A relative ratio greater than one indicates that the NADH specificity of the mutant enzyme exceeds that of wild-type NfsB. Little deviation from the wild-type ratios is seen for nitrofurazone in all of the enzymes, which indicates a similar preference for nitrofurazone with each cofactor in each enzyme. However, compared to the wild-type, F70S yielded similar ratios for the cofactors, while G158S and VAFS exhibited significantly greater specificity for NADH. G158S displayed approximately 4.5 times greater specificity for NADH than NADPH, which is about 4.2 times greater than that demonstrated by wild-type NfsB (Figure 3.18d). The catalytic specificity for NADH is essentially similar for all four enzymes (approximately $180 \mu\text{M}^{-1}\text{s}^{-1}$). The change in cofactor specificity for G158S and VAFS is attributable to a lower specificity for NADPH rather than a heightened one for NADH (Figure 3.18a).

For G158S, the decrease in catalytic specificity for NADPH is a consequence of a higher K_m value and lower turnover number than the wild-type enzyme (Table 3.4). A higher K_m value for NADPH is also indicated for VAFS. The apparently unaffected turnover number of VAFS may be attributable to the increased turnover number contributed by the F70S mutation that could mask a decrease imposed by the V154A mutation (Table 3.4). However, it may be an oversimplification to treat each of these mutations as exerting mutually exclusive and independent effects on the double mutant. The unaffected catalytic specificity for NADH and decreased specificity for NADPH in

VAFS and G158S, which possess mutations within the target region, suggests that this region is involved in the utilization of NADPH.

By comparison to wild-type NfsB, the decrease in the catalytic specificity of G158S for nitrofurazone is primarily due to a reduction in turnover number; there is little change in K_m values. A turnover number can reflect the transition energy of a reaction (Plapp, 1995), and, especially when there is little change in the K_m value (Rescigno and Perham, 1994), this smaller k_{cat} value may indicate that the transition energy of G158S is greater than that of the wild-type.

3.4 Discussion

3.4.1 Wild-type characterization

3.4.1.1 Expression and purification of NfsB

To date, the purification scheme for NfsB developed in this study is the only one that uses an affinity tag; other researchers have used the more traditional biochemical approach involving a succession of chromatography columns. As discussed in section 3.3.1, a number of problems were encountered during the development of an affinity purification scheme for *E. coli* NfsB. Most of the problems were a consequence of the apparent stickiness of NfsB: aggregation during overexpression, particularly at elevated temperatures; aggregation on the affinity column; and loss of purified protein unless stored in glass tubes with at least 40% glycerol. By manipulation of expression temperature and buffer constituents, these problems were largely overcome. The purified NfsB protein obtained by this method has the additional advantage of having no affinity tag.

3.4.1.2 Structural integrity

NfsB, a homodimer, is a flavoprotein with a bound FMN molecule in each of its subunits. The tightly bound FMN molecule is at the core of the active site as it accepts electrons from NAD(P)H and uses them to reduce the nitro-substituted substrate. Thus, the sensitivity of fluorescence spectra to disturbances in the solvent polarity near the fluorophore is an invaluable tool to assess the structural integrity of NfsB, especially at the active site. The fluorescence excitation spectrum of NfsB exhibits a characteristic flavoprotein peak at 464 nm. Excitation of NfsB at this wavelength results in two

emission peaks, 525 nm and 550 nm, that are characteristic of flavoproteins (Munro and Noble, 1999).

Excitation of tryptophan residues at 290 nm yields a peak at 345 nm, which is typical of buried Trp residues (Creighton, 1993). The Trp spectrum provides an indication of the protein's overall structural integrity. Furthermore, as one Trp residue, W138, is in the FMN binding pocket of NfsB, it may reflect the integrity of the binding site. However, changes in the Trp spectrum can be attributable to any combination of the three Trp residues.

3.4.1.3 Reaction mechanism

A previous study concluded that NfsB reduces nitro-containing compounds according to a ping-pong Bi-Bi mechanism (Figure 3.17) (Zenno et al., 1996b). Evidence for a ping-pong Bi-Bi mechanism is usually, as in the study by Zenno and coworkers, based upon parallel double reciprocal plots. Parallel double reciprocal plots are obtained for NfsB with both NADH and NADPH in this study as well; however, such plots are not definitive of a ping-pong mechanism. For example, an ordered Bi-Bi mechanism with a 'sticky' first substrate will also yield parallel lines (Segel, 1975). Detailed studies including inhibitor kinetics and isotope exchange experiments on the NfsB homologue of *E. cloacae*, NEC, indicate that it does conform to a ping-pong mechanism (Koder and Miller, 1998b), but the researchers did not unambiguously identify a Bi-Bi mechanism either.

The kinetic mechanism of NfsB must also explain the four-electron reduction of nitro-substituted compounds. Figure 3.17 illustrates the release of a nitroso intermediate

after the initial two-electron transfer. It is still not clear whether this intermediate is released and rebound, or whether the nitro compound is reduced by four electrons without an intermediate being released. Zenno and coworkers (1996b) state that there is evidence (unpublished) for a two electron transfer constituting the first half-reaction of NfsB's mechanism. Koder and Miller (1998b) addressed this question with NEC by attempting to identify nitroreduction products and intermediates of the simple nitroaromatic compound nitrobenzene. The only compound detected was the four-electron reduced hydroxylaminobenzene; yet the possible two-electron reduced intermediate, nitrosobenzene, reduces so quickly under the same assay conditions that it was impossible to determine whether the intermediate was released and preferentially rebound (as in a ping-pong Bi-Bi mechanism), or if it remained bound to the enzyme. There are instances in which a three-substrate (terreactant) system can yield parallel plots (Fromm, 1979; Segel, 1975). It is thus conceivable that NfsB may follow a terreactant mechanism in which two of the substrates (NADH or NADPH) are electron donors (Koder and Miller, 1998b). For example, a hexa uni Bi-Bi reaction mechanism yields parallel plots for all substrates (Fromm, 1979; Segel, 1975).

3.4.1.4 Kinetic parameters

Table 3.4 reveals the kinetic parameters, k_{cat} and K_m , obtained for the wild-type and mutant proteins. The $K_{m_{NADH}}$ (25.1 μM) and $K_{m_{NF(NADH)}}$ (174 μM) determined for NfsB in this study are very close to the values, 22 μM and 153 μM respectively, obtained in a prior study by Zenno and coworkers (1996b). The $K_{m_{NADPH}}$ (31.8 μM) and $K_{m_{NF(NADPH)}}$ (275 μM) values are greater than those for NADH. Zenno et al. (1996b),

who conducted experiments only with NADH, found that the $K_{m\text{NADH}}$ was lower (5.4 μM) when FMN was used as an electron acceptor instead of nitrofurazone. Rejecting the possibility of a different reaction mechanism for flavin reduction, they proposed that NfsB underwent a conformational change after substrate binding. This is a possibility, and some conformational changes are apparent upon binding of the cofactor (see Section 3.4.4 and Chapter 4 for more details); however, the K_m value is not a reliable indicator for the comparison of interactions between substrates and an enzyme (Plesner, 1986). It is the k_{cat}/K_m ratio, or catalytic specificity, that is the critical parameter for the comparison of substrate specificity (Copeland, 2000), and thus it is a variable slope on the primary double reciprocal plot that can signify a difference in substrate-enzyme interactions and reaction mechanisms. In fact, different K_m values are observed for various cofactors and substrates of NEC while their k_{cat}/K_m values are similar to one another, which suggests that this value is independent of the other substrate (Koder and Miller, 1998b). Conformational changes after cofactor binding in NfsB may account for the difference in $K_{m\text{NF(NADPH)}}$ and $K_{m\text{NF(NADH)}}$, as changes to the FMN binding pocket upon the binding of the nicotinamide analogue NIO are observed (Loverling et al., 2001; Chapter 4 this thesis). Another explanation could be that the reaction mechanism of NfsB is actually terreactant, with two of the substrates being identical (i.e. NADH or NADPH) (refer to Section 3.4.1.3, Figure 3.17).

The present study appears to be the only one that has compared the cofactor catalytic specificities of NfsB, but in accordance with a previous study that compared specific activities (Bryant et al., 1981), the catalytic specificities established here

demonstrate that wild-type NfsB has a slightly greater specificity for NADH than for NADPH. Figure 3.18 (a-c) reveals a slight preference for NADH, which is primarily due to a lower K_m value for nitrofurazone when NADH is used as a cofactor (refer to Table 3.4).

3.4.2 Mutagenesis

Targeted PCR mutagenesis was used in this study to evaluate the association of seven amino acids in NfsB, Gly-153 to Leu-159, with NAD(P)H. Another mutation at residue Phe-70 was fortuitously introduced by PCR: base substitutions occur at a frequency of approximately 1.1×10^{-4} base substitutions per base pair with *Taq* polymerase (Tindall and Kunkel, 1988). This mutagenesis approach has the advantage of simultaneously obtaining numerous unique mutations within the target region. Thus it avoids the more costly and perhaps more time-consuming alternative approach of introducing each mutation independently. For instance, 14 unique mutations were analyzed in this study, which in a traditional site-directed mutagenesis strategy would have meant the use of 13 more primers to achieve the same number of mutations. The introduction of multiple mutations can be viewed as both an advantage and a disadvantage. A possible advantage is that it allows analysis of combinations of mutations; however this often represents a disadvantage since the majority of multiple mutations lead to enzyme inactivity and thus selection of single mutations becomes more time-consuming. Furthermore, two key problems were encountered in the development of this mutagenesis strategy. First, when only a single degenerate primer was used to incorporate mutations, mostly wild-type genes were obtained. Second, inefficient

restriction endonuclease cleavage and ligation led to inaccurate subclones of the mutant genes. The latter problem was solved by the introduction of an intermediate cloning step into a pUC derived vector, which eliminated the need to use the *SapI* restriction endonuclease.

When this study was in progress a structure for *E. coli* NfsB was not available, so selection of the target region was primarily based on some similarity with the most commonly known consensus sequence for NAD-binding, the GXGXXG motif (Wierenga et al., 1985). The first glycine of this motif is highly conserved, but in some cases the second glycine has been replaced by valine, serine and alanine (Carugo and Argos, 1997). In *E. coli* NfsB an alanine is substituted for the second glycine. The substitution of an alanine at this position has been associated with effects on the binding of the adenine ribose (Rescigno and Perham, 1994). In *E. cloacae* NEC the complete GXGXXG sequence is present, which has been noted by other researchers (Bryant et al., 1991), although studies have not confirmed any involvement with NAD(P)H binding. These residues also align with the unverified NAD(P)H binding region in FRaseI, even though the two protein sequences are dissimilar at this location (Figure 3.19; an alignment of the proteins can be seen in Chapter 4, Figure 4.1) (Koike et al., 1998). Interestingly, shortly after this study was completed, the SWISS-PROT database (ExPASy) listed the 153-158 residue region of NfsB as the NAD(P)H binding site 'by similarity'. It is not clear what it has been compared to; as motif searches by PSITE (Solovyev and Kolchanov, 1994) and tools within the PredictProtein server (Rost, 1996), including ProDom (Corpet et al., 2000) and ProSite (Bairoch et al., 1997) do not identify this region (or any other in NfsB)

as a potential binding site. Akin to the reasoning that motivated the mutagenesis approach used in this study, the identification was probably founded on similarity to the most commonly cited GXGXXG motif.

3.4.3 Residues in target region may influence NADPH utilization

The mutagenesis target region spanning residues Gly-153 to Leu-159 does conform to the imprecise description of an NAD(P)H binding site, but the 3D structure of NfsB (Parkinson et al., 2000) demonstrates that these residues are unlikely to come into contact with any substrate of the enzyme, and that they are not in direct contact with bound nicotinic acid (NIO) (Loverling et al, 2001). However, the present study demonstrates that mutations introduced in this region do affect NfsB catalysis and cofactor preference. Two key explanations may account for these apparent incongruities. First, NADPH differs from NADH solely by the presence of the 2' phosphate on the AMP moiety, which is not present in nicotinic acid, and thus the NIO-bound structure of NfsB is not a complete representation of the cofactor contacts. Second, many residues in an enzyme can modulate catalysis without any actual direct contact, for example, by long-range electrostatic effects and repositioning of residues at the reaction centre (Mittl et al., 1994; Plapp, 1995), so it is possible that this region is directly involved in catalysis. Indeed, the contributions of distant residues to the active site of *E. coli* glutathione reductase were demonstrated by the switch in cofactor dependence from NADP⁺ to NAD⁺ through the introduction of seven point mutations (Scrutton et al., 1990), which were as distant as 15 Å from the actual cofactor (Mittl et al., 1993, 1994). Of course, any missense mutation will result in some level of local structural change, so analysis of

mutations must make a distinction between gross/indirect structural changes and direct effects on enzyme catalysis.

Most missense mutations examined within the target region resulted in the elimination of NR activity. The tempting implication is that this region is crucial for enzyme activity, but while this may be true, it may also be an oversimplification. It is possible that the amino acid substitutions made here were too drastic and that other substitutions could result in an active enzyme. Even though any amino acid change can potentially affect enzyme structure and/or catalysis, some interchanges are relatively conservative. A mutation data matrix compiled by Dayhoff et al. (1983), derived from comparisons of homologous proteins and consistent with chemical intuition, illustrates how some amino acid substitutions are relatively more frequent, or evolutionarily accepted, than others. According to this matrix, most of the mutations acquired in this study are usually not evolutionarily accepted (see Table 3.8). The exceptions are the Gly to Ser (positions 153 and 158) and Ala to Pro (positions 155 and 156) mutations. Indeed, the two Gly to Ser mutations obtained in this study, G153S and G158S, yielded the only mutant proteins that retained measurable NR activity. It is not surprising that the Ala to Pro substitutions disrupted NfsB function despite their general evolutionary acceptance. A proline substitution considerably decreases the carbon backbone's flexibility, which in an α -helix may result in major structural change. This structural modification is possibly

Table 3.8 Evolutionarily accepted amino acid substitutions based upon the mutation data matrix compiled by Dayhoff et al. (1983). Those residues in bold represent the two types of missense mutations that were obtained in this study. Only the Ser substitution resulted in an active enzyme, as discussed in the text.

Target residue	Frequently accepted mutation
Gly	Ala, Ser
Val	Ile, Leu, Met
Ala	Ser, Gly, Thr, Pro
Leu	Met, Ile, Val, Phe
Phe	Tyr, Leu, Ile

the reason for the distinct shape of the A156P FMN fluorescence spectrum (Figure 3.8b, Table 3.3), the only enzyme for which peak I is smaller than peak II. The diminished NR activity of G153S and G158S suggests that these residues are directly involved in enzyme catalysis. Unfortunately, the NR activity of G153S is too low to allow kinetic analysis (see Section 3.3.4.3), but analysis of the other mutants with mutations in the target region, G158S and VAFS, demonstrated that this region apparently has an effect on NADPH usage.

The significantly decreased NADPH preference of G158S and VAFS is reflected in the low k_{cat}/K_m ratios for this cofactor, about $50 \mu\text{M}^{-1}\text{sec}^{-1}$, compared to over $150 \mu\text{M}^{-1}\text{sec}^{-1}$ for NADH (Figure 3.18b). This effect is primarily due to the increased K_m values for NADPH (Table 3.4), an indicator of decreased affinity, which is interesting as NADP coenzymes are usually more flexible than NAD in their complexes with enzymes. In fact, few enzyme/cofactor interactions are uniquely associated with NADPH (Carugo and Argos, 1997a). Despite numerous studies, a general explanation for NAD/NADP discrimination does not exist. There are some cases in which a loop between an α -helix and β -strand has been shown to interact with the diphosphate moiety of NADP, with the dipole of the α -helix thought to help stabilize the interaction (Carugo and Argos, 1997b). Gly-158 may participate in this type of interaction as it is located in the loop region between α G and β 3 (refer to Figure 3.19), disruption of linkages involving Gly-158 could lead to decreased affinity for NADPH. Structural alterations here may disturb contact between other residues and the cofactor; the disruption of structural contacts is more thoroughly explored in Chapter 4.

The Trp fluorescence spectrum of G158S shows evidence of some structural changes in the mutant enzyme: the low intensity 375 nm peak emitted by this mutant is shifted markedly from the 345 nm peak apparent for wild-type NfsB (Table 3.3). However, the FMN spectrum is similar to that of the wild-type indicating an undisturbed environment near the FMN molecule. Thus, the Trp spectrum probably reflects changes at the 2 residues (W46 and W94) that are furthest from the putative substrate binding site, rather than at the W138 residue that is near the FMN binding pocket. As the FMN spectrum of G158S reflects no major conformational changes, it seems likely that Gly-158, and/or residues influenced by a mutation at Gly-158, is involved in NADPH utilization. A more detailed examination of the structural effect of G158S on NfsB is included in Chapter 4. The structure of NfsB is shown in Figure 3.21 with the residues discussed in this chapter indicated in orange. Modeling of the mutant structures using SwissPDBviewer suggests that the Val to Ala substitution of VAFS does result in some disruption of the wild-type structure. Unlike G158S, the Trp fluorescence spectrum of VAFS is similar to the wild-type and does not have a peak at 375nm (Table 3.3), but the FMN fluorescence spectrum exhibits significantly greater intensity than the other enzymes, especially for peak I (Figure 3.8b, Table 3.3). This increased fluorescence intensity is probably indicative of a structural disturbance in the mutant enzyme, which is not surprising considering that the V154A mutation alone significantly disrupts NR activity. Indeed, the spectrum of V154A indicates a structural disturbance near FMN, which is best reflected by the increase in the intensity of peak II compared to peak I (see Table 3.3 and Figure 3.8c). The fluorescence data obtained for the other mutant with no

Figure 3.21 NfsB structure. Phe-70 and the mutagenesis target region from Gly-153 to Leu-159 in one chain of the homodimer structure are shown in orange. (a) Monomer of NfsB. (b) The NfsB homodimer.

observed NR activity, A156P, indicates that FMN is bound in this enzyme, although in a different environment, as peak II exhibits a slight red shift to 555 nm. Spectra were only obtained for those enzymes included in Table 3.3 and Figure 3.8b as protein concentrations of other mutants were too low for analysis.

The turnover numbers of G158S are significantly less (about half) than for the wild-type. This could indicate an increase in the transition state energy for catalysis (Plapp, 1995; Rescigno and Perham, 1994), perhaps due to incorrect orientation of electron transfer resulting from distorted substrate binding. Small decreases in $K_{m\text{NADH}}$ and $K_{m\text{NF(NADPH)}}$ for G158S are evident. These may not be signs of increased affinity, but rather may reflect that those reactions of the ping-pong mechanism have become less rate limiting due to the decreased catalytic velocity (V_{max}) with the other substrate(s) (Rescigno and Perham, 1994). This may also explain the electron acceptor dependent discrepancy in the $K_{m\text{NADH}}$ values for NfsB observed by Zenno and coworkers (1996b). In that study, the K_{m} value for NADH was much lower with FMN (5.4 μM) than with nitrofurazone (22 μM) as the electron acceptor.

It is plausible that nitrofurazone exerts substrate inhibition in G158S, and even wild-type NfsB (Figure 3.19). Competitive inhibition by a substrate is not uncommon in ping-pong reactions as the slightly altered forms of the enzyme can often accommodate a substrate out of reaction order (Segel, 1975). Furthermore, the physiological substrate of NfsB is not known; therefore, while nitrofurazone may cause inhibition, it is unlikely that the natural substrate would, especially at physiological concentrations. However,

experiments with higher concentrations of nitrofurazone would be necessary to confirm any such inhibition.

3.4.4 F70S has no effect on cofactor specificity

A Phe to Ser substitution is evolutionarily an infrequently accepted mutation (Dayhoff, 1983), and yet the F70S mutant exhibits full NR activity, although with altered kinetic characteristics. Unlike the residues in the target region, Phe-70 is in proximity to the putative substrate binding site and does have contact with four atoms of the bound FMN molecule (Lovering et al., 2001; Parkinson et al., 2000), but not with nicotinic acid (Lovering et al., 2001). According to the 3D structure of NfsB, Phe-70 is adjacent to the entrance of the putative substrate binding pocket. Introduction of the F70S mutation into the 3D structure of NfsB with SwissPDBviewer indicates that by removing the bulky phenyl group of Phe and replacing it with the smaller Ser side chain, this area is sterically less restrictive (Figure 4.7), which may help accommodate substrates.

There is no apparent shift in cofactor specificity with the F70S mutant. Indeed, as expected for a ping-pong mechanism, the k_{cat}/K_m values for all of the substrates are similar to the wild-type values. However, there is some variability in the k_{cat} and K_m values. An apparent decrease in affinity for the cofactors is implied by greater K_m values; and increased turnover numbers may reflect a decreased transition state energy. Interestingly, Phe-70 apparently shifts conformation upon binding of nicotinic acid (NIO) (refer to Chapter 4, Section 4.4.2). It appears that a shift of Phe-70, and of Phe-124, opens an access channel (opening B in Figure 4.5) to the main active site pocket. In the NIO-bound structure Phe-70 only maintains contact with one of the four original FMN

contacts. Figure 4.7 illustrates that the substitution of Ser at this location mimics the shift of the aromatic sidechain of Phe-70, which possibly facilitates access to the main pocket from this direction. This could be the reason for the increase in turnover number of the F70S mutant enzyme, as well as for the decrease in affinity for the cofactors since more conformations (e.g. both oxidized and reduced) could bind.

CHAPTER FOUR: COMPUTER ANALYSIS OF THE NFSB ACTIVE SITE

Implications for structure-based drug development.

4.1 Introduction

Enzymes bind and orient substrates in an active site to facilitate a chemical transformation. Composed of amino acid residues that modulate activity of the enzyme, the active site includes a reaction centre as well as residues that are not in direct contact with the substrate(s) (Mittl et al., 1994; Plapp, 1995). The catalytic specificity of an enzyme is dependent upon multiple interactions that define the size and shape of the active site. An enzyme catalyzes a reaction by affecting the environment at this site; for example, by facilitating proton transfers or altering the electrostatic environment.

A goal of biotechnology is the ability to create a made-to-order enzyme, particularly for medicinal and pharmaceutical purposes. To date, large scale drug discovery efforts have focused less on redesign of enzymes with known structures, by site directed mutagenesis for example, than on *de novo* and rational design of biomolecules. A *de novo* approach attempts to form an biomolecule that folds and catalyzes a substrate within a specific environment (Blackburn, 2000), while rational design refers to a combinatorial chemistry approach that entails the selection of a particular enzyme from a pool of randomly generated ones using high-throughput screening techniques (Gane and Dean, 2000). Until recently, rational design was thought to be the most promising strategy for drug development (Amzel, 1998); however, with the technological advancements in biology and bioinformatics, structure-based drug development is now emphasizing the integration of clinical, cellular, biochemical, structural and biophysical knowledge of the target protein (Pattabiraman, 2002). The increasing ease and speed of

protein structure determination has led to over 18,000 protein entries, and therefore potential drug targets, in the Protein Data Bank (there were about 7,000 in the year 2000) (www.rcsb.org), and the computational techniques to analyze these structures are also rapidly growing in number, accessibility, and utility (Joseph-McCarthy, 2002). The advent of the information age in biology thereby allows the rational redesign of an existing enzyme to be a viable option for large scale structure-based drug development (Blackburn, 2000). Indeed, the redesign of enzyme substrate and cofactor specificity based on 3D structure information is becoming more common and accurate (refer to Cedrone et al., 2000 for some impressive examples), and the future of drug development probably lies in the cooperation of knowledge-based enzyme redesign with combinational high-throughput screening methods (Cedrone et al., 2000).

The first step in structure-based drug development is the identification of functionally relevant binding sites on the target enzyme (Sottriffer and Klebe, 2002). This is facilitated by computational analysis, which can aid in the recognition of protein pockets by geometric analyses (Liang et al., 1998), docking scans for ligand complementarity (Gane and Dean, 2000; Sottriffer and Klebe, 2002), structure comparison, and similarity searches of protein cavities (Sottriffer and Klebe, 2002). With the increasing number of freely available programs, which are often powerful web-based applications, the ability to meaningfully analyze proteins for the purposes of drug development is becoming more accessible to most researchers; although docking programs and compound libraries are still largely restricted to industrial institutions (Gane and Dean, 2000).

In this study, the active site of NfsB was analyzed with computational tools freely available from the Internet. The purpose of this study was two-fold: first, to elucidate key elements of NfsB's active site by means of computer analysis; second, to use this information to predict regions of NfsB that can be recruited for drug development. To achieve these goals, four primary research objectives were undertaken. First, the six available 3D structures from the nitroreductase family were obtained, and a structural alignment using NfsB as a template was generated. Second, protein pockets of these structures were identified and the structural contacts were determined. Third, a thorough structural comparison of NfsB and NfsA was accomplished. And fourth, the mutant structures of NfsB from the previous studies (Chapters 2 & 3) were modeled and compared with the wild-type structure. This information, combined with insights acquired in previous chapters of this thesis and from the literature, provides a more complete representation of the NfsB active site, forms the basis for enzyme redesign experiments, and reveals some promising targets for drug development.

4.2 Methods and Materials

4.2.1 Identification and visualization of NfsB homologues

NfsB and five homologues (Table 4.1) were identified in the Structural Classification of Proteins (SCOP) database (<http://scop.mrc-lmb.cam.ac.uk/scop>) (Murzin et al., 1995) and the structural files (.pdb) were downloaded from the Protein Data Bank (PDB) (www.rcsb.org) (Berman et al., 2000). The 3D structures of the enzymes were visualized in Protein Explorer (Martz, 2000), obtained from <http://proteinexplorer.org>, unless otherwise noted. Protein Explorer requires the Chime plug-in that is available at <http://www.mdlnchime.com/chime/> (MDL Information Systems).

Several approaches were used to identify additional homologues of NfsB including a text search of the PDB with 'nitroreductase', a PSI-BLAST search (Altschul et al., 1997) with default parameters on the ConSurf server (<http://bioinfo.tau.ac.il/ConSurf/>) (Glaser et al., manuscript in preparation), and a search using the Combinatorial Extension method for protein structure comparison (<http://cl.sdsc.edu/ce.html>) (Shindyalov and Bourne, 1998). A 3D structure of NfsB complexed with NIO, and of *Vibrio harveyi* Flavin reductase P (Frp) bound to NAD⁺ were also downloaded for further analysis. Three resolutions (1.7 Å, 1.8 Å and 2.4 Å) of the NfsB-NIO structure were available, so 1ICR (1.7 Å), the structure with the lowest R-factor and best resolution, was used.

Table 4.1 The proteins analyzed in this study. All structures were resolved by X-Ray crystallography.

Organism	Protein name	PDB ID	Total # of residues in monomer	Sequence identity	RMSD C ^{α,3}	Reference
<i>Escherichia coli</i>	*NfsB	1DS7	217			Parkinson et al., 2000
<i>E. coli</i>	NfsB+NIO ¹	*1ICR	217	100%	0.40Å	Loverling et al., 2001
<i>E. coli</i>	*NfsA	1F5V	240	16%	1.78Å	Kobori et al., 2001
<i>Enterobacter cloacae</i>	NfnB	*INEC	217	88%	0.42Å	Hecht et al., to be published
<i>Thermus aquaticus</i>	NADH oxidase	*1NOX ²	205	21%	1.42Å	Hecht et al., 1995
subspecies <i>thermophilus</i>						
<i>Vibrio fischeri</i>	FRaseI	*1VFR	218	34%	1.16Å	Koike et al., 1998
<i>V. harveyi</i>	Flavin Reductase P	1BKJ	240	16%	1.52Å	Tanner et al., 1996
	*Frp					
<i>V. harveyi</i>	*Frp+NAD ⁺	2BKJ	240	16%		Tanner et al., 1999

Notes: The * indicates the nomenclature used throughout the text.

¹ NfsB + NIO = NfsB complexed with nicotinic acid, or niacin (see Figure 4.3).

² All of the enzymes are physiological homodimers, but the 1NOX file only contains coordinates for the monomer.

³ Root mean square deviation (RMSD) of each structure as compared to NfsB. Alignments are for the C^α atoms of the dimer structures, except for NOX in which only the monomer was aligned with Chain A of NfsB. The structure file of 1NEC contains two dimers, so chains C and D were aligned with NfsB.

4.2.2 Structural alignment

A 3D alignment of NfsB with the other five structures was generated in Swiss-PdbViewer v.3.7b2, which was obtained from the ExPASy molecular biology server (<http://www.expasy.ch/spdbv/>) (Guex and Peitsch, 1997). NfsB was used as the template upon which each structure was independently aligned. A detailed description of this procedure is available in the tutorial material, indexed as 'superimposition', provided on the ExPASy site. Briefly, the PDB structure files were opened in the Swiss-PdbViewer v.3.7b2 program and the 'magic fit' tool was used to roughly align each structure with NfsB. The 'iterative magic fit' tool subsequently optimized each alignment, and a structural alignment was created with the 'generate structural alignment' tool. The alignments were viewed and manually adjusted as necessary. The root mean square deviation (RMSD) of each alignment was determined using the 'calculate RMS' option in the tools menu. The RMSD is calculated according to the following equation: $(\Sigma(\text{dist})^2/\text{nbAtoms})^{1/2}$, where dist is the distance (in angstroms) between a pair of related atoms. The Swiss-PdbViewer program was also used to analyze the protein structures, including the determination of B-factors (tools menu).

4.2.3 Determination of interatomic contacts

The structures of the six NR family proteins were resolved with bound FMN. Identification of the FMN-protein contacts was accomplished with the Ligand-Protein Contact (LPC) program available on <http://bioinfo.weizmann.ac.il:8500/oca-bin/lpccsu> (Sobolev et al., 1999). The contacts of nicotinic acid (NIO) in 1ICR, and of NAD⁺ in 2BKJ were also determined in this way. The associated Contacts of Structural Units

(CSU) software (Sobolev et al., 1999) identified contacts made by other residues in the proteins. Some contacts were also identified with the Swiss-PdbViewer and Protein Explorer programs.

4.2.4 Identification of protein pockets and cavities

Binding sites are often associated with structural pockets in a protein. The CASTp server (<http://cast.engr.uic.edu/cast/>) calculates exterior pockets and interior cavities of a protein, measures the area and volume of the pockets and the number and size of the pocket mouth openings based on geometric analyses (Liang et al., 1998). This server was used to identify the structural pockets of NfsB and the other enzymes.

4.2.5 Modeling of mutations

The mutants recovered in chapters 2 and 3 have mutations in NfsB that alter its NR activity. The missense mutations listed in Table 4.2 represent mutants described in previous studies; the substitutions were introduced into the 3D structure of NfsB using the mutation tool of Swiss-PdbViewer v.3.7b2, which selects the least stressful rotamer of the introduced residue. Analysis of the mutant enzymes was accomplished with the same programs described above for the wild-type enzymes.

Table 4.2 NfsB mutations, and one NfsA mutation, that were modeled in this study. Mutations were based on the mutants obtained in previous studies. Alterations in the structural and ligand contacts of the mutant structures were assessed by comparison with the wild-type structure.

Mutation ^a	Source ^b
L33P	Ch. 2
L34R	Ch. 2
S37R	Ch. 2
S40C	Ch. 2
I42Q	Ch. 2
P45E	Ch. 2
*F70S	Ch. 3
V83D	Ch. 2
D105Y	Ch. 2
*F124S	Zenno et al., 1996c
G148S	Ch. 2
G153S	Ch. 3
G153R	Ch. 3
G153C	Ch. 3
*V154A	Ch. 3
V154E	Ch. 3
V154G	Ch. 3
A155S	Ch. 3
A155P	Ch. 2, Ch. 3
A156P	Ch. 3
L157Q	Ch. 3
L157R	Ch. 3
L157P	Ch. 3
*G158S	Ch. 3
L159Q	Ch. 3
Y183D	Ch. 2
*V154A & F70S	Ch. 3
*E99G (NfsA) ^c	Zenno et al., 1998a

^a The number indicates the residue number in the protein (refer to Figure 4.1). The first letter is the wild-type amino acid and the second letter is the one that is substituted in the mutant enzyme. See Appendix I for amino acid codes.

^b Indicates where this mutant was originally obtained and studied. Ch. 2 and Ch. 3 refer to Chapters 2 and 3, respectively, of this thesis.

^c This mutation was obtained in *E. coli* NfsA.

* These mutant structures were analyzed in more detail, particularly with the CASTp program to determine protein pockets and cavities.

4.3 Results

4.3.1 Structural alignment of NfsB and its homologues

Alignments of primary protein sequences do not always reflect structurally conserved regions in proteins. This is particularly true in the case of NAD(P)H binding sites, which often share a conserved structural domain despite little sequence similarity (Carugo and Argos, 1997a,b). A principal division in the nitroreductase family is between the NfsA-like enzymes that exclusively use NADPH as a cofactor (NfsA, Frp), and the NfsB-like enzymes that can use both NADH and NADPH as cofactors (NfsB, VFR, NEC and NOX). A structural comparison of the six protein structures that have been resolved in the nitroreductase family can thus lead to a more complete conceptualization of the NfsB active site; and a comparison of the NfsA-like and NfsB-like enzymes can provide an indication of which elements might be important for NAD(P)H discrimination.

The structural alignment of one monomer from NfsB and its five homologues is shown in Figure 4.1. All of the enzymes are physiological homodimers, although the resolved *T. thermophilus* NOX structure is for a monomer. As expected for homologous enzymes, all of the alignments yielded RMSD values of less than 2 Å (Gille et al., 2000; Rost et al., 1997). Not surprisingly, the largest deviations, 1.78 Å and 1.52 Å, are for the NADPH dependent enzymes, NfsA and Frp, respectively.

4.3.2 NfsB: Protein pockets and structural contacts

The CASTp program calculated 61 pockets and cavities within the NfsB dimer structure, 30 per monomer plus one pocket (IV) (Table 4.3, Figure 4.2) that is

Figure 4.1 Structural alignment of NfsB and homologues. See Table 4.1 for protein identifiers, and refer to the text for details. Elements of secondary structure for NfsB are identified above the protein sequence as boxes (grey for α -helices, black for β -strands). These are nearly identical for the NfsB homologues, including NfsA and Frp. The differences for NfsA are shown below the protein sequences as white boxes with dotted outlines (see also Figure 4.9). The β -strands are the same size and in the same location in all of the proteins. **Bold** = in major pocket; single underline = FMN contact; double underline = NIO or NAD⁺ contact; ***bold italics*** = in major pocket, but has no observed contacts with FMN or nicotinamide cofactor.

		αA		αB		β1	αC
NfsB	1	--MDIISVAL <u>K</u> <u>R</u> <u>H</u> <u>S</u> <u>T</u> KAFDA	SKKLTPEQAE	QIKTLLQYSP	- <u>S</u> <u>S</u> <u>T</u> <u>N</u> <u>S</u> QPDWH	FIVASTEERGK	
1NEC	2	---DIISVAL <u>K</u> <u>R</u> <u>H</u> <u>S</u> <u>T</u> KAFDA	SKKLTAEAE	KIKTLLQYSP	- <u>S</u> <u>S</u> <u>T</u> <u>N</u> <u>S</u> QPDWH	FIVASTEERGK	
1VFR	2	-THPIIHDL <u>N</u> <u>R</u> <u>Y</u> <u>T</u> <u>S</u> KKYDP	SKKVSQEDLA	VLLEALRLSA	- <u>S</u> <u>S</u> <u>I</u> <u>N</u> <u>S</u> QPDWK	FIVIESDAK	
1NOX	6	PVLDAKTAAL <u>K</u> <u>R</u> <u>R</u> <u>S</u> <u>I</u> RRYRK	-DPVPEGLLR	EILEAALRAP	-SAWNLQDWR	IVVVRDPATK	
NfsA	1	-MTPTIELI <u>C</u> <u>G</u> <u>H</u> <u>R</u> <u>S</u> <u>I</u> RHFT-	-DEPISEAQR	EAIINSARAT	<u>S</u> <u>S</u> <u>S</u> <u>S</u> <u>F</u> <u>L</u> QCSS	IIRITDKALR	
FRP	2	--NNTIETIL <u>A</u> <u>H</u> <u>R</u> <u>S</u> <u>I</u> RKFT-	-AVPITDEQR	QTIIQAGLAA	<u>S</u> <u>S</u> <u>S</u> <u>S</u> <u>M</u> LQVVS	IVRVTDSEKR	
							

		αC	αD	β2	αE	αF
NfsB	58	ARVAKSAAGN YV <u>F</u> <u>N</u> <u>E</u> <u>R</u> <u>K</u> <u>M</u> <u>L</u> <u>D</u>	ASHVVVFCAC	TAMDDVWLKL	VVDQ <u>E</u> ADAGR	FATPEAKAAN
1NEC	57	ARVAKSAAGT YV <u>F</u> <u>N</u> <u>E</u> <u>R</u> <u>K</u> <u>M</u> <u>L</u> <u>D</u>	ASHVVVFCAC	TAMDDAWLER	VVDQ <u>E</u> ADAGR	FNTPEAKAAN
1VFR	60	QRMHDSFANM HQ <u>F</u> <u>N</u> <u>Q</u> <u>P</u> <u>H</u> <u>I</u> <u>K</u> <u>A</u>	CSHVILFANK	LSYTRDDYDV	VLSKAVADKR	IT-EEQKEAA
1NOX	64	RALREAA--- FGQ-- <u>A</u> <u>H</u> <u>V</u> <u>E</u> E	APVVLVLYAD	LEDALAHLE	VIHPGVQGER	-----R
NfsA	58	EELVTLT--- GGQ-- <u>K</u> <u>H</u> <u>V</u> <u>A</u> Q	AAEFWVFCAD	<u>F</u> <u>N</u> <u>R</u> <u>H</u> <u>L</u> <u>Q</u> <u>I</u> <u>C</u> --	-----	-----
FRP	58	NELAQFA--- <u>G</u> <u>N</u> <u>Q</u> -- <u>A</u> <u>Y</u> <u>V</u> <u>E</u> S	AAEFLVFCID	YQRHATIN--	-----	-----
		..	..	..	..	..

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		β4		αI	β4		
NfsB	175	EFGLKEKGYT	SLVVVPVGHH	SVE-DFNATL	PKSRLPQNIT	LTEV	
1NEC	174	EFGLKEKGFT	SLVVVPVGHH	SVE-DFNATL	PKSRLPLSTI	VTEC	
1VFR	175	IFADELKGYE	CHVALAIGYH	HPSEDYNASL	PKSRKAFEDV	ITIL	
1NOX	168	ILGLPSR-AA	IPALVALGY <u>P</u>	<u>A</u> EEG----- <u>Y</u>	<u>P</u> SHRLPLERV	VLWR	
NfsA	142	LLKLQP-HVL	PL <u>F</u> GLCLGWP	ADNP----- <u>D</u>	<u>L</u> KPRLPASIL	VHENSYQPLD	KGALAQYDEQ
FRP	142	LLGLPE-NSA	V <u>L</u> FGMCLGHP	DQNP----- <u>E</u>	<u>V</u> KPRLPAHVV	VHENQYQELN	LDDIQSYDQT
			*		*		** *
							**

		α H			α I	
NfsA	196	LA <u>E</u> <u>Y</u> <u>Y</u> <u>L</u> <u>T</u> <u>R</u> <u>G</u> <u>S</u>	NNRRDTWSDH	IRR <u>T</u> <u>I</u> <u>I</u> <u>K</u> <u>E</u> <u>S</u> <u>R</u>	PFILDYLHKQ	GWATR
FRP	196	MQA <u>Y</u> <u>Y</u> <u>A</u> <u>S</u> <u>R</u> <u>T</u> <u>S</u>	NQRCSTWSQE	VTG <u>K</u> <u>L</u> <u>A</u> <u>G</u> <u>E</u> <u>S</u> <u>R</u>	PHILPYLNSK	GLAKR
		. . **	. ***	***	* * * * * . .	* * . *
		α I	α J	α K		

		αI	αJ	αK
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Figure 4.2 Solvent accessible pockets of NfsB from the perspective of ‘looking’ into mouth opening A of the major pocket (i.e. opening B is viewed within the major pocket through mouth opening A). Refer to Tables 4.3 and 4.4 for volumes and residue composition of the pockets.

Table 4.3 Volume of the major pocket (I) and other pockets referred to in this study. The number of solvent accessible mouth openings to each pocket is indicated in parentheses.

	NfsB	NfsB+NIO	F70S ^a	G158S ^b	V154A	F124S ^d	VFR	NfsA	E99G	FRP	FRP+NAD
I	1302.5(2)	2520(3)	1518.8(3)	wt	wt	1998.7(3)	1437.8 (3)	1021.7(4)	1089.1	943.3	1043.8
II	557.4(1)	N/A	wt	wt	wt	N/A	750.4(2)	(247.8) ^e (0)	wt	N/A	N/A
III	138.5(1)	118.9(1)	wt	wt	148.3(1) ^c	wt	263.8(1)	(75.8) ^e (2)	wt	N/A	N/A
IV	171.1(2)	149(1)	wt	211.8(2)	wt	wt	N/A	(22.4) ^e (1)	N/A	N/A	N/A

NOTES:

wt = Identical to wild-type NfsB.

N/A = Not applicable as there are no pockets that are comparable to those in NfsB.

Changes to the compositions of the altered pockets as compared to wild-type NfsB are:

^a. Addition of Asn-67 to pocket I.

^b. Ala-155 and Ser-158 from one monomer are included in pocket IV.

^c. Pocket contains same residues as wild-type NfsB, even though it has a greater volume.

^d. Similar to the NIO-bound structure of NfsB, the major pocket of F124S is a combination of pockets I and II in unbound NfsB.

^e. These pockets, illustrated in Figure 4.9, are not comparable to the pockets in NfsB, but do contain residues that may be involved in the binding of NADPH. See text for details, particularly Sections 4.3.3.2 and 4.4.3.

symmetrically composed of equivalent residues from each chain. Many of these pockets are small, with molecular volumes of less than $20 \text{ \AA}^3$, but seven have volumes exceeding $100 \text{ \AA}^3$. The largest pocket of NfsB and its homologues contains FMN and is thus designated the major pocket, while the other pockets are referred to as ancillary pockets. The CASTp data are considered within the context of other information including a comparison with the NIO-bound structure of NfsB, singular features of the structural alignment (Figure 4.1), especially with regards to NfsA (Section 4.3.3), and the experimental results obtained from previous studies, particularly the mutants analyzed in chapters 2 and 3 (Section 4.3.4).

4.3.2.1 The major pocket: FMN, NAD(P)H and substrate binding

All of the structures in this study were resolved with bound FMN (see Figure 4.3b for the structure of FMN). All of these enzymes are physiological homodimers, with the two bound FMN molecules positioned within crevices formed at the dimer interface (refer to Figure 4.4). In NfsB these two major pockets are V-shaped, hold the bound FMN at the narrow part of the cleft, and are similar to each other in volume and composition; therefore, unless otherwise noted, only one monomer will be referred to throughout this chapter. Access to the major pocket in NfsB is possible by means of one large (A) and one small (B) opening (Figure 4.5). Opening B is located between the major pocket (I) and pocket II (seen on Figures 4.2 & 4.5). The volume of the major pocket is approximately $1300 \text{ \AA}^3$, which is noticeably smaller than in the F70S mutant ($1519 \text{ \AA}^3$), and much less than in NfsB complexed with NIO ($2520 \text{ \AA}^3$) (see Section 4.3.2.2.2).

(a) NAD(P)H

Figure 4.3 Diagrams of the cofactor structures mentioned in this study. (a) Oxidized and reduced nicotinamide adenine dinucleotide (NAD⁺) and NAD phosphate (NADP⁺); (b) FMN; (c-e) cofactor analogues. Continued on next page.

Figure 4.3 (cont'd)

Figure 4.4 Side and front views of the FMN-containing major pocket in (A) NfsB (top) and (B) NIO-bound NfsB (bottom). The two monomer chains are in different colours.

For the ligands grey = carbon atoms, red = oxygen, blue = nitrogen.

Figure 4.5 Solvent accessible pockets of NfsB (left) and NIO-bound NfsB (right). Refer to Tables 4.3 & 4.4 and the text for details. Continued on the next page.

Figure 4.5 (cont'd) Solvent accessible pockets of NfsB (left) and NIO-bound NfsB (right). Residues Phe-70 and Phe-124 are shown to demonstrate the altered conformation from the unbound to bound structure.

In NfsB, all of the FMN contact residues reside within the major pocket, and the bound position and conformation of FMN is conserved in the homologous enzymes. FMN is in a butterfly conformation with the *si* face buried in the protein and the *re* face solvent accessible (see Figure 4.4). Parkinson et al. (2000) include a detailed description of the FMN binding pocket in NfsB and note that access to FMN is limited to the third ring and *re* face of the cofactor. The protein-ligand contacts derived with the LPC program (Sobolev et al., 1999) are underlined and in bold in Figure 4.1, while Figure 4.6a illustrates these contacts for NfsB. In Figure 4.1 all residues that are in contact with FMN are noted regardless of the contributing monomer, as only one monomer of the alignment is shown. Some of the apparently missing contacts in NOX on this figure are attributable to NOX being resolved as a monomer. Most noticeable is the absence of contact with Pro-38 to Asn-42 in NOX since these residues are contributed from the other monomer.

One structure of NfsB was resolved with NIO, an analogue of the NAD cofactor (see structure in Figure 4.3c). The protein contacts of NIO are double underlined on Figure 4.1, and are illustrated in a LIGPLOT diagram in Figure 4.6b. Many of the nicotinamide contact residues in NfsB are also in contact with FMN, as both cofactors are located within the major pocket of the enzyme. The exceptions are H128 and W128, both of which are located in pocket II (Table 4.4, Figure 4.2). In the NIO-bound structure of NfsB, this pocket is incorporated into the major pocket (see Section 4.3.2.2.2 and Figure 4.5). However, while the NIO-bound structure contributes valuable information about cofactor binding in NfsB, the entire cofactor is not represented (see Figure 4.3 for

(a) FMN contacts in NfsB

Figure 4.6 LIGPLOT diagrams showing most of the structural contacts in NfsB with (a) FMN, and (b) NIO. Dotted lines = hydrogen bonds. Arcs with spokes = hydrophobic contacts. A and B refer to the two chains in the NfsB dimer. Refer to Figure 4.1 to see all of the structural contacts.

(b) NIO contacts in NfsB

Figure 4.6 (cont'd)

Table 4.4 Amino acid composition of pockets I-IV in NfsB and NfsB complexed with NIO.

Pocket No. ^a	Description	Chain A	Chain A With NIO	Chain B	Chain B With NIO
I	FMN, NIO contacts	P38,S39,S40,T41,N42,E102, R107,F108,A113,A116,N117, K1119,G120,R121,F123,F124, Q142,L145	Like Chain A and also contains M127,H128, L132,D134,W138,K141	L8,K9,R10,H11,S12,K14,Y68,F70, N71,K74,Y144,V147,L151, V162,P163,I164,E165,G166, V187,F199,N200,A201,L203, P204,K205,S206,R207	Like Chain B except: No L8,K9,V147,A201, P204, and contains N67,V69,F167,D168,A169, A170,S185,L186,F199
II	FMN contacts	F124,M127,H128,L132,D134, W138,K141	N/A	Y68,Y144,E165,F167,D168,A169, A170,S185,L186	N/A
III	FMN, NIO contacts and target region from Chapter 3	R10,H11,L151,V154,A155,L159, D160,A161,P163	Identical to Chain A	N/A	N/A
IV	Symmetrical pocket	M1,D2,I3,V6,A156	Identical to Chain A	Same residues as in Chain A	Same residues as in Chain A

^a Each monomer has pockets I-III, and thus there is two of each in the homodimer structure, but there is only one pocket IV in the complete protein.

cofactor structures) and therefore a complete picture of NAD(P)H binding still does not exist.

A number of residues, notably the K117-F123 tract are located in the major pocket of NfsB but do not have contact with FMN or NIO. These residues are probably relevant for substrate specificity and/or the accommodation of cofactors as they are part of a less conserved helix that has residues associated with low B factors, indicating that this region is highly mobile; furthermore, these residues align with the large gap introduced in NfsA (see Section 4.3.3; Figure 4.1).

4.3.2.2 Ancillary pockets

Ligand binding pockets usually have a molecular volume within the range of 10^2 - $10^3 \text{ \AA}^3$, and one to two mouth openings (Liang et al., 1998). Pockets of this size adjacent to the primary active site pocket represent potential additional binding surface for ligands (Liang et al., 1998). Although smaller than the FMN binding pocket in NfsB, there are two pockets (II and III) in each monomer that are greater than $100 \text{ \AA}^3$, plus there is one symmetrical pocket that is formed from equivalent residues in each monomer (IV) (Table 4.4, Figure 4.2). Pocket II is composed of residues from each monomer, and has two mouth openings, one of which is the shared small opening B of the main pocket (Figures 4.2&4.5). Pocket III is completely formed within each monomer, has one mouth opening, is adjacent to the main pocket, on the opposite side from pocket II, and contains many of the residues that comprise the mutagenesis target region of Chapter 3. Pocket IV is sandwiched between pockets III of each monomer, and has two mouth openings. The

size and location of these pockets suggests that they provide ligand contacts for NfsB and thus may be considered a part of the native active site.

In addition to size and proximity, conserved residues and cofactor induced conformational changes can provide signs that protein pockets may be considered part of the NfsB active site. Other regions of importance are implicated on the basis of comparison with the NADPH dependent enzymes (Section 4.3.3), and the structural alterations of the mutant proteins (Section 4.3.4).

4.3.2.2.1 Conserved residues

Most of the conserved residues revealed in Figure 4.1 have contact with FMN, or are located within the major pocket. However, there are two residues, Gln-44 and Gly-192 in NfsB, that are conserved in both the NfsB- and NfsA-like enzymes, but do not have contact with FMN, and are not located in the major pocket. Gln-44 is within a tract of residues that are probably an important domain of NfsB-like proteins, as discussed below, and substitution of this residue results in the elimination of NR activity (Chapter 2). Gly-192 is located within a small cavity of NfsB that is not surface accessible, and the 8 residues with which it has contact (Phe-16, Lys-20, Leu-22, Ser-79, Leu-159, Asp-160, Val-191 and His-193) are not located within the major pocket, nor do they have any contact with FMN or NIO. A low B-factor is associated with Gly-192 suggesting that its function is not to contribute flexibility, but rather to maintain structural integrity.

The stretch of residues from Gln-44 to Val-50 is highly conserved in NfsB homologues, as well as in the many putative homologues that have been identified from the burgeoning number of genome projects (for examples see Chapter 2, Table 2.5 and

Figure 2.5). With the exception of Pro-45, all of these amino acids have contact with C-terminus excursion domain residues of the other monomer in the dimer structure. The residues in the excursion domain have relatively low B-factors, suggesting low mobility of this region. The low mobility associated with most of the conserved residues suggests a role in maintaining structural integrity in the enzymes rather than flexibility for the accommodation of substrates and cofactors.

4.3.2.2.2 Conformational changes in NIO-bound NfsB

An alignment of NfsB (1DS7) and NfsB bound with NIO (1ICR) reveals little change (RMSD of 0.4 Å) between the C^α atoms of the two forms (Table 4.1). However, the RMSD of only the sidechains is 1.34 Å, and of all atoms is 0.98 Å. Upon examination of ICR it is apparent that the major pocket of ICR is actually a rough combination of pockets I and II in unbound NfsB (Table 4.4, Figure 4.5), which largely explains the much greater volume of the major pocket in ICR (2520 Å³) compared to unbound NfsB (1302 Å³) (Table 4.3). An increase in the size of opening B (Figure 4.5) is also noticeable in ICR, which accounts for the CASTp program including both pockets as a single entity in this structure. In addition, there are numerous slight conformational changes in NIO-bound NfsB, some of which are mentioned in other sections, plus the altered side chain conformations of Phe-70 and Phe-124 seen in Figure 4.7 that appear to be particularly relevant.

4.3.3 NfsA and Frp

In the NfsA dimer structure, 60 pockets were identified, 30 per monomer, and 10 with volumes greater than 100 Å³. Similarly, 10 pockets exceeding 100 Å³ were

Figure 4.7 (a) Major pocket of NfsB and NIO-bound NfsB. Phe-70 and Phe-124 are indicated to illustrate the shift in sidechain position when NfsB is complexed with NIO. (b) Aligned FMN molecules, Phe-70 and Phe-124 in NfsB and NIO-bound NfsB. Green = conformation in NfsB; Blue = conformation in NIO-bound NfsB. Yellow and red illustrate the F70S and F124S mutations, respectively.

identified in Frp, which has 48 pockets in total, or 24 per monomer. Thus, each monomer of NfsA and Frp contained five pockets with volumes exceeding $100 \text{ \AA}^3$.

4.3.3.1 Major pocket

The major pocket of NfsA is depicted in Figure 4.8 and the residues within this pocket are denoted for NfsA and Frp in bold italics on Figure 4.1. Notably, the volume of the major pocket is almost one-third less in the NfsA-like proteins (NfsA and Frp), approximately $1000 \text{ \AA}^3$, as compared to the NfsB-like proteins (NfsB, NEC, VFR), about $1400 \text{ \AA}^3$ (Table 4.3) despite the composition of the pocket being similar. The most notable difference in composition is the lack of any structure that aligns with αE and αF in NfsB (see Figures 4.1 & 4.9), including 11 residues that are located within the major pocket of NfsB.

All of the FMN contact residues are located within the major pocket of NfsA and Frp, and the majority of these align with the FMN contact residues in the NfsB-like proteins. The position of FMN is similar to the NfsB-like proteins, and is in the conserved butterfly conformation with the *re* face solvent accessible.

The major pocket of NfsA includes most of the NAD^+ contacts of Frp, many of which align with the NIO contacts of NfsB. The amino acids in the extended C-terminus domain, the excursion domain, of NfsA and Frp (see Figures 4.1 & 4.9) can reasonably be expected to contribute to their exclusive use of NADPH. Two residues in this region of Frp, K219 and R225, do have contact with NAD^+ , and these align with a stretch of residues in NfsA that are located within its major pocket.

Figure 4.8 Structural pockets of NfsA. FMN is bound in the major pocket (red). The second largest pocket (II) is shown in grey. Refer to Table 4.3 for pocket volumes and mouth openings.

Figure 4.9 Two views of the aligned NfsA (red) and NfsB (blue) structures. Only one monomer is shown. Unless noted, the labels refer to NfsB. Refer to Figure 4.1 for sequence details.

4.3.3.2 Ancillary pockets

In Frp, a few residues have contact with NAD⁺ but are not within the major pocket. The associated residues in NfsA are mostly located within two pockets, labeled III and IV (Figure 4.8), that are near the mouth of the major pocket and are less than 100 Å³ in volume. Pocket III is within the excursion domain specific to NfsA and Frp. Pocket II is the second largest pocket in NfsA and is adjacent to the major pocket, but in this structure there are no solvent accessible mouth openings.

4.3.4 Analysis of the mutant structures

4.3.4.1 Residues that influence NAD(P)H use

In Chapter 3, the stretch of amino acids from Gly-153 to Leu-159 were analyzed for their contribution to the utilization of NAD(P)H in NfsB. The substitutions introduced at Val-154, Ala-155 and Leu-159, which are located within pocket II (Table 4.4), resulted in the elimination of NR activity. Pocket II also includes 3 amino acids, Arg-10, His-11 and Pro-163, that have contact with FMN.

Two mutants described in Chapter 3, G153S and G158S, retained some NR activity, with G153S exhibiting less NR activity than G158S. Although neither of these amino acids resides within a discernible pocket of wild-type NfsB, it should be noted that in the mutant structure of G158S, the introduced Ser residue of one monomer is located within pocket IV along with another extra residue, Ala-155, from the same monomer. Gly-153 has contact with Leu-33, Phe-149, Leu-152, Val-154, Ala-156 and Leu-157 in NfsB. It may be that the lower NR activity of G153S relative to G158S is attributable to the contact of Gly-153 with Leu-33, a residue that has been implicated with cofactor

utilization in the NfsB-like homologue *Salmonella typhimurium* CNR (Watanabe et al., 1998), and that results in the elimination of NR activity upon mutation in NfsB (Chapter 2).

The remaining amino acid of this mutagenesis target region, Leu-157, is not associated with a pocket in the native FMN oxidized structure of NfsB (Parkinson et al., 2000), but is in the structure resolved with bound NIO (ICR) (refer to Section 4.3.3.2.4). Furthermore, this residue also has contact with Leu-33.

4.3.4.3 Residues that affect NR activity

In Chapter 2, a number of missense mutations in NfsB resulted in a loss of NR activity (Table 2.2). Some of these mutations have been revealed in the preceding sections to affect residues that are highly conserved, have a putative role in cofactor utilization, and/or have contact with FMN. However, those reasons cannot be used to explain the influence of seven residues that were mutated: Leu-33, Leu-34, Ser-37, Val-83, Asp-105, Gly-148 and Tyr-183. The effect on NR activity possibly is attributable to the following structural contacts. Leu-33, Ser-37, Val-83 and Gly-148 share pockets with FMN contact residues. As mentioned in Section 4.3.4.1, Leu-33 also has contact with Gly-153 and Gly-157, both of which are implicated in NAD(P)H utilization (Chapter 3), and is probably involved in cofactor utilization by CNR, the NfsB homologue of *S. typhimurium* (Watanabe et al., 1998). Asp-105 is situated between the flexible α E and α F helices that are specific to the NfsB-like proteins (Figure 4.9), and although this residue is not located within a pocket in native NfsB, it does share a pocket with the conserved FMN contact residues Lys-205 and Arg-207 in NIO-bound NfsB. Likewise,

Leu-34 and Tyr-183 are in different environments in NIO-bound NfsB, as compared to the unbound structure, and they share pockets with residues of the excursion domain, including the conserved region between Gln-44 and Val-50.

4.4 Discussion

4.4.1 Characterization of the oxidized NfsB active site

NfsB contains two bound FMN molecules, one per monomer, and each is the foundation of an independently functioning active site (Parkinson et al., 2000). The FMN molecule is necessarily the reaction centre as it receives a hydride from the donating cofactor, NADH or NADPH, which it then uses to reduce the nitro-containing substrate in a ping-pong Bi-Bi reaction mechanism (see Chapter 3, Figure 3.17). A ping-pong mechanism usually occurs in an enzyme with one primary ligand-binding site such that two ligands cannot bind at the same time, obliging the donor to leave before the acceptor can bind. In most enzymes the primary ligand-binding site is the largest surface accessible pocket of the protein (DesJarlais et al., 1988; Laskowski et al., 1996; Liang et al., 1998). In NfsB, the largest pocket (approximately 1300 Å³) is at the interface of the two monomers, and FMN is bound at the narrow end of the triangular cleft that they form (Figure 4.4).

The oxidized, bound FMN molecule is in a “butterfly” conformation with the *re* face exposed (Loverling et al., 2001; Parkinson et al., 2000). A butterfly conformation describes the bending of the two end rings of the isoalloxazine in the same direction, compared to the planar form of the free isoalloxazine ring system (Edmondson and Ghisla, 1999; Haynes et al., 2002). The FMN molecule is firmly bound in NfsB and its homologues with numerous protein contacts (Haynes et al., 2002; Koike et al., 1998; Loverling et al., 2001; Parkinson et al., 2000), and the protein interactions with FMN are barely disturbed upon binding of other ligands in NfsB (Loverling et al., 2001) or NEC

(Haynes et al., 2002). The overall bend of oxidized FMN in NfsB is approximately 15° (Loverling et al., 2001), and 16° in *E. cloacae* NEC (Haynes et al., 2002), both in the direction of the protein occluded *si* face. This is a large angle, approximately only 10% of reported structures have similar or more extreme bends (Lennon et al., 1999). Haynes and coworkers suggest that the energetically unfavourable bend of FMN in the NR enzymes may be a tradeoff for an increase in the two-electron reduction potential of the oxidized flavin. The oxygen-insensitive NR enzymes are unusual in that there is no known one-electron reduced, or semiquinone, state (Mason and Holtzman, 1975). Semiquinone FMN is planar, similar to free oxidized flavin, and thus the obligatory two-electron reduction of the Type I enzymes may be imposed by the severe bend in the bound flavin as it would be unable to stabilize the semiquinone form. Consequently, nitro compounds would be fully reduced rather than form oxygen radical species that cause cellular oxidative stress (Haynes et al., 2002). Evidence for NfsA (Liochev et al., 1999; Paterson et al., 2002) and NfsB (Liochev et al., 1999; Chapter 2, this thesis) having roles in the SoxRS response to oxidative stress indicates that this may be a key physiological role of the oxygen-insensitive NR enzymes in *E. coli*.

Based on the structure of native NfsB (Parkinson et al., 2000) in which access to the oxidized FMN molecule is restricted to the third ring of the isoalloxazine structure, the site of hydride transfer was originally predicted to be at FMN N1. However, as described in Section 4.4.2.1, the actual site of transfer is probably FMN N5, which suggests that upon binding of the cofactor there is a conformational change in NfsB.

4.4.2 Catalysis and the reduced form of NfsB

4.4.2.1 NAD(P)H

In ICR, the structure of NIO-bound NfsB (Loverling et al., 2001), the planar nicotinamide ring stacks above the central FMN ring to allow hydride transfer (Figure 4.4). This stacking is also apparent for the planar inhibitors benzoate and acetate in *E. cloacae* NEC (Haynes et al., 2002). In ICR the C4 atom of the nicotinamide ring is 3.4 Å from the FMN N5, which is close to the average distance of 3.5 Å for hydride transfer at this atom (Fraaije and Mettevi, 2000), and places the *pro-R* hydrogen facing FMN. Transfer to the FMN N5 atom is in accordance with a NMR study with the *V. fischeri* VFR homologue (Inouye and Nakamura, 1994), but it is at odds with the prediction derived from the oxidized form of NfsB that FMN N1 is the probable site of hydride transfer (Parkinson et al., 2000). This contrast between the bound and unbound structures of NfsB strongly suggests that there is a conformational change in NfsB after binding of the nicotinamide cofactor, such that hydride transfer occurs at the FMN N5 atom, as observed in VFR.

The structural alignment of oxidized NfsB with NIO-bound NfsB demonstrates that the conformational change in NfsB after binding is mainly due to alterations in the amino acid sidechains (RMSD 1.34 Å) rather than in the C α backbone (RMSD 0.4 Å). However, a key structural change in NIO-bound NfsB, illustrated in Figure 4.5, is the incorporation of the two largest pockets (I and II) seen in unbound NfsB into one large pocket as a consequence of the widening of mouth opening B. Therefore, the ancillary pocket II, containing NIO contacts, can reasonably be expected to contain additional

cofactor and substrate contacts that are made available to a substrate after cofactor induced conformational change. Furthermore, this larger pocket of NIO-bound NfsB has three mouth openings, compared to the two in unbound NfsB.

If the complete NAD(P)H cofactor is bound in NfsB in a similar orientation as NIO, then the remainder of the molecule, including the adenine and ribose moieties, would be mostly solvent exposed. Consequently, there would be little interaction of the protein with the cofactor, which may explain the ability of NfsB to use the cofactors with comparable efficiency. In agreement with this is the indication that interaction of NEC with NAD(P)H is largely mediated by the amide moiety of the nicotinamide ring and that there is little protein-adenine interaction (Koder and Miller, 1998a). However, there are two indications of more extensive NfsB-cofactor interactions. First, the decreased NADPH specificity of the G158S mutant (discussed below) suggests that protein factors do play a role in NADPH utilization. And second, reduced ribotide cofactors tend to be active in NfsB, while reduced ribosides are not, which suggests that the 5' phosphate is required for cofactor recognition (Knox et al., 1995). The interesting exceptions to this cofactor specificity are 1-methylnicotinamide (Figure 4.3d), which does not have a ribose moiety, and reduced nicotinic acid riboside (Figure 4.3e), which does not have a 5'phosphate. However, both of these analogues exhibit decreased specificity for NfsB, and have K_m values exceeding 5-10 times those obtained for NADH or NADPH (Knox et al., 1995). It may be that the 5' phosphate moieties aid in the binding and orientation of the complete cofactor, but due to less steric hindrance the smaller analogues do not

require large conformational shifts to accommodate binding. This binding may approximate that of competitive inhibitors such as dicoumarol, benzoate and acetate.

The most common protein element that is involved in the binding of NADH is a Rossman fold, or mononucleotide-binding domain, which is composed of two parallel β -strands linked by an α -helix such that the loops at the carboxyl termini of the β -strands interact with the phosphate group of the nucleotide (Carugo and Argos, 1997a; Wierenga et al., 1985). However, this motif is not present in NfsB or its homologues. Another common interaction specific to NADH is the presence of an aspartate or glutamate residue that hydrogen bonds with the diol group of the ribose near the adenine, and is followed by an apolar residue that interacts with the adenine (Carugo and Argos, 1997a). In the NfsB-like enzymes, Asp-168 is conserved and is often followed by an apolar residue. These residues follow the highly conserved FMN contact region from Val-162 to Gly-166 in NfsB, which includes two residues, Glu-165 and Gly-166, that bind NIO; therefore, it may be that Asp-168 and Ala-169 are involved in binding of the complete nicotinamide cofactor in NfsB, depending on the conformation that the NAD(P)H cofactor adopts (discussed below). The N1 atom of NIO interacts with Gly-166 of NfsB, which indicates that the cofactor is oriented towards the small opening B in NfsB and is largely solvent exposed, although it might still have numerous other protein contacts within pockets I and II of NfsB.

NAD(P)H can adopt a variety of conformations in a protein (Figure 4.10) (Bell et al., 1997; Bellamacina, 1996; Carugo and Argos, 1997a; Tanner et al., 1998), so the actual conformation cannot be predicted from the binding of NIO alone.

Figure 4.10 Some conformations of NAD. Adapted from Tanner et al., 1999.

Protein interaction with the adenine component of NAD(P)H is minimal in NEC (Koder and Miller, 1998a), and thus this may be the case for NfsB too; nevertheless, as mentioned previously, there is some evidence for protein interaction with the 5' phosphate of the cofactor. The effect of the G158S mutation on NADPH utilization implicates protein-NADPH contact, even though Gly-158 probably does not interact with the cofactor directly as it is about 18 Å from the FMN molecule. In the G158S mutant structure, Ser-158 and Ala-155 from one monomer are included in, and increase the volume of, pocket IV while having no apparent effect on the neighbouring pockets. This altered pocket or the disrupted contacts of Gly-158 could be responsible for the mutant enzyme's decreased specificity for NADPH. The most consistent feature of NADPH binding sites is the presence of an arginine that is able to face the adenine plane of NADPH; however, disruption of an arginine residue is not apparent in G158S. Gly-158 is associated with a low B-factor in the 3D structure of NfsB, which suggests that this residue contributes stability rather than flexibility to the protein. The fact that Gly-158 is a long distance from the putative binding site of the nicotinamide cofactor does not negate its putative influence on cofactor specificity. In fact, residues as distant as 15 Å from the bound NAD(P)⁺ cofactor in *E. coli* glutathione reductase were shown to change the enzyme's cofactor specificity from NADP⁺ to NAD⁺ (Mittl et al., 1993,1994; Scrutton et al., 1990). Furthermore, one of these distant mutations resulted in the introduction of a residue that was hitherto not included in the known active site and yet helped alter the specificity for the 'wrong' cofactor (Scrutton et al., 1993). This is

perhaps analogous to the introduction of Ala-155 and Ser-158 into pocket IV as seen for the G158S mutant of NfsB.

It is clear from the ICR structure and from the inhibitor bound structures of NEC (Haynes et al., 2002) that there is little room for any other molecule to bind when the cofactor is bound in the main pocket (Figure 4.7). This makes sense in the context of a ping-pong reaction mechanism, as it means that cofactor must leave before the substrate can bind.

4.4.2.2 FMN

A structure of the reduced form of the NfsB homologue NEC was recently resolved (Haynes et al., 2002). The coordinates of this structure are not yet released, but Haynes and coworkers report an increase in the isoalloxazine bend to 25° from the 16° of the oxidized form. At this severe angle, the structure is one of the largest bend angles reported for a protein bound FMN (Lennon et al., 1999), and is close to the approximated bend in free reduced flavin (27-29°) (Cavelier and Amzel, 2001). However, the protein contacts of reduced FMN in this structure are relatively undisturbed, as the bend is mainly about the N5-N10 axis (Haynes et al., 2002). Given the high degree of homology with NEC (88% protein sequence identity), it is reasonable to assume that reduced FMN in NfsB adopts a similar conformation. Indeed, despite obvious shifts in some of the amino acid sidechains of the major pocket in the NIO-bound structure of NfsB, FMN itself does not appear to change conformation (Figure 4.7b).

4.4.2.3 Substrate binding

NfsB exhibits a broad substrate specificity for compounds of various sizes (McCalla et al., 1978; Zenno et al., 1996b), which suggests that the active site of this enzyme is quite flexible. In the structure of oxidized NfsB, helix αF is relatively unordered and contains residues with high B-factors, which implicates this region as being highly mobile. The helix becomes more ordered upon the binding of inhibitor (Parkinson et al., 2000) or NIO (Loverling et al., 2001). There is also a high degree of sequence variability between the NfsB-like enzymes within this region, especially within the stretch from Asn-117 to Phe-124 that corresponds to the large alignment gap in NfsA and Frp (Figures 4.1 & 4.8). The variability, mobility and presence of certain residues (discussed below) within the major pocket suggest that this region is involved in substrate specificity.

The stretch of residues from Pro-45 to Val-50 is highly conserved in the NfsB-like enzymes. In Figure 4.1, it can be seen that the QPW sequence is completely conserved, followed by a positively charged residue (H/K/R) and three apolar residues. Although these residues are not within the major pocket, FMN and NIO contact residues within the major pocket directly precede this region in the primary sequence. However, the residues between Pro-45 and Val-50 are part of the domain that includes C-terminus residues from the other monomer of the dimer structure, which help form the mouth opening of the major pocket. The associated B-factors indicate that the Pro-45 to Val-50 region is not very mobile, a rigidity that is probably largely attributable to the conserved Pro-45 residue, and therefore it is unlikely that these residues shift to accommodate

substrates. However, as Pro-45 to Val-50 are directly adjacent to the major pocket and NIO contact residues, it may be that the conserved QPWHFIV region of NfsB is responsible for the conformation of the NADH binding region and perhaps has contact with the cofactor itself.

Conformational changes that are obvious in NIO-bound NfsB include shifts in the aromatic sidechains of Phe-70 and Phe-124, which implicate their importance in substrate and/or cofactor binding. Notably, Phe-124 is within the flexible helix α_F mentioned above. Examination of the 3D structures suggest that the shifts of Phe-70 and Phe-124 in NIO-bound NfsB are crucial conformational changes (Figure 4.7) that allow access to either pocket I or II through opening B (Figure 4.5). There is also evidence in other studies for the participation of these residues in substrate binding. In the structure of VFR complexed with dicoumarol, Phe-124 shifts to accommodate the inhibitor (Koike et al., 1998), and Zenno et al. (1996b) demonstrated that the substitution of Phe-124 with a Ser residue alters the substrate specificity of NfsB such that it becomes an even more effective flavin reductase than its flavin reductase homologue VFR. Indeed, the major pocket of the F124S mutant is similar to NIO-bound NfsB in composition and volume (Table 4.3).

However, despite the structural similarity with NfsB, pocket analysis of VFR reveals a number of deviations that are probably attributable to the different substrate specificities of these enzymes. The equivalent pockets in VFR are larger than in NfsB, and the two largest pockets contain an extra mouth opening (Table 4.3). Flavins are large molecules and the ability to accept FMN as a substrate suggests that a large pocket

volume is necessary. It can thus be reasoned that the F124S mutant of NfsB should have a greater volume in the binding site pocket(s) if the ability to catalyze flavin reduction is primarily a function of pocket volume rather than specific protein contacts in this enzyme. Indeed, the major pocket of F124S has about 50% greater volume than wild-type NfsB, and, like VFR, has three mouth openings instead of the two observed in wild-type NfsB. F124S is a temperature-sensitive mutant with respect to nitrofurazone reduction, which indicates that the mutation destabilizes the nitrofurazone-binding site. This destabilization appears to be due to altered protein contacts with nitrofurazone rather than a function of pocket volume, as a positively charged residue (R/H) does not decrease substrate reduction, while a negatively charged residue (D) drastically reduces the enzyme's ability to use nitrofurazone as a substrate (Zenno et al., 1996c).

Phe-70 is a solvent accessible residue in the stretch of residues that separates the mouth openings A and B of the major pocket in NfsB (Figures 4.5 & 4.7). Figure 4.1 reveals that this stretch of residues is also not well conserved, which may point toward a role in substrate specificity. Haynes et al. (2002) propose that this residue in NEC, along with Tyr-68 and Tyr-123, participates in substrate specificity by adjusting the side-chain conformation according to the substrate. In Chapter 3 of this thesis it was shown that NfsB exhibited an increased turnover number upon the substitution of Phe-70 with Ser. Cofactor specificity was not affected, so it could be that this residue is altered primarily to accommodate the subsequent substrate. If this change in conformation serves to reduce physical constraints on the incoming substrate, then the F70S mutant should reflect this as a greater pocket volume. Indeed, the major pocket of F70S does have a

pocket volume that is approximately 200 Å³ greater than wild-type NfsB, as well as having 3 mouth openings to the pocket akin to the NIO-bound structure of NfsB.

4.4.3 *NfsA and NfsB: A revealing comparison*

The key biochemical distinction of NfsA compared to NfsB is its NADPH dependence. Otherwise, NfsA and NfsB share similar substrate specificity, although they reveal different efficiencies at reducing many substrates. NfsA has been designated as the major oxygen-insensitive nitroreductase component of *E. coli* due to its more rapid reduction of nitrofurazone: NfsA reduces nitrofurazone at a rate over five times that of NfsB (McCalla et al., 1978; Zenno et al., 1996a), even though there are some substrates, such as menadione, that are more efficiently reduced by NfsB (Doi et al., 1983; Zenno et al., 1996a). Other differences in substrate specificity between NfsA and NfsB include the lack of significant inhibition of NfsA by dicoumarol, and the inhibition of NfsA tartrazine azoreductase activity by exogenous FMN (Zenno et al., 1996a).

NfsA and NfsB share little sequence identity, about 16%, but do have similar 3D structures (Figure 4.9). However, there are five fundamental structural differences between these enzymes that logically extend to their functional disparities, especially their different abilities to discriminate between NADPH and NADH. First, the major pocket of NfsA is about three-quarters of the volume of NfsB's major pocket (Table 4.3), which may contribute to the NADPH dependence of NfsA. The structure of NAD⁺-bound Frp shows the NAD⁺ molecule bound in a unique folded conformation, with the nicotinamide and adenine rings stacked in parallel (Figure 4.10), that is usually associated with NAD in solution (Tanner et al., 1999). In order to enter the narrow pocket of

NfsA/Frp, the cofactor probably enters the pocket ‘backwards’ with the 2’ phosphate moiety stacked next to the flavin (Tanner et al., 1999). In NOX, VFR and NfsB a water molecule is bound at the same location as the phosphate in Frp. This folded conformation places the nicotinamide C4 atom 10 Å from FMN N1, and as the required distance for hydride transfer is less than 4 Å (Fraaije and Mettevi, 2000; Mittl et al., 1994; Tanner et al., 1999), the cofactor probably unfolds for catalysis. Recognition of the 2’ phosphate may promote unfolding of the cofactor into the extended active form, and thus discriminate NADPH from NADH (Tanner et al., 1999).

The second key difference between NfsA and NfsB is the extended length of NfsA. The structural alignment shown in Figures 4.1 and 4.9 displays the 61 residue C-terminus extension of NfsA that comprises the excursion domain, which includes some residues (Thr-219 to Arg-225) that are part of the FMN binding pocket as well as the ancillary pocket III (Table 4.3, Figure 4.9). In the NAD⁺ bound structure of Frp, three of the 13 NAD⁺ contacts are within this domain. In pocket III of NfsA, Arg-203 may contribute to the discrimination of NADPH, perhaps by recognition of the 2’-phosphate, as substitution with an Ala residue at this location results in a 33-fold increase in the *K_m* value for NADPH (Kobori et al., 2001). However, NADP⁺ is normally more flexible in binding to enzymes than NAD⁺ (Carugo and Argos, 1997a), and it could be that the NADPH dependency of NfsA/Frp is due to a lack of specific recognition factors for NADH, rather than a specific recognition factor for NADPH itself.

Third, both Arg-133 and Asn-134 are unique to NfsA/Frp as they are located within a 3_{10} helix that is not present in the NfsB-like enzymes (refer to Figures 4.1 &

4.9). In all of the NfsB-like structures a negatively charged Asp residue occupies the aligned Arg-133 position, followed by a small alignment gap. Asn-134 of Frp forms a hydrogen bond with the ribose near the adenine of NAD^+ , which, according to the LPC software, is a destabilizing contact. This contact could stabilize NADPH, which may help explain the specificity for this cofactor. In the NADP^+ -dependent 6-phosphogluconate dehydrogenase, an Asn residue adjacent to an Arg has been shown to interact with the diol group of the ribose near the adenine of NADP^+ , an interaction that has not been observed with NAD^+ , and thus this region may represent a NADP(H) discriminating factor (Carugo and Argos, 1997a).

The fourth major difference between these enzymes is the alignment gap introduced in NfsA that overlaps helices αE and αF in NfsB (refer to Figures 4.1 & 4.9). In particular, the flexible helix αF of the NfsB-like enzymes has been implicated in the accommodation of substrates (see Section 4.4.2.3), while residues within and between these helices are involved in FMN and NIO binding (Figure 4.1). The alignment gap in NfsA and Frp also suggests that this region could play a role in the ability to accommodate both cofactors in NfsB. Indeed, four of the 12 NIO contacts in NfsB (Phe-108, Ala-113, A116 and Phe-124) are within this region, and the absence of these contacts in NfsA may be key to the inability of this enzyme to utilize NADH. NOX has a ten-fold preference for NADH over NADPH as a cofactor (Hecht et al., 1995), and it may be that the smaller gap introduced within this region of NOX, spanning residues Phe-108 to Ala-116 of NfsB (see Figure 4.1), is a further indication that this region has a role in the accommodation of both cofactors.

The fifth distinguishing feature of NfsB compared to NfsA is the NfsB-specific PW(HFIV) region that includes residues 45 to 50 in NfsB. This stretch of conserved residues is obvious in the NfsB homologues, but is lacking in the NfsA-like proteins. The positively charged H/K/R residue in the NfsB-like enzymes is aligned with the polar Ser residue in NfsA, and it could be that this positive charge in NfsB acts to stabilize the negative diphosphate moiety of NADH. Such an interaction is often required for NADH binding, but has so far not been shown to be a requirement for NADPH binding.

It is interesting to note that, like the F124S mutation in NfsB, the substitution of Glu-99 with Gly alters the substrate specificity of NfsA such that it becomes an effective flavin reductase similar to its homologue Frp (Zenno et al., 1996a). The researchers that observed this mutant suggest that it is an increased pocket volume that leads to the altered substrate specificity; however, the major pocket only reveals a slight increase in volume, about $70 \text{ \AA}^3$, and none of the other pockets significantly increase in volume (Table 4.3). It may be that this slight volume increase is sufficient for the altered specificity, or that the pocket volume in the FMN reduced conformation of NfsA is substantially different, but it could also be that other factors are responsible for the altered substrate specificity. The more drastic increase in pocket volume observed for the NfsB F124S mutant suggests that the mode of altered substrate specificity is different for NfsA.

The small volume of the major pocket in NfsA and Frp suggests that either a dramatic change in conformation takes place after cofactor binding, or the adjacent pockets of the enzymes have the space and contacts to accommodate bulky substrates.

Pocket analysis of NfsA reveals an above average number of mouth openings (four) in the major pocket (Liang et al., 1998), which could be an indication that adjacent pockets contribute protein contacts for ligands. Unlike NfsB, a large increase in pocket volume is not observed upon binding of a cofactor analogue in Frp (NAD^+), or in the NfsA flavin reductase mutant, E99G, that must accommodate large flavin molecules in its active site. It is possible that, like pocket IV in NfsB, other pockets affect catalysis despite not being directly adjacent to the major pocket, but it is difficult to assess these effects without mutant analysis or binding experiments. The second largest pocket of NfsA is adjacent to the major pocket but has no mouth openings. Perhaps, similar to pocket II of NfsB, cofactor binding provokes a conformational change that results in this pocket being included in the active site. Two smaller pockets that are likely involved in the active site of NfsA are near the major pocket. Pocket IV contains some of the equivalent NAD^+ contacts in Frp, while pocket III is situated near the mouth opening and contains Arg-203, a residue that has been implicated in the use and/or binding of NADPH (Kobori et al., 2001). It is possible that most bound substrates are necessarily solvent exposed due to the small volume of the major pocket, which could help explain the broad substrate specificity of these enzymes, especially the ability to bind such large substrates.

4.4.4 The implications for structure-based drug development

4.4.4.1 NfsB

Pockets adjacent to the primary active site pocket present additional binding surface for ligands. Ligand contacts in ancillary pockets and cavities have been successfully predicted for some proteins using the CAST software, including elastase,

FK506 and HIV-1 protease (Liang et al., 1998). Furthermore, the recruitment of nearby pockets that do not have contact with substrate can provide additional protein contacts for designed ligands (Mattos et al., 1994; Mattos and Ringe, 1996; Shuker et al., 1996). The prospect of ligand design in NfsB is important for the utilization of modified cofactors (Knox et al., 1995) and novel prodrugs (Denny, 2002) in targeted cancer therapy approaches (see Section 1.3.1.2), as well as for antimicrobial drug development. Prediction of additional binding sites in NfsB could aid in the development of highly specific substrates that would be less vulnerable to the development of resistance or activation by host enzyme activity. The incorporation of pocket II with the main active site pocket in NIO-bound NfsB suggests the importance of this region for enzyme function, while pocket III is a good candidate for recruitment into a rational redesign approach for drug development.

4.4.4.2 NfsA

Whereas NfsB is being actively explored as a promising candidate in A/GDEPT cancer therapy approaches, NfsA is not, which may simply be a consequence of the earlier characterization of NfsB. However, there is some investigation into the use of NfsA in A/GDEPT strategies (Bennett and Lambert, manuscript in preparation) and genotoxicity assays (Carroll et al., 2002), and given its more efficient NR activity with some compounds of commercial and environmental importance, it is a promising candidate for such applications. Here, then, as in NfsB, the ability to predict potential binding sites, and design specific ligands is a valuable asset for drug development. With four mouth openings in the major pocket of the oxidized state of NfsA, and a number of

adjacent pockets of reasonable size, it appears that there are numerous candidate sites that could be exploited for drug development. For example, pocket II (Table 4.2), the second largest pocket in NfsA, is probably part of the active site, and is a promising candidate for potential ligand contacts. Smaller pockets that are adjacent to the main pocket (e.g. pockets III and IV) may also be involved in catalysis, and/or may be recruited for ligand design in NfsA.

CHAPTER FIVE: CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

Oxygen-insensitive nitroreductases are becoming recognized as a widespread family of enzymes. Over 140 putative enzymes have been identified that span the three main domains of life: Bacteria, Eukaryota and Archaea. The NfsB protein of *E. coli* is involved in the bioactivation of nitro substituted compounds, and its activity is being exploited for a number of applications including genotoxicity assays and cancer therapy. However, the physiological role of NfsB and other nitroreductases remains a mystery. The work reported in this thesis contributes to the rapidly accumulating knowledge of NfsB, and thus of the nitroreductase family, in three main areas: its role in the development of 5-nitrofur resistance; its structure-function relationships; and its possible physiological role.

The investigations reported in this thesis give rise to a number of additional questions and emphasize the importance of several issues that preexisted this research. Here, the unifying and pertinent conclusions that arose from these studies are described along with directly related questions that would represent the most interesting and logical continuation of this work.

5.1 Development of 5-nitrofur resistance in *E. coli*

The nitrofurazone reductase activity of a large number of nitrofur resistant mutants was determined and the *nfsB* gene was genetically characterized in order to test the hypothesis that the second step of *E. coli* resistance to furazolidone and nitrofurazone is due to the mutational inactivation of the *nfsB* gene. Koziarz (2000) determined that the first step of resistance correlates with the mutational inactivation of the *nfsA* gene in these mutants.

5.1.1 Conclusions

1. 5-Nitrofurantoin resistance is acquired through a sequential mechanism that involves the obligatory mutational inactivation of *nfsA* (first step mutant), followed by the inactivation of *nfsB* (second step mutant).
2. Mutant cross resistance is observed with furazolidone and nitrofurazone. Mutants that display an inconsistent third step of resistance to furazolidone exhibit second step levels of resistance to nitrofurazone. This phenomenon, previously demonstrated with nitrofurantoin (Obaseiki-Ebor, 1984), does not appear to be a factor of decreased permeability for furazolidone.
3. The *nfsB* gene contains integration hotspots for the IS elements IS1, IS5 and perhaps IS2.

5.1.2 Suggestions for further research

1. The third step of resistance that is associated with furazolidone and nitrofurantoin but not with nitrofurazone (Section 2.3.1) warrants further investigation. One possibility is that other nitroreductase activities are involved in the metabolism of these compounds. There is some biochemical evidence for a third oxygen-insensitive nitroreductase component in *E. coli* (Bryant et al., 1981), and double *nfsA/nfsB* mutants do retain a low level of nitroreductase activity (Chapter 2, Table 2.2). It could be that one or both of the two *E. coli* sequences identified by the Pfam database as putative nitroreductase genes represent this minor nitroreductase component (see Chapter 1, Section 1.2). The sequences of these genes in the third step mutants, as well as in the second step mutants for which no

mutation in *nfsB* was found (Section 2.3.2; Table 2.2), could be compared with the parental strains. Any observed variation between the mutant and parental strains would make the cloning of these new genes, and characterization of their products, even more compelling.

2. The unanticipated number of IS element integrations in *nfsB* opens an appealing avenue of research. Interestingly, a large number of IS elements were also observed in the *nfsA* gene of first step mutants (Koziarz, 2000). The putative IS element hotspots in *nfsB* (and *nfsA*) may implicate a horizontal gene transfer mechanism by which the nitroreductase family could have evolved to what now appears to be a widespread family of enzymes. The notion that nitroreductase genes were horizontally transferred by means of transposable elements (TEs) is plausible, especially given the putative role of nitroreductases in the bacterial response to environmental stress (Section 5.3). In addition, TEs are often activated under conditions of environmental stress (Chao et al., 1983; Kidwell and Lisch, 1997; McClintock, 1984), including exposure to DNA damaging agents (Eichenbaum and Livneh, 1998). Thus, two related questions arise from this phenomenon that would be interesting to investigate:
 - i. Do 5-nitrofurans, which are DNA damaging agents, stimulate TE activity in *E. coli*?
 - ii. Does the *nfsB* gene contain hotspots for IS element integration, or are the large number of IS element integrations observed in this study simply a consequence of furazolidone stimulated TE activity?

To test the hypothesis that 5-nitrofurans stimulate TE activity it would be necessary to compare IS element activity of untreated and nitrofurantreated cells, preferably within the context of the whole genome. A comparison of whole genome IS element activity may perhaps be achieved by the technique of bacterial comparative genomic hybridization (BCGH) (Malloff et al., 2001). Essentially, this technique compares the genome of two related bacterial strains by digesting and combining their genomic DNA, resolving the DNA on a 2D gel, transferring it to a membrane, and probing with a total genomic DNA probe from each strain. For the purposes of this study, the genomic variance associated with particular IS elements could be identified by probing with specific IS element probes. Thus, genomic variance as a consequence of nitrofurantreatment can be compared between strains, particularly that which is attributable to the activity of specific IS elements.

The hypothesis that *nfsB* contains hotspots for IS elements can be tested by introducing *nfsB* on a plasmid, as a TE trap, into a $\Delta nfsA\Delta nfsB$ host. Selection of nitrofurant resistant mutants, followed by Southern blot analysis of the extracted plasmid DNA would facilitate determination of mutant frequencies and analysis of the number and type of IS elements.

5.2 Structure and function of NfsB

In this study, the structure and function of NfsB was assessed in two ways: first, by mutagenesis and kinetic analysis; and second, by computer analysis of the 3D crystal structure of NfsB and its homologues. Ideally, these studies would have been approached

in the reverse order, but the mutagenesis study was completed prior to the availability of the 3D structure. In addition to these two studies, information on the structure and function of NfsB was obtained through the investigation of 5-nitrofurantoin resistance development which identified residues whose mutation eliminated nitroreductase activity.

5.2.1 Conclusions

1. A number of features of NfsB that had been determined, or suggested, in other studies were confirmed here:
 - i. The *nfsB* gene has a promoter within 148 bp of the initiation codon (Chapter 2).
 - ii. The NfsB protein is about 24 kD and appears as approximately 27 kD on a denaturing SDS-PAGE gel (Chapter 3); it can use both NADH and NADPH as a source of reducing equivalents, with a slight preference for NADH (Chapter 3); and reduces nitrofurantoin by means of a mechanism that is consistent with a ping-pong Bi Bi reaction type (Chapter 3).
2. Unique to this study is the identification of numerous residues that are essential for nitroreductase activity. These are revealed by the single missense mutations that confer a nitrofurantoin resistant phenotype (Chapter 2), and by those introduced through mutagenesis that resulted in null or altered nitroreductase activity (Chapter 3). Many of these residues are involved in FMN binding, but several others appear to be associated with NAD(P)H and/or substrate binding.
 - i. The region from Pro-45 to Val-50 is probably involved in substrate specificity and is also a key characteristic of the NfsB-like proteins that

distinguishes them from the NfsA-like proteins (Chapter 4). The *H. pylori* RdxA protein, which is more similar to NfsB than NfsA in terms of sequence and activity, contains this conserved region, but uses NADPH exclusively, so it is unlikely that this region specifies NADH utilization.

- ii. Gly-158 appears to be significant for the utilization of NADPH, but not NADH (Chapter 3).
 - iii. Phe-70 does not affect NAD(P)H utilization, but does have an effect on the catalytic efficiency of NfsB, most likely by influencing the transition energy of catalysis (Chapter 3). Positioned between the two main pockets of NfsB, Phe-70 may be key for shifting conformation to accommodate the binding of bulky substrates (Chapter 4).
 - iv. The region from approximately Phe-108 to Asp-135 is largely comprised of a long, flexible α -helix (α F) and is not well conserved. This region is probably a major contributor to the ability of NfsB and its homologues to accommodate the wide range of bulky substrates characteristic of these proteins (Chapter 4).
3. NfsB has seven key surface accessible pockets that can reasonably be expected to be involved in catalysis for reasons of size, proximity to FMN, and/or inclusion of critical residues (i.e. conserved or indicated by mutation analysis) (Chapter 4). There are three similar pockets in each monomer, for a total of six pockets per dimer, and one pocket that is symmetrically comprised of residues from each

monomer. These seven pockets represent important structural elements to be considered in the design of drugs and ligands for NfsB.

4. There are five main features that distinguish the NfsB-like proteins from the NfsA-like members of the nitroreductase family (Chapter 4, Section 4.4.4):
 - i. The main active site pocket of NfsB is about one third larger than NfsA.
 - ii. NfsA is larger than NfsB by 23 amino acids and contains an extended C-terminus domain of about 60 residues.
 - iii. NfsA contains a 3_{10} helix that is not present in NfsB.
 - iv. NfsA does not have the flexible α helix (α_F) mentioned in 5.2.1, 2(iv) above that is implicated in the substrate binding of NfsB.
 - v. As mentioned in 5.2.1, 2(i) above, the NfsA-like proteins lack the highly conserved Pro-45 to Val-50 region of the NfsB-like proteins.

5.2.2 Suggestions for further research

1. The kinetic analysis of this investigation confirms that NfsB reduces nitrofurazone according to a ping-pong reaction mechanism. However, it is not clear that this is a Bi Bi mechanism, as the data is also consistent with a Hexa Uni reaction mechanism. To determine the specific reaction type of NfsB, isotope exchange experiments and kinetic analysis of the reverse reactions of NfsB could be performed. Identification of the actual reaction mechanism of NfsB would be facilitated by the determination of the reaction intermediates of the reaction, particularly whether the first released intermediate is reduced by 2 or 4 electrons.

2. The mutations analyzed in this study suggest that the mutated residues influence nitroreductase activity. However, it is possible that at least some of the mutations were simply too drastic and that substitution with similar residues may yield information regarding the influence of the residue(s) on catalysis. For example, some substitutions of the amino acids that were mutated in Chapters 2 and 3 could be made with more evolutionarily accepted residues (for example with those listed in Table 3.8).
3. Some combinations of mutations could be informative. For example, Gly-158→Ser combined with Phe-70→Ser produces an enzyme with high activity along with increased specificity for NADH.

5.3 The Physiological Role of *nfsB*

Mutants lacking a functional NfsB enzyme are easily cultured under normal conditions, which indicates that this enzyme is not an essential constituent of *E. coli*, at least under laboratory conditions. The only known activity of NfsB is the reductive metabolism of nitro containing compounds. These features are both consistent with the view that NfsB has a role in the defense of the cell against nitro substituted xenobiotic compounds.

The high frequency of IS element insertions observed in nitrofurant resistant mutants also concurs with this notion, as TEs are often associated with (groups of) genes that are involved in environmental stress responses. For example, the rapid transmission of a variety of genes associated with the catabolism of aromatic environmental pollutants is often facilitated by TEs (for reviews see Tan, 1999; Wyndham et al., 1994). A few

more examples of environmental response genes that are likely transmitted by transposons include mercury resistance genes (Osborn et al., 1997), catalases (Klotz et al., 1997) and the histidine kinases of Archaea (Kim and Forst, 2001).

Recent studies have indicated that NfsA is part of the SoxRS response to oxidative stress (Liochev et al., 1999; Paterson et al., 2002), and that both NfsA and NfsB are members of the associated MarRAB regulon (Barbosa and Levy, 2000). To test for the participation of NfsB in these regulons, induction with paraquat (SoxR specific) and salicyclate (MarA specific) was demonstrated (Chapter 2), suggesting that NfsB is part of the SoxRS/MarRAB regulated global response to environmental stress.

5.3.1 Suggestions for further research

1. The regulatory regions of *nfsB* should be characterized. While no marbox consensus sequence has been identified to date, DNA footprinting and gel shift assays using the regulatory elements of the operons (i.e. SoxR and MarA) would reveal binding site(s) for these proteins.
2. The concept of NfsB as a member of a global response to environmental stress can be expanded and tests for other environmental factors such as pollutants, UV light and starvation could help to reveal the role of NfsB in the defense mechanisms of *E. coli*.
3. Identification of the cell cycle expression profile of NfsB may also yield clues as to the physiological role of the enzyme. For instance, in *Caulobacter crescentus* enzymes involved in the oxidative response are mainly expressed in G1, which is suggested to be a way that the organism protects against DNA damage during

replication (Grünenfelder et al., 2001). This would be most easily facilitated by microarray analysis for gene expression such that a global representation of the cell's entire gene expression profile would be observed.

APPENDIX I:

The one and three letter codes of the 20 amino acids found in proteins.

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown/unspecified	Xaa	X

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