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**MOLECULAR ADAPTATION TO ANOXIA AND RECOVERY FROM ANOXIA
IN THE FRESHWATER TURTLE TRACHEMYS SCRIPTA ELEGANS**

by

WILLIAM GLEN WILLMORE

B.Sc University of Guelph, 1992

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

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario, Canada

August 1997

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"MOLECULAR ADAPTATION TO ANOXIA AND RECOVERY FROM ANOXIA IN
THE FRESHWATER TURTLE TRACHEMYS SCRIPTA ELEGANS"

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ABSTRACT

The responses to stresses associated with oxygen extremes, namely anoxia and oxidative stress associated with recovery from anoxia, were studied in an organism that must tolerate both extremes. The model organism used was the turtle Trachemys scripta elegans that can survive complete anoxia for up to 18 weeks at lower temperatures. Metabolic adaptations that support survival of oxygen extremes in this organism that were examined included: a) tissue-specific changes in the maximal activities of enzymes of intermediary metabolism, b) an assessment of the metabolite and enzymatic antioxidant defenses utilized to combat the oxidative stress encountered upon recovery from anoxia, c) adaptations in specific enzymatic antioxidant defenses and d) adaptations to anoxia and recovery from anoxia of a specific protease. Of the key enzymes of intermediary metabolism, 14 instances of reduced and only 3 instances of increased enzyme activities were observed in tissues during anoxia as compared to controls. Brain had changes related to both metabolic rate depression and neurotransmitter release. Maximal activities which increased during anoxia included G6PDH in heart, MDH in white muscle and CPT in kidney. Anoxia stress and aerobic recovery produced relatively few changes in organ antioxidant enzyme activities and levels of lipid peroxidation products. Changes included tissue specific decreases in antioxidant enzymatic activities during anoxia, particularly SOD and CAT. The levels of enzymatic antioxidant activities were high in turtles in comparison with other vertebrates and non-vertebrates. Turtles maintained high levels of total glutathione in tissues in comparison to other ectotherms. Tissue-specific changes in the maximal activities of the glutathione-related enzymes occurred, the most dramatic being a decrease in γ -GTPase during anoxia to 2 % of

control values. Turtle liver contained one homodimeric Alpha class and a unique heterodimeric Alpha-like GST. Turtle liver GR revealed a high affinity for GSSG. No new isoforms of either GST or GR were formed during anoxia. MPC displayed an increase in peptidylglutamyl-peptide bond hydrolyzing activity during recovery from anoxia, possibly as a result of oxidative damage to proteins in this tissue. Overall, T. s. elegans maintains continually high defenses against the stresses of anoxia and oxidative stress upon recovery from anoxia.

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LIST OF ABBREVIATIONS

- ADP – adenosine diphosphate
- AHR – alkyl hydroperoxide reductase
- AK – adenylate kinase
- ALD – aldolase
- AMP – adenosine monophosphate
- ANOVA – analysis of variance
- ATP – adenosine triphosphate
- BHT – butylated hydroxytoluene
- β -MeSH – β -mercaptoethanol
- β DH – β -hydroxybutarate dehydrogenase
- CAT – catalase
- CDNB – 1-chloro-2,4-dinitrobenzene
- CK – creatine kinase
- CL – citrate lyase
- CM – carboxymethyl
- COT – carnitine octanoyltransferase
- CPT – carnitine palmitoyltransferase
- CS – citrate synthase
- DEAE – diethylaminoethyl
- DMSO - dimethylsulfoxide
- DTNB – 5,5'-dithiobis-2-nitrobenzoic acid

E_a – energy of activation

EDTA – ethylenediamine tetraacetate

EGTA – ethyleneglycol bis (β -aminoethyl ether) tetraacetate

FBP – fructose-1,6-bisphosphate

FBPase – fructose-1,6-bisphosphatase

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GK – glucokinase

GOT – glutamate-oxaloacetate transaminase

G3PDH – glycerol-3-phosphate dehydrogenase

G6PDH – glucose-6-phosphate dehydrogenase

GPOX – glutathione peroxidase

GPT – glutamate-pyruvate transaminase

GR – glutathione reductase

GRN – glutaredoxin

GS – glutathione synthetase

GSH – reduced glutathione

GSH_{eq} – total glutathione equivalents

GSSG – oxidized glutathione

GST – glutathione S-transferase

γ -GTPase – gammaglutamyl transpeptidase

HED – hydroxyethyldisulfide

HK – hexokinase

HOAD – 3-hydroxyacyl-CoA dehydrogenase

HPLC – high performance liquid chromatography

I_{50} – concentration of inhibitor that reduces $V_{\max}$ activity by one half

I.E.F. – isoelectric focusing

KADH – branched chain ketoacid dehydrogenase

KGDH – α -ketoglutarate dehydrogenase

K_m – Michaelis-Menten constant

KPi – potassium phosphate

LDH – lactate dehydrogenase

MDA – malondialdehyde

MPA – Microplate Analysis

MPC – multicatalytic proteinase complex

mRNA – message ribonucleic acid

MW – molecular weight

NAD^+ – nicotinamide adenine dinucleotide, oxidized form

NADH – nicotinamide adenine dinucleotide, reduced form

NADMDH – malate dehydrogenase (NAD dependent)

NADPMDH – malate dehydrogenase (NADP dependent)

$NADP^+$ - nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

IDH – isocitrate dehydrogenase

O_2^- – superoxide anion radical

$OH^\cdot$ – hydroxyl radical

PEG – polyethylene glycol

PEP – phosphoenolpyruvate

PEPCK – phosphoenolpyruvate carboxykinase

PFK – phosphofructo-1-kinase

pI – isoelectric point

6PGDH – 6-phosphogluconate dehydrogenase

PK – pyruvate kinase

PMSF – phenylmethylsulfonyl fluoride

R_f – relative mobility

ROS – reactive oxygen species

SDH – serine dehydratase

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNK – Student-Newman-Keuls

SOD – superoxide dismutase

Suc-LLVY-AMC – N-succinyl-LEU-LEU-VAL-TYR 7-amido-4-methylcoumarin

TBA – thiobarbituric acid

TBARS – thiobarbituric acid reactive substances

Tris – Tris[hydroxymethyl]aminomethane

U – international unit of enzyme activity

$V_{\max}$ – maximal enzyme velocity

XDH – xanthine dehydrogenase

XO – xanthine oxidase

Z-ARR-MNA – N-CBZ-ALA-ARG-ARG 4-methoxy β -naphthylamide

Z-LLE-MNA – N-CBZ-LEU-LEU-GLU β -naphthylamide

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CHAPTER ONE

GENERAL INTRODUCTION

Hypotheses

The red eared slider (Trachemys scripta elegans), one of the champions of vertebrate facultative anaerobes, provides a useful model with which to study vertebrate survival of stresses associated with oxygen extremes; namely anoxic stress (oxygen lack) and oxidative stress (the production of reactive oxygen species when aerial breathing resumes after anoxia). Anoxic survival involves metabolic depression in order to match energy demand to decreased energy production. The maximal activities of enzymes provide an indication of which pathways are adjusted during metabolic depression. Adjustments to the $V_{\max}$ activities of enzymes of various metabolic pathways were investigated to determine which pathways are most affected by anoxia in this anoxia tolerant vertebrate. As well, anoxia/reoxygenation of tissues provides a second stress commonly seen in vertebrate ischemia/reperfusion injury; oxidative stress from reactive oxygen species. The maximal activities of antioxidant enzymes reveals the type of strategy which anoxia tolerant vertebrates use to deal with this second stress. Examination of some of the glutathione related enzymes in their pure state will determine if production of new isoforms of these enzymes during anoxia is part of the strategy in dealing with oxidative stress. Finally, the activity of a protease which targets oxidatively damaged proteins will be examined to determine if removal of damaged proteins occurs during recovery from anoxia.

The following hypotheses were tested:

- A. That metabolic adaptations supporting turtle survival of anoxia include tissue-specific changes in the maximal activities of enzymes of intermediary metabolism.
- B. That the reintroduction of oxygen after anoxia stress is associated with the potential for oxidative stress (similar to that seen during reperfusion after ischemia in mammalian systems).
- C. That the potential for oxidative stress after anoxia is minimized or counteracted by metabolic adaptations of both metabolite and enzymatic antioxidant defenses.

D. That adaptation of enzymatic antioxidant defenses will include anoxia- and/or recovery-induced changes in one or more of the following: maximal activities, isozymic forms, or kinetic properties of enzymes.

E. That oxidatively damaged proteins are dealt with by adaptive changes in the activity or kinetics of a specific protease.

Adaptation

Stress can be defined as "a change in homeostasis that must be reversed by biochemical mechanisms". The maintenance of homeostasis within specific environments is known as adaptation. Adaptation involves the modification of the characteristics of organisms that facilitates an enhanced ability to survive and reproduce in a particular environment (Hochachka and Somero 1984). The key functions of biochemical adaptation are to a) preserve the structural integrity and function of macromolecules and macromolecular ensemblages, b) provide adequate supplies of energy currency (ATP), reducing power (NADH, NADPH), and metabolic intermediates for the synthesis of storage compounds, nucleic acids and proteins and c) the maintenance of mechanisms for regulation of metabolic rates and directions of metabolic flow in response to a changing environment (Hochachka and Somero 1984). Adaptations can be a) genetic which occur over many generations of organisms, b) acclimations or acclimatizations which occur during the lifetime of the organism, and c) immediate which occur too rapidly to involve the restructuring of existing cellular systems. Immediate adaptations generally involve the modulation of the activities of preexisting enzymes. Adaptation can therefore be defined as a return to homeostasis during a change in environment and may involve short or long term changes in cellular function.

Turtle Anoxia and Freeze Tolerance

For many animals, the best defense against harsh environmental conditions is a retreat to a hypometabolic or dormant state that allows the animal to survive until conditions favourable to the resumption of normal activities return. For example, seasonal dormancy occurs amongst both endotherms and ectotherms in northern latitudes and may permit energy savings of up to 88 percent (in the case of mammals) compared to maintaining euthermia over the winter months (Storey and Storey 1990). Metabolic depression is also key to survival when oxygen is limiting and occurs widely among species that experience cyclic or periodic bouts of oxygen deprivation in their environment. Gill-breathing intertidal invertebrates, for example, suppress anoxia metabolic rate to only 5-10 % of the corresponding aerobic rate to deal with the cyclic oxygen deprivation that occurs with the rising and falling of the tides (Storey 1993). Various species of freshwater turtles also endure anoxia during breathhold diving or, more impressively, during prolonged submergences of many months while hibernating underwater at the bottom of ponds, lakes and rivers (Ultsch 1989). Turtles rely upon anaerobic glycolysis to generate ATP in the absence of molecular oxygen. Facultative anaerobiosis in turtles is supported by adaptive strategies including: (i) large tissue stores of fermentable fuel, (ii) a high tolerance of metabolic acidosis and compensatory ion changes that buffer and retard the development of acidosis, and (iii) metabolic rate depression to a level only 10-20% of the aerobic resting rate that permits homeostasis to be maintained by glycolytic ATP production alone (Clark and Miller 1973, Herbert and Jackson 1985, Jackson 1968, Penney 1974, Robin et al. 1964, Storey 1996).

The best facultative anaerobes among freshwater turtles include members of the genera Trachemys (pond slider turtles) and Chrysemys (painted turtles). These species are widespread over most of the United States and southern Canada. Species of Chrysemys range farther north than any other land or freshwater turtle in North America. Sliders and painted turtles are able to survive without oxygen for up to two weeks at

16°C to 18°C and for 12 to 18 weeks at 3°C (Ultsch and Jackson 1982, Ultsch 1985, Herbert and Jackson 1985, Gatten 1987). Mammalian tissues are notoriously sensitive to even brief episodes of anoxia whereas turtle tissues rapidly decrease their metabolic rates (to 10 to 20% of their normoxic resting rates) (Jackson 1968, Herbert and Jackson 1985) and can then survive for prolonged periods of time in this new, metabolically depressed state (Wasser et al. 1992). In turtle tissues, a transient disruption of ATP and energy charge during the hypoxic transition period but this is reversed and stabilized during long term submergence (Lutz et al. 1984, Kelly and Storey 1988). ATP turnover drops, as it is a measure of metabolic rate, but total adenylates and energy charge are quite stable in the suppressed state; this is one of the hallmarks of a successful metabolic rate depression (Lutz et al. 1984, Kelly and Storey 1988). Turtles are therefore superiorly adapted biochemically for anoxia tolerance.

The anoxia tolerance of turtles may also be an important component of winter freezing survival for hatchling turtles which spend their first winter in shallow terrestrial nests. Turtles spend weeks with over 50% of their body water frozen and without breathing or circulation of blood (Storey et al. 1988, Churchill and Storey 1992). Organs are cut off from oxygen rich haemoglobin as ischemia occurs. Many of the same metabolic adaptations that support anoxia tolerance may ensure survival of the ischemic state caused by the freezing of blood plasma.

Adjustments to Metabolic Pathways During Anoxia and Recovery From Anoxia

One of the ways to estimate flux through a metabolic pathway is to analyze the maximal rates of pathway enzymes in vitro. Enzyme maximal activities typically correlate positively with potential pathway flux both between tissues and between species and maximal pathway flux can be estimated from the maximal activity of the rate-determining (lowest activity) enzyme. Conversely, the rate-determining enzyme of a pathway can often be identified as that enzyme with the lowest maximal activity.

Changes in the maximal activities of rate-limiting enzymes arising from a stimulus are indicative of an adaptive response to the stimulus. In general, regulatory enzymes are low in activity and are therefore controlled by the total amount of enzyme present; either by a change in the rate of synthesis or degradation of the enzyme (Newsholme and Crabtree 1986). A small change in the amount of a key regulatory enzyme in a pathway can induce large changes in flux through that pathway. Synthesis of such enzymes during stress, especially one that initiates metabolic depression, is energetically costly and when such synthesis occurs, suggests the importance of the enzyme to organismal survival of the stress.

The second chapter of the thesis investigates the maximal activities of 32 enzymes from 6 different tissues of the turtle T. s. elegans under control, anoxic and anoxic recovered conditions. The enzymes selected compose the major enzymes of 5 different metabolic pathways. Changes in the maximal activities of these enzymes during anoxia indicate changes in carbon flux through these pathways. Increases in the $V_{\max}$ of an enzyme during stress may indicate that it is of importance to the survival of the stress by the organism.

Oxidative Stress Upon Recovery From Anoxia

Upon recovery from anoxia or freezing, glycolytic ATP production is replaced by ATP production from oxidative phosphorylation. Indeed, organs experience a relative hyperoxia during the early stages of recovery as metabolism recovers to rates above that of resting. The reintroduction of oxygen, however, brings about an increased production of oxygen reactive species. The occurrence of oxygen free radicals, resulting from cellular metabolism and taken in with atmospheric oxygen, is enhanced. Oxygen free radicals contain one unpaired electron in their outer orbital. Oxygen radicals can react with other molecules by (i) donating their unpaired electron (ii) removing an electron from another molecule or (iii) simply joining onto the molecule. The nonradical

molecule then becomes a radical and initiates a chain of radical-nonradical reactions. Damage to crucial macromolecules may be far removed from the initial radical reaction. For example, free radical production from mitochondrial processes may lead to peroxidation of polyunsaturated fatty acids in other organelles and plasma membranes. Free radical exposure may also result in the oxidation of sulfhydryl-containing enzymes, inactivating these. Damage may also occur to carbohydrates (polysaccharide depolymerization) and nucleic acids (single and double strand scissions). An increase in oxygen consumption by tissues increases free radical exposure. Free radical damage during perfusion with reoxygenated blood is frequently termed post-anoxic 'reperfusion injury' and is commonly seen during organ transplant and the destruction of coronary artery occlusions (Southorn and Powis 1992). Prevention of ischemic reperfusion injury is currently attracting much research and, if clinically applicable, will mitigate the effects of free radical damage in heart and stroke patients.

Production of reactive oxygen species during aerobic recovery may occur by various mechanisms. Proteolytic cleavage of the enzyme xanthine dehydrogenase during anoxia produces xanthine oxidase which acts upon one of its substrates (xanthine or hypoxanthine) to generate O_2^- and H_2O_2 in reperfused tissue (Gutteridge and Halliwell 1990). In mammals, the concentrations of xanthine increase during anoxia due to adenylate breakdown (Gutteridge and Halliwell 1990). During mammalian ischemia, the mitochondrial electron transport chain becomes highly reduced and transferal of electrons from the disrupted ETC to molecular oxygen occurs as soon as oxygen becomes available during reperfusion. Accumulation and activation of neutrophils in reperfused tissues also produces oxygen radicals. Sources of reactive oxygen species may also be associated with prostaglandin synthesis, oxidation of catecholamines, platelet activation and increases in oxygen radical production by the vascular endothelium (Marklund 1988, Halliwell and Gutteridge 1989).

Cytotoxic oxygen species include hydrogen peroxide (H_2O_2), the superoxide anion radical (O_2^-), the hydroxyl radical ($\text{OH}\cdot$) and the peroxide radical ($\text{ROO}\cdot$). Of these oxygen radicals, $\text{OH}\cdot$ is the most highly reactive and the least specific in the type of molecules it attacks. $\text{OH}\cdot$ may be produced from O_2^- through the Fenton reaction (with the aid of an iron catalyst):

or from H_2O_2 by the Haber-Weiss reaction:

Methods of eliminating H_2O_2 would prevent the formation of $\text{OH}\cdot$ in vivo and protect against its damaging effects.

All animals have enzymatic mechanisms to protect against reactive oxygen species (Figure 1). Superoxide dismutase enzymes (SOD) catalyze the dismutation of the O_2^- , proceeding at a rate approximately 10^4 faster than spontaneous dismutation at physiological pH (McCord and Fridovich 1969, 1977):

Two enzymes exist to catalyze the breakdown of hydrogen peroxide. At low concentrations, H_2O_2 is removed by the enzyme glutathione peroxidase (GPOX). In the process, reduced glutathione (GSH) becomes oxidized:

At high concentrations, H_2O_2 is removed by catalase (CAT):

Secondary antioxidant defenses include the enzymes glutathione S-transferase (GST) (catalyzes conjugation reactions with GSH) and glutathione reductase (GR) (reduces GSSG to replenish GSH). Antioxidant enzymes operate in concert to remove damaging radicals.

Anoxia tolerant animals may utilize one of three strategies to defend against oxidative stress occurring as the result of oxygen free radicals production during recovery. Organisms may make anticipatory changes in their antioxidant defenses during the anoxic period that allow them to deal with a burst of oxygen free radical generation during recovery. This strategy has been seen in anoxia tolerant garter snakes (Hermes-Lima and Storey 1993), estivating land snails (Hermes-Lima and Storey 1995a) and anoxia tolerant carp (Vig and Nemcsok 1989). A similar situation occurs during estivation (a dormant state of very low oxygen consumption) in desert toads (Scaphiopus couchii) with respect to liver and muscle SOD (Grundy 1996). Another possible strategy is to maintain high levels of antioxidant enzymes continuously, preventing oxidative damage from occurring during successive bouts of anoxia and recovery. In the freeze tolerant frog, Rana sylvatica (the freezing of blood plasma imposes ischemia on cells), the activities of many antioxidant enzymes were orders of magnitude higher than values in a freeze intolerant species (R. pipiens) (Joanisse and Storey 1996a). Similar results were found for the majority of the antioxidant enzymes in most tissues when comparing estivating and non-estivating desert toads (Grundy 1996). A third possible strategy is to allow (limited) damage to occur but to augment mechanisms for removal and replacement of damaged products. Thus, increased activities of proteolytic enzymes during recovery could remove damaged proteins whereas increased activity of membrane bound glutathione peroxidase would degrade the products of lipid peroxidation. Nuclease activity, responsible for DNA repair, may also be enhanced during recovery.

The third and fourth chapters of this thesis assess the strategy used by T. s. elegans in order to combat the potential for oxidative stress during recovery from anoxia. The effects of anoxia exposure and aerobic recovery on the maximal activities of enzymes involved in hydroperoxyl metabolism (Chapter 3) as well as those involved in glutathione metabolism (Chapter 4) were investigated. The products of lipid peroxidation were measured as well as the status of glutathione systems in six tissues. The strategy T.

s. elegans utilizes appears to be one of continual maintenance of protection against oxidative stress as opposed to stress-induced protection seen in other anoxia- or freeze-tolerant organisms.

Antioxidant Enzymes

The glutathione S-transferases are an important group of multifunctional enzymes involved in a variety of cellular processes, one of which is the detoxification of alkyl hydroperoxides. These enzymes conjugate the breakdown products of oxidative damage to reduced glutathione (GSH; gamma-glutamyl-cysteine glycine). GSTs may be grouped into three classes on the basis of their substrate and inhibitor properties. Kinetic characterization of all isozymes present in a tissue will allow classification of the enzymes into one of these three groups. New isozymes which are formed during stress can also be classified in this manner according to their kinetics.

Glutathione reductase is the enzyme responsible for glutathione status in the cell. The reduced glutathione provided by the enzyme is used to maintain the overall redox state of the cell, can directly scavenge prooxidants, and is used as a substrate by the enzymes GPOX and GST. The enzyme is tied to the pentose phosphate pathway in its requirement for NADPH and is important in maintaining the overall GSH/GSSG ratio. Indeed, the GSH/GSSG ratio is frequently used as an indicator of oxidative stress in tissues. The high ratio that occurs in cells is maintained by GR due to its high affinity for GSSG.

A high proportion of the total H_2O_2 generation in the body occurs in the liver (Barga de Quiroga et al. 1993). Since many of the responses of antioxidant enzymes to anoxia occur in this tissue, it provides an excellent source for purification of such enzymes for further study. To determine whether anoxia induced alterations in the properties of specific antioxidant enzymes, selected glutathione-related enzymes were purified from turtle liver and characterized. By analyzing the properties of the pure

enzyme, it could be determined if changes in activity during stress was due to a change in isozyme form of the enzyme which has either increased or decreased activity. Chapter 5 investigates the properties of turtle liver GSTs and compares the kinetic characteristics of these enzymes to those of other stress-tolerant and -intolerant organisms. Chapter 6 deals with the purification and characterization of turtle liver GR and determines whether the enzyme shows any unique properties when compared to its counterparts from other sources.

Protease Function During Anoxia and Recovery From Anoxia

Organisms which maintain an energy restricted, anoxic hypometabolic state must also suppress the rate of protein turnover. Protein metabolism is a major energy expenditure for cells and must be closely regulated according to energy availability during the hypometabolic state. Suppression of protein turnover could include both decreased synthesis and decreased proteolytic degradation.

A major cytosolic protease is the multicatalytic proteinase complex. This 24 subunit protease has at least three different proteolytic activities associated with it. One of these is regulated by reversible phosphorylation via a cAMP-dependent protein kinase that copurifies with the complex (Pereira and Wilk 1990). The protease is also partially responsible for the rapid degradation of proteins subjected to oxygen free radical damage (Rivett 1985, Pacifici and Davies 1988). The regulation of this enzyme could be key to both the depression of protein turnover in the anoxic state and the repair of oxidatively damaged proteins during recovery from the anoxia. Chapter 7 of the thesis deals with the partial purification of the MPC from turtle liver and analysis of its catalytic activities in the control, anoxic and anoxic recovered states.

Summary of Aims and Objectives

The first aim of this thesis is to assess changes in metabolic flux through various pathways of intermediary metabolism in T. s. elegans during anoxia and recovery from anoxia by examining changes to the $V_{\max}$ activities of key enzymes. Enzymes which increase in activity during the anoxic state are of potentially key importance to the survival of the turtles. Specific decreases in enzyme activities could reduce flux through pathways that need to be shut down during anoxia.

The second aim of this thesis is to determine the strategy which T. s. elegans utilizes to survive the second major stress which they encounter upon recovery from the anoxic state; namely oxidative stress. Via measurements of the $V_{\max}$ activities of primary and secondary antioxidant enzymes as well as elucidation of glutathione status and analysis of lipid peroxidation, the mechanisms which turtles use to survive this potential stress can be assessed. Purification and characterization of some of the key antioxidant enzymes which reveal changes during stress will further divulge the mechanisms which turtles use to control the activities of antioxidant enzymes.

The third aim of this study is to determine whether the activities of a major cytosolic protease are important in either decreased protein turnover during anoxia or increased proteolytic degradation of oxidatively damaged proteins during recovery from anoxia. Catalytic activities of the partially purified enzyme will give insight into whether this enzyme is activated or inhibited during anoxia or recovery from anoxia in T. s. elegans. Data collected on the activities of these enzymes will yield a clearer overall picture of how T. s. elegans survives the two stresses associated with the hypometabolic state; namely anoxia and oxidative stress.

Fig. 1. Pathways of production and reduction of reactive oxygen species (ROS).

Iron produces the hydroxyl radical via two pathways; the Haber-Weiss reaction and the Fenton reaction. Reactive oxygen species include: O_2^- superoxide anion radical; $OH^\cdot$ hydroxyl radical; H_2O_2 hydrogen peroxide.

CHAPTER TWO

CHANGES IN METABOLIC FLUX POTENTIAL DURING ANOXIA AND RECOVERY IN THE ANOXIA TOLERANT TURTLE TRACHEMYS SCRIPTA ELEGANS

INTRODUCTION

Mammalian tissues are notoriously sensitive to anoxia and acidosis. Freshwater turtles, however, are remarkably tolerant of these stresses both in vitro and in vivo. The red-eared slider (T. s. elegans) is one of the best facultative anaerobes among vertebrates (Ultsch 1985, Storey and Storey 1990). Turtles submerged in oxygen-depleted water can endure several days of anoxia at elevated temperatures and as long as 3 months during winter hibernation at low temperatures (0-5°C) (Jackson and Heisler 1982, Ultsch and Jackson 1982, Herbert and Jackson 1985). A decrease in ATP production in anoxia is matched by decreased ATP utilization (energy charge is maintained) as the result of a profound metabolic rate depression. Molecular adjustments to metabolic rate can be accomplished in a number of ways including via posttranslational modification of key regulatory enzymes, reversible aggregation of enzymes or binding to subcellular structures, and changes in the concentrations of selected enzymes as the result of altered gene expression or altered rates of enzyme biosynthesis/degradation. The role of reversible protein phosphorylation in coordinating the activities of key enzymes and functional proteins during metabolic depression is well known for turtles (Brooks and Storey 1989, Brooks and Storey 1991, Brooks and Storey 1993a, Mehrani and Storey 1995a, Mehrani and Storey 1995b, Mehrani and Storey 1996) and anoxia-induced changes in enzyme binding to the subcellular particulate fraction are also a part of the metabolic response to submergence anoxia in turtles (Duncan and Storey 1992). Recently, changes in protein synthesis, mRNA populations, and gene expression have also been identified as some of the metabolic adjustment for survival during oxygen deprivation (Brooks and Storey 1993b, Douglas et al. 1994, Cai and Storey 1996). Another approach to identify metabolic adaptations that support anoxia survival is to assess changes in the maximal activities of enzymes in response to the stress. By analysis of enzymes in multiple pathways of intermediary metabolism, an overall picture can be

developed of the pathways and types of cellular functions that are enhanced or suppressed in order to provide a coordinated metabolic response to low oxygen.

In vitro determination of enzyme maximal activities has long been employed to provide insight into metabolic flux potential in vivo (Newsholme and Crabtree 1986). The maximal activities of metabolic enzymes can be used to identify key sites regulated by the total amount of enzyme present in addition to assessing changes in potential carbon flux throughout metabolic pathways. Typically, non-regulatory enzymes exhibit relatively high activities and catalyze equilibrium reactions, whereas regulatory enzymes exhibit low activities and catalyze non-equilibrium reactions (Newsholme and Start 1973). Flux through a regulatory enzyme may be regulated by the amount of the enzyme present in tissues or by modification of the enzyme itself. Enzyme activity measurements are useful for two reasons: i) the subtlest of changes in enzyme concentration can induce large effects on metabolic pathways and ii) the synthesis of enzymes can consume a large fraction of the ATP equivalents that are available in the cell and hence enzymes that are synthesized under stress conditions when ATP is limiting should be those that are important to organismal survival of the stress (Newsholme and Crabtree 1986). This forms the basis of the Metabolic Control Theory (Newsholme and Start 1973) which makes predictions on rates of flux through individual enzymes in a metabolic pathway. In the past many enzymes have been considered to be rate-limiting, but without assays of enzymatic activities under optimal conditions, predictions cannot be made about flux. Only through the measurement of multiple enzymes along a metabolic pathway can the nonequilibrium regulatory enzymes be identified.

The present study examines the maximal activities of 32 metabolic enzymes in 6 tissues of T. s. elegans. These will be used as indicators of metabolic flux in the pathways of carbohydrate metabolism, citric acid cycle, fatty acid metabolism, adenylate metabolism and amino acid metabolism.

MATERIALS AND METHODS

Chemicals and animals.

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO., U.S.A.) or from Boehringer Mannheim Corporation (Montreal, Quebec) and were of the highest purity available. Winter acclimated adult red-eared sliders (*T. s. elegans*) were obtained from Wards Natural Science, Mississauga, Ontario and were maintained in large tanks of dechlorinated water at 7 °C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed ad libitum on a diet of trout pellets, lettuce and egg shells.

Control (normoxic) turtles were sampled directly from the tank. Anoxia was imposed by submerging turtles for 20 h at 5 °C in sealed tanks of 60 L deoxygenated water that had been previously bubbled with 100 % nitrogen gas for 1 hour. A wire mesh placed 20 cm below the surface of the water prevented turtles from surfacing. Some turtles were sampled immediately at the end of anoxia treatment whereas others were returned to aerobic conditions in a 5°C incubator and allowed to recover for 24 h before sampling. All animals were killed by decapitation and organ samples were quickly removed, frozen in liquid nitrogen and then transferred to -80 °C for storage.

Preparation of tissue extracts for enzyme assay.

Tissue samples were quickly weighed while still frozen and homogenized 1:5 (w:v) in ice-cold buffer containing protein kinase and protein phosphatase inhibitors (50 mM imidazole, pH 7.5, 10 % v/v glycerol, 30 mM β -MeSH, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, a few crystals of PMSF) with a Diamed Pro 200 homogenizer set to full power. Homogenates were centrifuged in a Heraeus Biofuge 15 at 20,000 x g for 25

minutes and then the supernatant was collected and stored on ice prior. Extracts for assay of carnitine-palmitoyl transferase, carnitine octanoyl transferase and citrate synthase were also prepared as above, except that the pH of the extraction buffer was set to 8.1 and NaF and β -MeSH were omitted.

Determination of enzyme activity.

Unless otherwise stated, all assays were performed at room temperature using a Dynatech MR5000 microplate reader in a final volume of 0.250 ml plus added extract at an absorbance of 340 nm. Optimal assay conditions were determined for each enzyme in turtle liver (these conditions were used for liver, kidney, brain and heart) and in turtle red muscle (these conditions were used for red muscle and white muscle). Blanks (measured in the absence of the most specific substrate) were subtracted to yield net activity values. All activities are reported as Units (one Unit is defined as the production of one μ mole of product per minute) per gram wet weight of tissue. All coupling enzymes were desalted by centrifugation through a small column of Sephadex G25 (Helmerhorst and Stokes 1980). Optimal assay conditions were as follows :

Enzymes of carbohydrate metabolism

Lactate dehydrogenase (LDH; E.C. 1.1.1.27): 50 mM imidazole buffer (pH7.0), 2 mM MgCl_2 , 2 mM pyruvate, 0.15 mM NADH.

Pyruvate kinase (PK; E.C. 2.7.1.40): 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM MgCl_2 , 6 mM PEP, 0.15 mM NADH, 1 mM ADP, 0.2% (v/v) rotenone saturated ethanol and 2 U LDH.

Phosphofructo-1-kinase (PFK; E.C. 2.7.1.11): 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM MgCl₂, 1 mM ATP, 8 mM fructose-6-phosphate, 0.15 mM NADH and 1 U each of ALD, G3PDH and triosephosphate isomerase.

Fructose-1,6-bisphosphatase (FBPase; E.C. 3.1.3.11): 25 mM imidazole buffer (pH 8.0), 5 mM MgSO₄, 0.2 mM NADP⁺, 1.4 mM FBP and 1 U each of G6PDH and phosphoglucose isomerase.

Phosphoenolpyruvate carboxykinase (PEPCK; E.C. 4.1.1.32): 100 mM imidazole buffer (pH 6.6), 6 mM β-MeSH, 50 mM sodium bicarbonate, 1.2 mM PEP, 1.25 mM inosine diphosphate, 1 mM MnCl₂, 0.15 mM NADH and 2.5 U malate dehydrogenase. All solutions were degassed prior to assay.

Aldolase (ALD; E.C. 4.1.2.13): 50 mM imidazole buffer (pH 7.0), 2 mM MgCl₂, 0.2 mM FBP, 0.15 mM NADH, 1 U triosephosphate isomerase and 2 U G3PDH.

Glucokinase (GK; E.C. 2.7.1.2): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM MgCl₂, 100 mM glucose, 0.2 mM NADP⁺, 1 mM ATP and 1 U glucose-6-phosphate dehydrogenase.

Hexokinase (HK; E.C. 2.7.1.1): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM MgCl₂, 10 mM glucose, 0.2 mM NADP⁺, 1 mM ATP and 1 U G6PDH.

Glycerol-3-phosphate dehydrogenase (G3PDH; E.C. 1.1.1.8): 50 mM imidazole buffer (pH 8.1), 30 mM glycerol-3-phosphate and 0.8 mM NAD⁺.

Enzymes of NADPH production

Glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49): 60 mM Tris-HCl buffer (pH 7.5), 3.3 mM glucose-6-phosphate, 0.4 mM NADP⁺ and 6 mM MgCl₂.

6-Phosphogluconate dehydrogenase (6PGDH; E.C. 1.1.1.43): 50 mM Tris-HCl buffer (pH 7.0), 0.5 mM 6-phosphogluconate, 0.4 mM NADP⁺.

Malate dehydrogenase, NADP⁺-dependent (NADPMDH; E.C. 1.1.1.37): 50 mM imidazole buffer, 10 mM MgCl₂, 6 mM oxaloacetate, 0.15 mM NADPH.

Isocitrate dehydrogenase, NADP⁺-dependent (IDH; E.C. 1.1.1.42): 50 mM imidazole buffer (pH 8.1), 5 mM MgCl₂, 6 mM isocitrate and 0.4 mM NADP⁺.

Enzymes of the citric acid cycle

α -Ketoglutarate dehydrogenase (KGDH; E.C. 1.2.4.2): 50 mM imidazole buffer (pH 7.5), 10 mM 2-oxoglutarate, 0.4 mM NAD⁺, 2 mM cocarboxylase, 0.1 mM CoA and 0.2% (v/v) rotenone saturated ethanol.

Malate dehydrogenase NAD⁺-dependent (NADMDH; E.C. 1.1.1.37): 50 mM imidazole buffer, 10 mM MgCl₂, 6 mM oxaloacetate, 0.15 mM NADH.

Citrate synthase (CS; E.C. 4.1.3.7): 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 1 mM oxaloacetate, 0.1 mM acetyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 410 nm.

Enzymes of fatty acid metabolism

Citrate lyase (CL; E.C. 4.1.3.8): 200 mM Tris-HCl buffer (pH 8.4), 10 mM β -MeSH, 0.2 mM CoA, 0.15 mM NADH, 20 mM MgCl₂, 20 mM potassium citrate, 10 mM ATP and 1 U malate dehydrogenase.

3-Hydroxyacyl-CoA dehydrogenase (HOAD; E.C. 1.1.1.35): 50 mM imidazole buffer (pH 8.0), 0.1 mM acetoacetyl-CoA and 0.1 mM NADH.

β -Hydroxybutyrate dehydrogenase (β OHBD; E.C. 1.1.1.30): 50 mM imidazole buffer (pH 8.1), 20 mM β OHBD and 0.4 mM NAD⁺.

Branched chain ketoacid dehydrogenase (KADH): 30 mM potassium phosphate buffer (pH 7.5), 2 mM MgCl₂, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100, 2 mM

ketoisovalerate, 0.2 mM NAD⁺, 0.4 mM cocarboxylase, 0.4 mM CoA and 10 U lipoamide dehydrogenase.

Carnitine palmitoyltransferase (CPT; E.C. 2.3.1.21): 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 5 mM L-carnitine, 50 μ M palmitoyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 410 nm.

Carnitine octanoyltransferase (COT; E.C. 2.3.1.21): 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 5 mM L-carnitine, 50 μ M octanoyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 410 nm.

Enzymes of adenylate and amino acid metabolism

Adenylate kinase (AK; E.C. 2.7.4.3): 50 mM potassium phosphate buffer (pH 7.4), 500 mM MgSO₄, 200 mM KCl, 2 mM AMP, 2 mM ATP, 0.15 mM NADH, 2 mM PEP and 2 U each of LDH and PK.

Creatine Kinase (CK; E.C. 2.7.3.2): 50 mM imidazole, pH 7.0, 2 mM NAD⁺, 2 mM MgCl₂, 1 mM ADP, 20 mM creatine phosphate, 5 mM glucose and 1 U each of HK and G6PDH.

Glutamate-oxaloacetate transaminase (GOT; E.C. 2.6.1.1): 500 mM imidazole buffer (pH 7.8), 30 mM β -MeSH, 15 mM 2-oxoglutarate, 250 mM L-aspartate, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U of malate dehydrogenase.

Glutamate-pyruvate transaminase (GPT; E.C. 2.6.1.2): 500 mM imidazole buffer (pH 7.3), 30 mM β -MeSH, 7.5 mM 2-oxoglutarate, 500 mM L-alanine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U LDH.

Glutamate dehydrogenase (GDH; E.C. 1.4.1.2): 100 mM triethanolamine buffer (pH 8.0), 5 mM EDTA, 200 mM ammonium carbonate, 7.5 mM 2-oxoglutarate, 0.15 mM NADH and 1 mM ADP.

Serine dehydratase (SDH; E.C. 4.2.1.13): 200 mM potassium phosphate buffer (pH 8.0), 2 mM EDTA, 200 mM L-serine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% (v/v) rotenone saturated ethanol, and 1 U LDH.

Protein

Soluble protein concentration was determined by Bio-Rad assay kit (Coomassie blue G-250 binding method; Bradford 1976) with bovine serum albumin as the standard. Spectrophotometric quantification was performed at 595 nm using a Dynatech MR-5000 microplate reader to a final well volume of 310 μ l.

Data Analysis and Statistics

Vmax enzyme activity levels provided from the microplate reader were interpreted by the use of the MPA program (Brooks 1994). All values were compared by one-way ANOVA followed by a two-tailed Dunnett's test or SNK. Calculations were performed using a statistical program for the analysis of enzyme kinetics (Brooks 1992).

RESULTS

Protein

Soluble protein content was measured in all tissues but did not change significantly during anoxia or aerobic recovery in any case. Protein contents (values pooled for all 3 conditions, n = 12) were 118 ± 19.8 , 91.0 ± 5.33 , 73.1 ± 16.9 , 58.7 ± 14.4 , 111 ± 16.3 and 69.3 ± 7.46 mg/g wet weight (gww) in liver, heart, red muscle, white muscle, kidney and brain, respectively.

Enzyme Maximal Activities

Activities of many enzymes were unchanged by anoxia exposure (20 h at 5°C) or aerobic recovery (24 h at 5°C) and the overall mean maximal activities of these, combining data from control, anoxic and recovered samples (n=12), are shown in Table 1. Of the enzymes of carbohydrate metabolism, FBPase and PEPCK could not be detected in muscle but had detectable activities in all other tissues; the highest levels of activity being in liver and kidney. GK activity was present in liver but not in other tissues whereas HK had the opposite distribution. The highest activities of ALD and LDH occurred in red and white skeletal muscle while the lowest activity of G3PDH occurred in these tissues. Of the enzymes of NADPH production, G6PDH activity was lowest in skeletal muscle while 6PGDH activity could not be detected in these tissues. IDH activity was only detectable in heart, red muscle and white muscle with heart containing the highest activity. NADPMDH activity was highest in heart. Of the enzymes of the citric acid cycle, KGDH and CS activities were not detectable in muscle and KGDH activity was not detectable in liver. KGDH activity was highest in heart while CS activity was highest in kidney and heart. Among the enzymes of fatty acid metabolism, CL activity was only detectable in kidney and brain and KADH activity was only detectable in heart. CPT activity, by far, was the highest in brain. COT activity was undetectable in liver and was highest in red muscle. Of the enzymes involved in adenylate metabolism, AK and CK activities were highest in brain and skeletal muscle, respectively. Of the enzymes which metabolize amino acids, only GPT and GDH were undetectable in heart and white muscle, respectively. GPT and SDH activities were highest in muscle while GOT and GDH activities were highest in heart and liver, respectively.

Enzymes of carbohydrate metabolism

Changes in the maximal activities of the enzymes of carbohydrate metabolism in turtle organs in response to anoxia exposure and/or aerobic recovery are presented in Figure 2. Of gluconeogenic enzymes, the only change noted was a 60 % increase in liver FBPase activity (compared with controls and anoxia) during recovery from anoxia. Enzymes of glycolysis were modified during anoxia in selected instances; ALD decreased significantly brain, HK was suppressed in kidney, and PFK activity fell to 68 and 70 % of control values in liver and brain, respectively. Brain ALD and liver and brain PFK remained suppressed after 24 h aerobic recovery whereas kidney ALD and HK increased again. The activity of G3PDH was strongly reduced during anoxia in heart, falling to one-fourth of control activity but activity returned to control levels during recovery.

Enzymes of NADPH production

The effects of anoxia exposure and aerobic recovery on the maximal activities of enzymes involved in producing NADPH reducing equivalents are shown in Figure 3A. The maximal activity of G6PDH, the initial enzyme of the pentose phosphate pathway, was differentially affected in turtle organs. In liver, activity decreased significantly during aerobic recovery compared with values in control and anoxic turtles whereas in brain, G6PDH activity rose by 26 % during recovery. In heart, activity increased by 52 % over control values during anoxia and remained elevated during recovery. The activity of 6PGDH increased in recovered as compared to anoxic turtle heart. NADPMDH activity in turtle white muscle increased enormously (7.9-fold) over control values during anoxia and remained high during recovery from anoxia. The maximal activity of IDH increased during recovery in both red and white skeletal muscle, rising by 87 and 47 %, respectively.

respectively, over control values for white muscle and control and anoxic values for red muscle.

Enzymes of the citric acid cycle

The effects of anoxia exposure and aerobic recovery on the maximal activities of enzymes involved in the citric acid cycle are shown in Figure 3B. CS activity increased in liver during recovery, rising by 47 % over control values. By contrast, KGDH activity was reduced from controls in anoxic brain by 28 % and remained low during the recovery period.

Enzymes of fatty acid metabolism

Changes in the maximal activities of the enzymes associated with fatty acid metabolism are shown in Figure 4. The activity of CL, an enzyme involved in fatty acid synthesis, decreased by 69 % in brain during anoxia and partially returned to control values during recovery; the recovered values not being significantly different than anoxic values. HOAD activity in red muscle decreased during anoxia to only 35 % of control values and returned to normoxic values during recovery from anoxia; recovered activities were significantly different from those found during anoxia. KADH activity decreased from anoxic levels during recovery from anoxia in heart. CPT activity changed in three organs. In kidney, activity increased by almost 3-fold during anoxia but was partially reduced to control levels again during recovery (recovered not significantly different than anoxic). In heart and brain, however, significant effects occurred during the aerobic recovery period, with a dramatic 13.5-fold increase over control and a 4.6-fold increase over anoxic activities in heart and a 31 % increase over control in brain. COT activity

changed only in liver, being undetectable in liver of control turtles but rising to 1.47 U/gww during recovery from anoxia.

Enzymes of adenylate and amino acid metabolism

Enzymes of adenylate metabolism were unaffected by anoxia exposure and recovery from anoxia. Effects of anoxia exposure and aerobic recovery on enzymes of amino acid metabolism in turtle organs are shown in Figure 5. GOT activity decreased by 32 % and 36 % during anoxia in heart and red muscle, respectively, but rose again to control activity after aerobic recovery. In liver, activity was unaffected during anoxia but rose by 28 %, compared with controls, during the recovery period. GPT activity decreased by 32 % in brain during anoxia but returned to control levels during recovery. In white muscle, however, GPT activity was unaffected during anoxia but increased by 2-fold from control and anoxic values during recovery. The activity of GDH decreased to less than half of control values during anoxia in brain but was restored again during recovery. In red muscle, however, the enzyme was strongly affected during recovery, rising by 3.5-fold over control and anoxic values. SDH activity was affected in three organs. In brain, activity was reduced by 40 % during anoxia and remained low during recovery. However, in heart and white muscle SDH activity increased 2.1- and 2.5-fold above control and anoxic values during the recovery period.

DISCUSSION

In general, maximal activities of most enzymes were not modified during anoxia exposure or aerobic recovery in turtles. This suggests that the turtle is well suited with constitutive enzyme activities to meet the demands of anoxic excursions whenever they

occur. Constitutive levels of enzymes may therefore be sufficient to deal with anoxic demands and metabolic rate depression may affect protein synthesis/degradation equally so that overall protein and enzyme levels do not change.

Selected specific changes in the maximal activities of some enzymes are made as indicated by the above results as well as recent gene expression studies (Cai and Storey 1996) and these, along with other studies that have shown anoxia-induced changes in the regulation of various other enzymes (via reversible phosphorylation, reversible binding with subcellular structures, or allosteric regulation) (Storey and Hochachka 1974, Storey 1985, Hochachka 1986, Duncan 1988, Storey 1988, Brooks and Storey 1988, Mehrani and Storey 1995c), all contribute to accomplishing the metabolic adjustments needed for long term anaerobic survival. Overall, there were 16 instances of enzyme changes between control and anoxic turtles, 21 differences between controls and recovery from anoxia, and 16 differences between anoxia and recovery from anoxia. Compared to controls, there were 13 instances of reduced enzyme maximal activities in anoxia and only 3 instances of increased activities. The preponderance of reduced enzyme activities during anoxia is consistent with an overall suppression of the rates of ATP-dependent protein synthesis during anoxia as has been suggested by the work of Land and Hochachka (1994) but the many instances of unchanged enzyme activities in anoxia would suggest that, in general, rates of protein synthesis and protein degradation must be suppressed in a coordinated fashion as part of the overall metabolic rate depression. Against a background of largely unchanged enzyme activities, the specific enzyme activities affected by anoxia exposure gain more significance. Via suppressing the activities of specific enzymes, overall suppression of specific pathways may be achieved

and contribute to the overall metabolic rate depression during anoxia. By contrast, in terms of enzyme activity changes, turtles showed much more metabolic activity in the recovered state versus the anoxic state. This is probably consistent with the return of aerobic metabolism and possibly with the clearance of anaerobic end products or other processes that were impeded when oxygen was limiting.

Of all tissues, brain showed the most changes in enzyme activities associated with anoxia/recovery. In fact, the responses seen in brain were quite distinct from those seen in other tissues. The reasons for the large number of changes in brain may be two-fold: 1) changes occur that are associated with metabolic rate depression and anaerobic energy production, or 2) changes occur that are associated with an altered metabolism of neurotransmitters. Anoxia in turtle brain is associated with an overall suppression of excitatory transmitters and enhancement of inhibitory transmitters, many of which are amino acids (Nilsson et al. 1990). Brain has an active response to anoxia in terms of suppressing a variety of enzymes that would help to reduce metabolic rate in anoxia and/or elevating activities of others in the recovery period. These include reduced ALD and PFK in glycolysis, KGDH (a regulatory enzyme of the citric acid cycle), and CL and CPT of fatty acid metabolism. In addition, activities of several enzymes of amino acid metabolism were also suppressed, particularly those linked with glutamate metabolism. Decreased activities of GDH and SDH in brain during anoxia would limit the catabolism of glutamate (through α -ketoglutarate) and serine (through pyruvate) respectively. The enzymes of glutamate metabolism could also link amino acid oxidation/synthesis to neurotransmitter metabolism which is also suppressed in anoxia. For example, in turtle

brain, the levels of the excitatory amino acid, glutamate, fall in anoxia (from 6 to 4 mM over 13 hours at room temperature), along with a comparable drop in glutamine levels, whereas levels of the inhibitory transmitter γ -aminobutyric acid increase by 2.3 fold (from 2.6 to 6 mM) over the same time (Nilsson et al. 1990). As well, both glycine and serine rise in parallel during 13 hours of anoxia, glycine from 1 to 7.5 mM and serine from 0.5 to 2.3 mM (Nilsson et al. 1990). Glycine is a well established inhibitory neurotransmitter in lower brain areas and spinal cord and hence, glycine changes can be viewed as part of metabolic rate depression in brain. Serine may rise in parallel during anoxia as it is the immediate precursor of glycine, and hence, the suppression of SDH in anoxia (about a 50 % decrease) may be key to letting both serine and glycine accumulate. It would be interesting to see whether serine/glycine also rise in other organs during anoxia and therefore, whether the large increases in SDH in both heart and white muscle during recovery are relevant. As well, elevated CS activity in brain during recovery from anoxia could enhance the oxidation of accumulated glycolytic end products.

The NADPH-producing enzymes increased in most organs during recovery; G6PDH in heart and brain, 6PGDH in heart, NADPMDH in white muscle (although this is also high in anoxia) and IDH in red and white muscle. These are biosynthetic enzymes which could provide NADPH for renewed synthesis/repair of fatty acids, steroids or DNA after the metabolic suppression of anoxia is removed. The NADPH produced may also be used to reduce GSSG through the action of GR. Since a decrease in glutathione reductase activity and the total glutathione pool has been found in anoxic turtle heart previously (Willmore and Storey 1997b), the later seems unlikely.

Liver showed responses that are common in other systems undergoing metabolic rate depression. In this tissue, regulation of glycolysis/gluconeogenesis at PFK and FBPase loci is very clear and the changes seen during anoxia/recovery support a major role for liver in gluconeogenesis from lactate during the recovery period. Elevated CS activity may also suggest that lactate oxidation is also enhanced during recovery. A decrease in the activity of the glycolytic enzyme PFK during anoxia exposure is key to metabolic rate depression in this tissue. An increase in FBPase activity with a corresponding decrease in PFK activity during recovery from anoxia suggests an inhibition of glycolysis and an activation of gluconeogenesis during recovery from anoxia. It also suggests that the two enzymes are regulated in an opposite manner. Liver is typically the primary organ that consumes the lactate accumulated during anoxia by converting it back to glucose/glycogen. By doing so, this restores the liver glycogen that was the primary source of the blood glucose that was used as an anoxic fuel by most organs. Inhibition of glycolysis and activation of gluconeogenesis is generally not a synthesis/degradation response, but is typically controlled by reversible protein phosphorylation with a stable covalent modification changing the maximal measurable amount of enzyme. Liver PFK and FBPase are well known to be regulated in this manner.

The changes in kidney included decreased GK and ALD activities with a subsequent return to normoxic activity during recovery from anoxia. This, again, indicates glycolytic control supporting metabolic rate depression.

Heart, red and white muscle all showed similar responses that would support metabolic rate depression and also have consequences for amino acid metabolism in

anoxia. Changes in the glutamate-related enzymes in these tissues could suggest that they utilize amino acids as fermentative substrates and resynthesize them during recovery from anoxia. GOT activity decreased in both heart and red muscle during anoxia, suggesting a decreased flow of aspartate into the citric acid cycle at oxaloacetate or into gluconeogenesis at phosphoenolpyruvate during anoxia in these tissues. An increase in the activities of SDH in heart and white muscle and GDH in red muscle during recovery from anoxia possibly indicates a utilization of amino acids as fuel for the citric acid cycle. On the other hand, if one accepts that an increase in activity in one direction of an equilibrium enzyme also means that activity in the opposite direction is similarly increased, then during recovery from anoxia, there is also increased potential for synthesizing aspartate and glutamate to replenish these amino acid pools that are depleted in anoxia as well as to enhance protein synthesis. Although not as well developed as in marine molluscs, turtles do make use of fermentable amino acids (aspartate, glutamate/glutamine) as fuels during anoxia and accumulate low amounts of end products (succinate) that result from them. These amino acids also provide the amino groups for alanine synthesis as a glycolytic end product. Although lactate is the main product of anaerobic glycolysis in turtle tissues, some alanine also accumulates. Aspartate fermentation to succinate can be coupled with pyruvate conversion to alanine via the actions of GOT and GPT or glutamate fermentation can be coupled to alanine synthesis via GPT alone. Both G3PDH activity in heart and HOAD in red muscle were strongly reduced in anoxia which corresponds to suppressed lipid oxidation in these tissues. CPT increased in activity during recovery from anoxia in heart (as well as during anoxia in kidney and recovery in brain). The enzyme is clearly responsive to stress, more so than

COT and other lipid related enzymes. This evidence points towards increased β -oxidation of fatty acids may occur in heart and brain during recovery from anoxia.

In conclusion, the changes in maximal activities of enzymes in turtle tissues during anoxia or aerobic recovery supports the unifying principles of metabolic control that underlie metabolic rate depression in turtles. Future studies will combine data on metabolite intermediates with $V_{\max}$ activities of enzymes in order to gain better insight into shifts in flux through various pathways.

Fig. 2. Enzymes of carbohydrate catabolism that showed significant changes in maximal activities (U/gww) over the course of anoxia and recovery from anoxia in Trachemys scripta elegans. Control (white bars), 20 h anoxia exposure (grey bars) and 24 h recovery from anoxia (black bars). Data are Units per gram wet weight, means $\pm$ S.E.M. (n = 4). The scientific notation refers to all 3 experimental conditions within a set of bars. Significant differences for one-way ANOVA are indicated when tested with two-tailed Dunnetts (a,b - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully) or Student-Newman-Keuls (c,d - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully; e,f - significantly different from anoxic at $P < 0.05$ and $P < 0.01$ respectfully).

Fig. 3. Enzymes of (A) NADPH production and (B) the citric acid cycle that showed significant changes in maximal activities (U/gww) over the course of anoxia and recovery from anoxia in Trachemys scripta elegans. Control (white bars), 20 h anoxia exposure (grey bars) and 24 h recovery from anoxia (black bars). Data are Units per gram wet weight, means $\pm$ S.E.M. (n = 4). The scientific notation refers to all 3 experimental conditions within a set of bars. Significant differences for one-way ANOVA are indicated when tested with two-tailed Dunnetts (a,b - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully) or Student-Newman-Keuls (c,d - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully; e,f - significantly different from anoxic at $P < 0.05$ and $P < 0.01$ respectfully).

Fig. 4. Enzymes of fatty acid metabolism that showed significant changes in maximal activities (U/gww) over the course of anoxia and recovery from anoxia in Trachemys scripta elegans. Control (white bars), 20 h anoxia exposure (grey bars) and 24 h recovery from anoxia (black bars). Data are Units per gram wet weight, means $\pm$ S.E.M. (n = 4). The scientific notation refers to all 3 experimental conditions within a set of bars. Significant differences for one-way ANOVA are indicated when tested with two-tailed Dunnetts (a,b - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully) or Student-Newman-Keuls (c,d - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully; e,f - significantly different from anoxic at $P < 0.05$ and $P < 0.01$ respectfully).

Fig. 5. Enzymes of amino acid metabolism that showed significant changes in maximal activities (U/gww) over the course of anoxia and recovery from anoxia in Trachemys scripta elegans. Control (white bars), 20 h anoxia exposure (grey bars) and 24 h recovery from anoxia (black bars). Data are Units per gram wet weight, means $\pm$ S.E.M. (n = 4). The scientific notation refers to all 3 experimental conditions within a set of bars. Significant differences for one-way ANOVA are indicated when tested with two-tailed Dunnetts (a,b - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully) or Student-Newman-Keuls (c,d - significantly different from controls at $P < 0.05$ and $P < 0.01$ respectfully; e,f - significantly different from anoxic at $P < 0.05$ and $P < 0.01$ respectfully).

Table 1. Control values of the maximal activities of the enzymes of carbohydrate, NADPH, citric acid, fatty acid, amino acid and adenylate metabolism (U/gww) from tissues of *Trachemys scripta elegans*. MDH, also an enzyme of the citric acid cycle is listed under carbohydrate metabolism for convenience. Values are means $\pm$ S.E.M. of pooled numbers from control, anoxic and recovered treatments $\pm$ S.E.M. (n = 12 pooled results for enzymes which revealed no change; * n = 4 control values only for enzymes which revealed changes). n.d.= not detected.

Enzyme	Tissue					
	Liver	Kidney	Heart	Brain	Red Muscle	White Muscle
Carbohydrate Metabolism						
FBPASE	0.772 $\pm$ 0.0600*	0.714 $\pm$ 0.0608	0.183 $\pm$ 0.0361	0.191 $\pm$ 0.0234	n.d.	n.d.
PEPCK	0.196 $\pm$ 0.0182	0.209 $\pm$ 0.0305	0.0821 $\pm$ 0.0366	0.113 $\pm$ 0.00427	n.d.	n.d.
ALD	0.837 $\pm$ 0.0674	1.28 $\pm$ 0.328*	7.49 $\pm$ 1.02	14.9 $\pm$ 1.27*	25.4 $\pm$ 0.790	21.0 $\pm$ 1.05
GK	0.140 $\pm$ 0.0154	n.d.	n.d.	n.d.	n.d.	n.d.
HK	n.d.	0.619 $\pm$ 0.0195	1.50 $\pm$ 0.295	1.98 $\pm$ 0.134	0.0775 $\pm$ 0.00286	0.0409 $\pm$ 0.00649
LDH	267 $\pm$ 12.0	369 $\pm$ 33.1	684 $\pm$ 116	555 $\pm$ 16.8	60.4 $\pm$ 1.10	69.2 $\pm$ 5.14
PK	19.4 $\pm$ 0.591	33.0 $\pm$ 0.674	61.5 $\pm$ 2.86	70.6 $\pm$ 0.668	69.2 $\pm$ 6.02	57.7 $\pm$ 3.73
PFK	0.683 $\pm$ 0.0444*	0.643 $\pm$ 0.0762	1.71 $\pm$ 0.410	3.58 $\pm$ 0.200*	3.33 $\pm$ 0.491	2.83 $\pm$ 0.499
NADMDH	37.9 $\pm$ 1.28	65.8 $\pm$ 5.39	84.0 $\pm$ 15.0	87.0 $\pm$ 1.77	16.5 $\pm$ 1.51	12.8 $\pm$ 0.980
G3PDH	0.146 $\pm$ 0.0178	0.106 $\pm$ 0.00596	0.234 $\pm$ 0.039*	0.174 $\pm$ 0.0141	0.0869 $\pm$ 0.00570	0.0692 $\pm$ 0.0114
NADPH Production						
G6PDH	1.70 $\pm$ 0.0750*	1.18 $\pm$ 0.0352	0.430 $\pm$ 0.0700*	0.914 $\pm$ 0.0240*	0.116 $\pm$ 0.00760	0.0923 $\pm$ 0.0151
6PGDH	0.620 $\pm$ 0.0461	0.752 $\pm$ 0.0585	0.343 $\pm$ 0.0330*	0.436 $\pm$ 0.0333	n.d.	n.d.
NADPMDH	2.46 $\pm$ 0.0970	5.18 $\pm$ 0.346	9.01 $\pm$ 2.26	4.71 $\pm$ 0.158	4.78 $\pm$ 0.450	1.96 $\pm$ 0.0938*
IDH	n.d.	n.d.	5.72 $\pm$ 1.27	n.d.	0.146 $\pm$ 0.0320*	0.199 $\pm$ 0.0360*
Citric Acid Cycle						
KGDH	n.d.	0.140 $\pm$ 0.0380	0.398 $\pm$ 0.0248	0.0807 $\pm$ 0.00390*	n.d.	n.d.
CS	39.3 $\pm$ 1.78*	135 $\pm$ 9.16	127 $\pm$ 13.0	100 $\pm$ 3.64	n.d.	n.d.
Fatty Acid Metabolism						
CL	n.d.	0.0818 $\pm$ 0.0146	n.d.	0.540 $\pm$ 0.0470*	n.d.	n.d.
HOAD	1.34 $\pm$ 0.0878	1.24 $\pm$ 0.0340	0.872 $\pm$ 0.189	0.544 $\pm$ 0.0312	0.964 $\pm$ 0.110*	0.300 $\pm$ 0.0612
KADH	n.d.	n.d.	0.457 $\pm$ 0.118*	n.d.	n.d.	n.d.
CPT	5.16 $\pm$ 1.27	3.35 $\pm$ 1.79*	0.582 $\pm$ 0.474*	31.0 $\pm$ 1.66*	1.23 $\pm$ 0.145	0.354 $\pm$ 0.0736
COT	n.d.*	1.94 $\pm$ 0.458	2.11 $\pm$ 0.374	0.659 $\pm$ 0.200	3.17 $\pm$ 0.564	1.06 $\pm$ 0.207
Adenylate and Amino Acid Metabolism						
AK	6.22 $\pm$ 0.741	22.9 $\pm$ 1.28	28.8 $\pm$ 5.71	45.5 $\pm$ 1.24	19.7 $\pm$ 1.15	17.8 $\pm$ 3.42
CK	18.1 $\pm$ 1.29	45.3 $\pm$ 2.87	225 $\pm$ 25.9	97.7 $\pm$ 3.00	742 $\pm$ 2.18	649 $\pm$ 60.8
GOT	19.8 $\pm$ 0.815*	15.3 $\pm$ 0.684	27.9 $\pm$ 3.74*	14.8 $\pm$ 0.177	12.9 $\pm$ 1.23*	2.71 $\pm$ 0.253
GPT	0.291 $\pm$ 0.0579	0.330 $\pm$ 0.0287	n.d.	0.0750 $\pm$ 0.00822*	1.84 $\pm$ 0.268	2.01 $\pm$ 0.762*
GDH	23.0 $\pm$ 0.491	12.7 $\pm$ 1.21	0.775 $\pm$ 0.117	6.89 $\pm$ 1.26*	0.594 $\pm$ 0.151*	n.d.
SDH	0.647 $\pm$ 0.0596	0.380 $\pm$ 0.0398	0.104 $\pm$ 0.0249*	0.547 $\pm$ 0.0544*	0.871 $\pm$ 0.217	0.820 $\pm$ 0.159*

CHAPTER THREE

ANTIOXIDANT SYSTEMS AND ANOXIA TOLERANCE IN A FRESHWATER TURTLE, TRACHEMYS SCRIPTA ELEGANS

INTRODUCTION

Since the advent of O₂-based aerobic metabolism, living organisms have required antioxidant defenses in order to combat the damaging effects of reactive oxygen species (ROS) (Halliwell 1987). ROS include the superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH·). Of these, OH· is the most reactive and destructive. Many cellular metabolic activities produce ROS including the enzymatic breakdown of small biological compounds (Jones 1986), the microsomal cytochromes P450 and b5, microsomal flavoprotein reductases and superoxide leakage from the electron transport chain (Halliwell and Gutteridge 1989, Kleinveld et al. 1989). Other sources of O₂⁻ and H₂O₂ can include cytosolic and peroxisomal enzymes. Of these, xanthine oxidase is purported to be a key source of toxic O₂⁻ in acute reoxygenation injury of anoxic tissues (Roy and McCord 1983, Terada et al. 1991, Terada et al. 1992). ROS attack many biological macromolecules including membranes (causing peroxidation of lipids), cellular proteins and enzymes (damaging functional properties and increasing proteolytic susceptibility) (Sies 1993, Stadtman 1992), and nucleic acids (causing strand breaks and altered bases) (Floyd 1990).

During prolonged diving in turtles blood in the heart is shunted from the right to the left ventricle, bypassing the lungs, and both cardiac output and systemic resistance to blood flow decreases (Millen et al. 1964, White and Ross 1966). Blood oxygen content drops to near zero values (Jackson 1968) after one hour of submergence. Within the first ten minutes of recovery from anoxia, blood oxygen rapidly returns to normoxic levels. Thus, tissues of anoxia tolerant turtles periodically undergo repeated bouts of deoxygenation and reoxygenation without encountering ischemia (Storey and Storey 1990). This situation is similar to ischemia/reperfusion in mammals in which the interruption of blood flow causes a lack of oxygenation of tissues and then a subsequent reoxygenation when flow is reestablished (e.g. during stroke (Floyd 1990)). Studies of

the effects of ischemia in mammalian systems have revealed that, in addition to the metabolic effects of oxygen deprivation, metabolic injuries are also incurred as a result of ROS formation during reperfusion (Halliwell 1992, Traystman et al. 1991). The sudden reintroduction of oxygen leads to an overproduction of ROS in tissues and these can have numerous deleterious effects (for reviews see Floyd 1990, Gutteridge and Halliwell 1990, Halliwell and Gutteridge 1989, Traystman et al. 1991). Under reperfusion conditions, these ROS are produced mainly from the mitochondria as anoxia results in the accumulation of reducing equivalents in the cytochrome chain (Dawson et al. 1993). Electrons are available from the cytochrome chain for formation of ROS when oxygen is reintroduced into anoxic tissues. Animals which naturally encounter anoxia/reoxygenation of tissues on a periodic basis should, therefore, possess mechanisms that deal with potential damage from ROS.

Animals have evolved antioxidant defense systems, with both enzymatic and nonenzymatic components (Sies 1993), to protect from the serious damaging effects of ROS on cellular macromolecules. Three levels of protection have been considered; (1) prevention of ROS formation, (2) termination of free radical chain reactions using radical scavengers or antioxidants and (3) repair of cellular components after damage has occurred. Specific enzymes directly detoxify ROS; SOD catabolizes O_2^- whereas CAT and GPOX degrade H_2O_2 (at high and low concentrations of H_2O_2 , respectively). AHR converts lipid hydroperoxides and other alkyl hydroperoxides to their corresponding alcohols, using either NADH or NADPH as the reducing agent (Jackson et al. 1989, Storz et al. 1989).

To investigate the role of antioxidant defenses in vertebrate anoxia tolerance, we chose to study the effects of anoxia exposure and aerobic recovery on the activities of antioxidant enzymes and the extent of lipid peroxidation damage in the red-eared slider turtle, *T. s. elegans*. This species, and related freshwater turtles, are without doubt among the best vertebrate facultative anaerobes. Turtles submerged in oxygen-depleted water can

survive as long as 3 months without breathing during winter hibernation at low temperatures (0-5°C) (Herbert and Jackson 1985, Ultsch 1989). The present study shows that constitutively high antioxidant enzyme activities in turtle organs minimize the danger of damage by ROS during transitions between anoxic and aerobic states.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were obtained from either Sigma Chemical Co., St. Louis, MO or Boehringer Mannheim Corp., Montreal, Quebec. Adult red-eared slider turtles (*T. s. elegans*) were obtained from Wards Natural Science, Mississauga, Ont. and were maintained in large tanks of dechlorinated water at 7°C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed ad libitum on a diet of trout food pellets, lettuce and egg shells.

Control (normoxic) turtles were sampled directly from the tank. Anoxia was imposed by submerging turtles for 20 h at 5°C in sealed tanks of deoxygenated water that had been previously bubbled with 100 % nitrogen gas for 1 h. Some turtles were sampled immediately at the end of anoxia treatment whereas others were returned to the aerobic holding tank and allowed to recover for 24 h before sampling. All animals were killed by decapitation and organ samples were quickly removed, frozen in liquid nitrogen and then transferred to -80°C for storage.

Preparation of tissue extracts for enzyme assay

Frozen tissue samples were quickly weighed and homogenized 1:5 (w:v) in ice-cold 50 mM potassium phosphate (KPi) buffer (pH 7.5) containing 1 mM EDTA (CAT and SOD), 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 5 mM dithiothreitol (XO and XDH) or 50 mM Tris (pH 7.5 to 8; AHR) and 1 mg/ml of

phenylmethylsulfonyl fluoride using an Ultra-Turrax (Tekmar) tissue homogenizer. Homogenates used for catalase and superoxide dismutase determination were then sonicated for 10 s on ice with a Kontes micro-ultrasonic cell disrupter. Homogenates were centrifuged at 16,000 x g for 15 min (25 min for XO and XDH) at 4°C using an Eppendorf microcentrifuge. Supernatants were removed and desalted by passage through a small column (1 x 5 cm) of Sephadex G-25 (equilibrated in homogenizing buffer) with centrifugation for 1 min in an IEC benchtop centrifuge at full speed (Helmerhorst and Stokes 1980). Desalting was omitted for catalase assay as the procedure resulted in high activity loss.

Xanthine oxidase/xanthine dehydrogenase (XO; E.C. 1.1.3.22/XDH; E.C. 1.1.1.204)

All measurements of enzyme activities were taken within a half an hour of homogenization. Fifty μ l of desalted supernatant was quickly added to a cuvette containing 500 μ l of 100 mM KPi (containing 1 mM EDTA) and 420 μ l of water and the fluorescence was measured using a Perkin-Elmer LS50 luminescence spectrophotometer (Perkin-Elmer, Beaconsfield, Buckinghamshire; excitation: 345 nm; emission: 390). After an initial blank rate was taken for 50 seconds, 10 μ l of 2 mM pterine (0.02 mM final concentration) was added and the rate of XO activity was taken over 300 seconds. Ten μ l of 1 mM methylene blue was then added and the combined rate of XO and XDH was measured over 350 seconds. The reactions were then stopped with the addition of 20 μ l of 2 mM allopurinol and 10 μ l of 0.01 mM isoxanthopterin (0.1 μ M final concentration) as an internal standard correcting for fluorescence quench in the assay. One unit of activity was defined as the amount of enzyme which produces one μ mole of isoxanthopterin per minute at 21 °C.

Catalase (CAT; E.C. 1.11.1.6)

CAT activity was monitored by following the consumption of 7.5 mM H₂O₂ in 50 mM KPi (pH 7.0) at 240 nm ($\epsilon = 0.0394 \text{ mM}^{-1}\text{cm}^{-1}$) (Aebi 1984). The reaction was initiated with 5 μL of a 1:5 v/v dilution of the original enzyme extract. Blanks were run in the absence of either H₂O₂ or enzyme. One unit of activity is defined as the amount of enzyme that reduces 1 μmol of H₂O₂ per minute at 21°C.

Superoxide dismutase (SOD; E.C. 1.15.1.1)

The activity of SOD was measured according to Paoletti et al. (1986). This method quantifies the inhibition of O₂⁻-induced oxidation of NADH caused by the addition of enzyme extract (monitored at 340 nm). The source of O₂⁻ is the reaction between 5 mM EDTA, 2.5 mM MnCl₂, 3.75 mM β -MeSH and atmospheric oxygen in media buffered with 50 mM KPi (pH 7.4) and containing 0.27 mM NADH. One unit of activity is defined as the amount of enzyme that inhibits the oxidation of NADH by 50% at 21 °C. A series of 10 cuvettes with increasing amounts of enzyme extract were used and inhibition curves were analyzed using a simple computer program to determine I₅₀ values (Buzadzic et al. 1990a).

Alkyl hydroperoxide reductase (AHR)

AHR activity was measured using the method of Jacobson et al. (1989) and Storz et al. (1989) using cumene hydroperoxide as the model hydroperoxide to measure enzyme activity. The reaction followed the breakdown of cumene hydroperoxide to cumene alcohol coupled to the oxidation of either NADPH or NADH. Reaction mixtures (0.25 mL) contained 50 mM Tris buffer (pH 7.5 to 8.0), 1.6 mM cumene hydroperoxide and 0.4 mM NADPH or NADH. Reactions were started with the addition of 10 to 50 μL of a 1:5 v/v dilution of the original enzyme extract. The rate of NADPH or NADH oxidation at 340 nm was measured ($\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$) using a Dynatech MR5000

microplate reader (Dynatech Laboratories Inc., Chantilly, Virginia). One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μ mol of NADPH or NADH per min at 21 °C. A blank minus cumene hydroperoxide showed little or no activity.

Glutathione peroxidase (GPOX; E.C. 1.11.1.9)

Both selenium-dependent and independent GPOX activities were assayed by the coupled enzyme assay of Ahmad and Pardini (1988). Consumption of 0.2 mM NADPH was followed in media containing 50 mM KPi (pH 7.4), 1 mM EDTA, 2 mM sodium azide, 10 mM GSH, 5 IU of glutathione reductase and 20 μ l of the enzyme extract. Selenium-dependent activity was measured by initiating the reaction with 0.25 mM H_2O_2 whereas the sum of selenium-dependent and independent activities were measured by initiating the reaction with 2.4 mM cumene hydroperoxide. Blanks were run in the absence of enzyme. One unit is defined as the amount of enzyme that oxidizes 1 μ mol of NADPH per min at 21°C.

Conjugated dienes

Conjugated dienes were detected using second order derivative spectroscopy according to Corongiu and Milia (1983). Frozen (less than 4 wk) tissue samples were homogenized 1:20 (w:v) in ice cold chloroform:methanol (2:1 v/v) using a glass-Teflon homogenizer and centrifuged at 1000 x g using an Eppendorf microcentrifuge. The organic layer was removed and proteins were precipitated with a second extraction using 0.5 vol 3 mM HCl. The organic phase was transferred to preweighed glass test tubes and the solvent was then removed with a stream of 100% N_2 gas. Extracted lipids were weighed and then reconstituted in hexane at a final concentration of 2 mg/ml. A second derivative wavelength scan (220-300 nm) was performed using a Perkin-Elmer Lambda 4B spectrophotometer (Norwalk, Connecticut) with butanol as the blank. The change in

absorbance between 233 and 240 nm was measured and values are reported in relative second derivative absorbance units (d^2A/mg lipid).

Lipid hydroperoxides

Lipid hydroperoxides were quantified via their ability to induce the formation of a xynol orange- Fe^{+3} complex at acidic pH values as described by Jiang et al. (1991) and modified for tissue extracts by Hermes-Lima et al. (1995). Frozen (less than 3 mo) tissues were homogenized 1:5 (w:v) in HPLC grade methanol and centrifuged at 1000 x g in a benchtop centrifuge. Ten μ l of supernatant was then added to a reaction medium containing 0.25 mM $FeSO_4$, 25 mM H_2SO_4 and 0.1 mM xynol orange. Samples were incubated at room temperature until the reaction was complete (30 min to 2 h). Samples and blanks (no homogenate) were read at 580 nm against a water blank on a Gilford 250 spectrophotometer and then 5 μ l of 1 mM cumene hydroperoxide was added to all cuvettes and the A_{580} was measured again. Values for lipid hydroperoxides are expressed in cumene hydroperoxide equivalents.

Thiobarbituric acid reactive substances (TBARS)

TBARS were quantified by the method of Uchiyama and Mihara (1978). The reaction of malonaldehyde (MDA) with thiobarbituric acid (TBA) forms a complex which has an absorbance peak at 532 nm. Frozen (less than 3 mo) tissues were homogenized 1:5 (w:v) in 0.2% v/v phosphoric acid. A second volume of 2% phosphoric acid was then added to the mixture resulting in a final concentration of 1.1%. An aliquot of homogenate (0.4 ml) was then mixed with 0.4 ml of TBA solution (1% w/v TBA) in 50 ml 50 mM NaOH containing 0.5 ml 10 mM butylated hydroxytoluene (BHT) and 0.2 ml of 7% phosphoric acid. Individual blanks were prepared by replacing the TBA solution with 3 mM HCl. The pH of each sample was adjusted to 1.6 with either 10 N NaOH or 12 N HCl and samples and blanks were heated at 100°C for 15 min. After

cooling to room temperature, 1.5 ml of butanol was added to both samples and blanks and these were then vortexed vigorously for 40 s. Tubes were then centrifuged at 1000 x g for 5 min using a IEC benchtop centrifuge. Wavelength scans (400-600 nm) were performed using a Lambda 4B spectrophotometer and glass cuvettes. The difference $A_{532}(\text{sample} - \text{blank}) - A_{600}(\text{sample} - \text{blank})$ was measured and TBARS were quantified using the TBA-MDA extinction coefficient (156 mM^{-1}).

Protein assay

Protein was measured by the Coomassie blue dye-binding method (Bradford 1976) using the Bio-Rad prepared reagent and the micro assay procedure with bovine serum albumin as the standard.

Statistical analysis

Values are reported as means $\pm$ S.E.M. Statistical testing used one-way analysis of variance followed by the Dunnett's two-tailed test to analyze the data (Brooks 1992).

RESULTS

Xanthine oxidase/xanthine dehydrogenase (XO/XDH)

XO and total (XO + XDH) activities are presented in Table 2. Both XO and total activities were highest in heart, somewhat lower in liver and kidney, and lowest in other tissues. XO activity increased significantly compared with control values during anoxia in brain (from 7.39 ± 0.83 mUnits/mg protein in controls to 22.25 ± 3.36 mUnits/mg protein during anoxia) but this was the only significant change noted in any of the tissues. XO comprised approximately 50-75 % of the total activity in most tissues with the exception of kidney where it was 36-50 % of the total activity.

Antioxidant enzymes

Activities of CAT in turtle organs are shown in Figure 6. In organs from control turtles, CAT was highest in kidney and liver (299 ± 12 and 229 ± 8 U/mg, respectively) and was about 50 U/mg in other organs. Anoxia exposure resulted in a strong decrease in CAT activity in kidney (by 68%) and brain (by 80%) and a lesser decrease (by 31%) in heart but did not affect the enzyme activity in other organs. During aerobic recovery, CAT activity was not significantly different from controls in heart but remained suppressed in brain. In kidney a partial recovery of CAT activity occurred but values were still lower than controls.

SOD activity in turtle organs is shown in Figure 7. Activities ranged from about 15-50 U/mg in different organs. Anoxia exposure led to a significant decrease in SOD in liver and brain, values being reduced by 30% and 15% respectively. Enzyme activity remained low in brain during aerobic recovery but showed a return to near-control values in liver. In heart, SOD activity rose by 45% during the recovery period. SOD activity was unaffected by experimental state in the other three organs.

Activities of AHR in turtle tissues are shown in Figure 8. Activities in control tissues were highest in heart followed by liver and red muscle. All other tissues had lower amounts of AHR activity. Activity increased two-fold in the heart and 3.5 fold in kidney during anoxia and returned to control values during aerobic recovery from anoxia. By contrast, AHR activity rose in skeletal muscle during aerobic recovery; activities rose to 200% and 168% of control values in red and white muscle respectively. Activity during recovery from anoxia decreased to 35% of control values in brain.

Activities of GPOX in turtle organs are shown in Table 3; both selenium-dependent GPOX and total GPOX (sum of Se-dependent and independent activities) were measured. Total GPOX was highest in red skeletal muscle and kidney with intermediate activities in liver, heart, and brain, and very low activities in white skeletal muscle. Se-dependent GPOX activity was not significantly affected by either anoxia exposure or

recovery in any organ but total GPOX activity was affected by experimental state in some instances. Anoxia resulted in a 41% decrease in total GPOX activity in kidney whereas in heart and white skeletal muscle total GPOX decreased during the recovery period, by 75 and 77 %, respectively. Table 3 also shows the percentage of total GPOX that was selenium-dependent. Under most conditions in liver, heart and white skeletal muscle, the Se-dependent activity was equal to the total activity, suggesting that GPOX activity in these organs may be due solely to the Se-dependent enzyme. In red muscle, kidney and brain, however, substantial amounts of Se-independent activity occurred.

Products of lipid peroxidation

One form of metabolic damage by reactive oxygen species is the peroxidation of cellular lipids. Three different products of lipid peroxidation were quantified as indicators of oxidative stress during anoxia or recovery. Each analyzes damage at a different stage in the peroxidation process. Conjugated dienes are one of the initial products of lipid peroxidation. Analyses of samples from all six organs showed that these were detectable only in liver. Values for conjugated dienes were $13.1 \pm 1.78 \text{ d}^2\text{A/mg lipid} \times 10^{-5}$ (n=11) in control liver and significantly lower, $8.15 \pm 1.11 \text{ d}^2\text{A/mg lipid} \times 10^{-5}$ (n=11; $P < 0.05$), in anoxic liver. After aerobic recovery the value was $9.39 \pm 1.21 \text{ d}^2\text{A/mg lipid} \times 10^{-5}$ (n=12), not significantly different from controls. Table 4 shows measurements of two other products of lipid peroxidation in four organs: lipid hydroperoxides (measured using the xyleneol orange- Fe^{3+} assay) and thiobarbituric acid reactive substances (TBARS), malonaldehyde being one of the common degradation products (Halliwell and Gutteridge 1989). Both methods agreed in showing highest levels of lipid peroxidation products in liver and kidney and overall lower amounts in red and white skeletal muscles. As measured by these methods there were no significant changes in the amounts of lipid peroxidation products in either liver or white muscle during anoxia and recovery. However, red muscle showed a significant decrease in TBARS under both experimental

conditions. Anoxia did not affect either parameter in kidney but after recovery anomalous results were found; the xylenol orange-Fe³⁺ method indicated reduced lipid peroxidation whereas a significant increase in TBARS was found.

DISCUSSION

Anoxia tolerant animals need to deal with the potential for oxidative stress when oxygen is reintroduced to their bodies after extended periods of anaerobic life. Various strategies might be taken to deal with a burst of ROS production during reoxygenation. These might include: 1) maintenance of consistently high antioxidant defenses in order to immediately quench or limit the formation of ROS, 2) stress-induced adjustments to antioxidant defenses during the anaerobic period which prepare the system for the oxidative stress that will accompany reoxygenation, or 3) tolerance of higher levels of free radical damage and/or enhancement of mechanisms for the removal of damage products. Each of these strategies has their own advantages and each might serve different circumstances of natural anoxia exposures. For example, species that experience cyclical daily anaerobic excursions (e.g. intertidal bivalve molluscs) might require a different antioxidant defense strategy than species that experience anoxia only rarely or seasonally. A recent study of the effects of anoxia or freezing exposures on garter snakes found that each stress induced increases in the activities of different enzymes (anoxia stimulated SOD, freezing stimulated CAT and GPOX) that could serve to deal with oxidative stress during recovery (Hermes-Lima and Storey 1993). These stress-induced changes (strategy 2) appear to be effective for a species that probably experiences anoxia/ischemia only rarely. Severe hypoxia also stimulated large increases in organ SOD activities in carp *Cyprinus carpio* (Vig and Nemcsok 1989). Hibernating ground squirrels also showed a similar elevation of antioxidant defenses during hibernation, particularly in brown adipose tissue, apparently in anticipation of the burst of ROS generated by the

intense metabolic activity of thermogenesis in this tissue during the arousal process (Buzadzic et al. 1990b).

Freshwater turtles, as diving species, normally experience wide variation in tissue oxygen levels. Most normal diving is aerobic but oxygen tensions fall throughout the dive (Jackson 1968) and circulatory readjustments, particularly during extended dives, may lead to severely hypoxic conditions in some organs as oxygen is preferentially shunted to others (Davies 1989). Winter hibernation underwater is characterized by extended severe hypoxia or even complete anoxia and turtles can endure this (at low temperatures) for weeks or months at a time (Herbert and Jackson 1985, Ultsch 1989). Thus, turtles live under conditions where blood and organ oxygen tensions vary over wide ranges naturally and where episodes of hypoxia/anoxia exposure can be frequent and unpredictable in time or duration. The strategy that probably suits this lifestyle is to maintain antioxidant defenses at constant high levels continuously in order to limit the production of ROS after any episode of hypoxia or anoxia.

Indeed, this seems to be the strategy indicated by the present results. T. s. elegans exhibited high activities of antioxidant enzymes in all organs tested. For example, liver CAT, liver SOD, and liver and white muscle selenium-dependent GPOX were 3, 6, and 2-7 fold higher than these enzyme activities in the same tissues of the anoxia tolerant garter snake (See Figures 1-4, Table I in Hermes-Lima and Storey 1993). All antioxidant enzyme activities were 2-7 fold higher in turtle red muscle than they were in red muscle of garter snakes. Liver GPOX was 2 and 4 fold higher and red muscle SOD was 1.7 and 5 fold higher than in the corresponding organs in wood and leopard frogs respectively (Joanisse and Storey 1996a). Likewise red muscle CAT was 40 fold higher than that found in frogs. SOD activities in liver and brain of turtles were also each about 2-fold higher than the corresponding activities in carp organs (Vig and Nemcsok 1989). Comparisons can also be made between turtle liver and mammalian liver. GPOX activities were similar in turtle and rat liver, both being about 6-fold higher than in

ground squirrels (Buzadzic et al. 1990a). Activities of SOD in turtle liver were also very similar to those of mammalian liver but CAT activity was lower than in mammals (Buzadzic et al. 1990a). Specific activities of AHR in liver and red muscle were comparable to specific activities found in cells engineered to overexpress a number of proteins involved in protection from oxidative damage, including AHR (*oxyR* mutants of Salmonella typhimurium; 8.3 mU/mg protein (NADPH), 6.7 mU/mg protein (NADH); (Jackson et al. 1989)). AHR in control heart of turtles had twice the specific activity of the same enzyme found in S. typhimurium. It appears, then, that despite the much lower aerobic metabolic rate of ectothermic turtles compared with endothermic mammals, that anoxia-tolerant turtles have antioxidant enzyme activities in the ranges characteristic of mammalian tissues. These activities are, in general, considerably higher than those seen in other ectothermic lower vertebrates. This would provide turtle organs with a high constitutive level of antioxidant defenses that would serve to prevent or buffer damage due to bursts of ROS generation during reoxygenation after submergence.

Given the high constitutive antioxidant defenses of turtle organs and the suppressed metabolic rate during anoxia, it is perhaps not surprising that, in general, anoxia stress and aerobic recovery produced relatively few changes in organ antioxidant enzyme activities. Nonetheless, several significant effects were noted in specific organs. Liver, heart, kidney, and brain all showed decreased activities of selected enzymes after 20 h anoxia exposure: SOD decreased in liver, CAT decreased in heart, CAT and total GPOX decreased in kidney, and CAT and SOD decreased in brain. All of these changes were consistent with a reduced potential for damage by ROS in the anoxic state and organ-specific differences may be related to the particular metabolism of the individual organs. For example, both enzymes that catabolize peroxides, catalase and GPOX, decreased dramatically in kidney during anoxia.

After 24 h of aerobic recovery, anoxia-induced changes in brain CAT and SOD persisted but in other organs the changes that occurred during anoxia had been reversed

either partially (kidney CAT) or completely (all others). Activities of several enzymes also showed specific changes during the aerobic recovery period; these included elevated SOD in heart and reduced total GPOX in heart and white muscle. The controlling factors for these changes remain to be explored but elevated enzyme levels in heart could help to deal with an increase in ROS generated during reoxygenation of this tissue.

Mammalian studies have shown that ischemia results in the catabolism of ATP which raises cellular AMP levels. AMP is, in turn, catabolized to a variety of products including adenosine, inosine and finally hypoxanthine and xanthine (McCord 1985). Accumulated xanthine and hypoxanthine provide the substrates for a burst of O_2^- formation by XO as soon as O_2 becomes available again upon perfusion with reoxygenated blood. An increase in Ca^{2+} during hypoxia in mammalian tissues activates Ca^{2+} dependent proteases which take part in the transformation of XDH to XO (Roy and McCord 1983). By contrast with the mammalian situation, freshwater turtles have previously been shown to maintain cellular ATP levels and to buffer changes in Ca^{2+} ion concentration during anoxia (Storey and Storey 1990). Correspondingly, tissues did not show any changes in the XO/XDH status during anoxia and recovery. However, a high percentage of the total (XO + XDH) activity in turtle tissues was XO activity (between 35 and 75 %; average approximately 50%). The ratio of XO/Total represents the potential for oxidative stress from XO; the higher the ratio, the higher the potential for XO production of O_2^- . The ratio in turtle tissues is much higher than that found in rat heart (Elsayed and Tierney 1989), isolated rat hepatocytes (de Groot and Littauer 1988) or isolated Chinese hamster V79 cells (Hasan et al. 1991). This ratio in turtle tissues is also higher than in tissues of most molluscs (Dyken and Shick 1992, Hermes-Lima and Storey 1995b). The reason for such high XO/Total ratio in the absence of significant AMP catabolism remains unclear. Freshwater turtles have high potential for oxidative stress from this enzymatic source upon the reintroduction of oxygenated blood to tissues

if significant concentrations of xanthine are present in tissues. Future studies will focus on the levels of this intermediary metabolite in tissues.

Fatty acids are one of the primary targets of damage by ROS and various assays have been developed that assess different stages in the chain of events in the lipid peroxidation process. The present study measured conjugated dienes, lipid hydroperoxides and TBARS which represent the initial, middle and final stages of the lipid peroxidation process respectively. The data show two key points. Firstly, the levels of lipid peroxidation products varied between organs. Overall, liver and kidney had the highest levels of these products. Liver was the only organ in which conjugated dienes could be detected and values for TBARS were as much as 20-fold higher than in white muscle. However, liver TBARS values were within the wide range of about 20-150 nmol/gww reported for livers from a range of vertebrate species (Lopez-Torres et al. 1993). Apparently, then, metabolic conditions in liver create the greatest scope for lipid peroxidation damage among the four organs tested. Secondly, it is clear that anoxia and aerobic recovery occurred with very little change in the levels of lipid peroxidation products in any organ. A similar situation is found in freeze tolerant frogs which show no accumulation of lipid peroxidation damage products either during freezing or during recovery from a frozen state (Joanisse and Storey 1996a). ROS may not be formed in the initial stages of recovery due to the efficient radical scavenging systems in place. This indicates that the aerobic recovery process for turtles can be accomplished with very little oxidative stress and supports the idea that the high levels of antioxidant enzymes in turtle organs are effective in preventing lipid peroxidation damage during reoxygenation.

Exceptional facultative anaerobes, therefore, deal with the potential of ROS generation during the anoxic to aerobic transition by maintaining constitutively high activities of antioxidant enzymes and a large glutathione pool (Joanisse and Storey 1996a, Willmore and Storey 1997a,b). Under these circumstances, the formation of ROS during reperfusion may be effectively quenched so that no net oxidative damage (and therefore

no oxidative stress) occurs. By contrast, animals that only infrequently experience oxidative stress appear to initiate adaptive changes in their antioxidant defenses during the stress period that minimize oxidative damage during recovery (Hermes-Lima and Storey 1993). Biochemical adaptation for anoxia tolerance in hibernating freshwater turtles must therefore include continuously high activities of the antioxidant enzymes to deal with the potential for ROS formation during the reoxygenation of organs after anoxia submergence.

Fig. 6. Effect of anoxia exposure and aerobic recovery on catalase activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., $n = 3-4$, expressed as units per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia.^a - Significantly different from the corresponding control value, $P < 0.05$ ^b - $P < 0.01$.

Fig. 7. Effect of anoxia exposure and aerobic recovery on superoxide dismutase activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., $n = 3-4$, expressed as units per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia.^a - Significantly different from the corresponding control value, $P < 0.05$ ^b - $P < 0.01$.

Fig. 8. Effect of anoxia exposure and aerobic recovery on alkyl hydroperoxide reductase activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., n = 3-4, expressed as milliunits per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia.^a - Significantly different from the corresponding control value, $P < 0.05$ ^b - $P < 0.01$.

Table 2. Activities of xanthine oxidase and total (xanthine oxidase and dehydrogenase) in *Trachemys scripta elegans* tissues during anoxia and recovery from anoxia.

Tissue	Condition	n	Xanthine Oxidase mUnits/mg protein	Total mUnits/mg protein	% XO
Liver	control	9	66.1 ± 9.40	102.4 ± 12.86	65.0 ± 5.24
	anoxic	8	56.5 ± 17.3	106.0 ± 28.20	50.2 ± 3.52
	recovered	8	90.3 ± 19.7	153.4 ± 25.04	57.1 ± 5.12
Red muscle	control	6	9.72 ± 2.62	24.2 ± 12.7	56.6 ± 9.47
	anoxic	6	19.2 ± 5.67	48.8 ± 18.3	68.6 ± 18.6
	recovered	6	31.5 ± 14.6	65.5 ± 28.1	62.9 ± 19.8
White muscle	control	6	19.6 ± 14.3	38.0 ± 13.6	35.8 ± 14.7
	anoxic	6	45.1 ± 13.9	87.1 ± 23.4	72.6 ± 24.9
	recovered	6	46.4 ± 22.5	140.5 ± 60.04	42.1 ± 14.1
Kidney	control	5	53.9 ± 12.8	140.9 ± 42.67	41.8 ± 3.32
	anoxic	6	35.8 ± 4.81	82.0 ± 21.5	49.3 ± 6.00
	recovered	7	51.8 ± 5.53	114.2 ± 19.00	49.2 ± 4.43
Heart	control	6	123.1 ± 40.35	162.0 ± 47.85	74.9 ± 5.65
	anoxic	7	159.7 ± 60.98	226.2 ± 69.26	67.7 ± 7.50
	recovered	8	117.4 ± 25.26	196.3 ± 35.69	61.1 ± 7.96
Brain	control	7	7.39 ± 0.83	13.1 ± 0.93	57.0 ± 6.02
	anoxic	8	22.3 ± 3.36 *	40.0 ± 9.54	69.1 ± 9.50
	recovered	8	17.8 ± 6.10	42.9 ± 16.8	49.1 ± 12.6

Note. Data are means ± SEM with n values as indicated. * Significantly different from controls as determined by the Dunnett's test ($P < 0.05$).

Table 3. Effect of anoxia exposure and aerobic recovery after anoxia on selenium-dependent and total glutathione peroxidase activities in Trachemys scripta elegans organs.

Tissue	Condition	Se-GPOX mU/mg	Total GPOX mU/mg	% Se-GPOX
Liver	control	298 ± 2.0	299 ± 71	99
	anoxic	287 ± 46	198 ± 30	100
	recovered	314 ± 56	237 ± 22	100
Heart	control	152 ± 55	222 ± 57 (3)	68
	anoxic	191 ± 28	113 ± 43	100
	recovered	75 ± 25	56 ± 25 ^a	100
Red muscle	control	244 ± 31	463 ± 84 (3)	53
	anoxic	363 ± 92 (3)	575 ± 116	63
	recovered	188 ± 13	320 ± 31	59
White muscle	control	32 ± 1	13 ± 4	100
	anoxic	31 ± 1	9 ± 2	100
	recovered	29 ± 2	3 ± 2 ^a	100
Kidney	control	282 ± 82	591 ± 63	48
	anoxic	328 ± 77	346 ± 30 (3) ^a	95
	recovered	371 ± 52	502 ± 39	74
Brain	control	79 ± 12	103 ± 17	77
	anoxic	70 ± 2 (3)	107 ± 10	65
	recovered	64 ± 14 (3)	61 ± 4	100

Note. Data are milliunits/mg protein, means ± SEM with n = 4 unless indicated in brackets. ^a - Significantly different from the control values, P < 0.05.

Table 4. Products of lipid peroxidation in organs of control, 20 h anoxic and 24 h aerobic recovered Trachemys scripta elegans.

Tissue	Condition	Lipid Hydroperoxides nmol/gww	TBARS nmol/gww
Liver	control	5230 ± 131 (3)	71.0 ± 14.1 (12)
	anoxic	4216 ± 86 (3)	85.9 ± 16.7 (11)
	recovered	5624 ± 783 (3)	89.0 ± 22.9 (10)
Red muscle	control	843 ± 67 (4)	9.54 ± 0.90 (3)
	anoxic	557 ± 92(5)	6.57 ± 0.81 (3) ^a
	recovered	709 ± 78 (6)	3.83 ± 0.52 (4) ^b
White muscle	control	2901 ± 145 (3)	4.19 ± 0.84 (4)
	anoxic	3260 ± 391 (3)	3.60 ± 0.98 (4)
	recovered	3133 ± 174 (3)	3.92 ± 1.67 (4)
Kidney	control	6506 ± 259 (3)	20.7 ± 1.44 (4)
	anoxic	4852 ± 212 (3)	24.8 ± 0.92 (4)
	recovered	3967 ± 919(3) ^a	29.2 ± 1.62 (3) ^b

Note. Values for lipid hydroperoxides (measured by the xylene orange-Fe³⁺ method and reported in cumene hydroperoxide equivalents) and for thiobarbituric acid reactive substances are reported as nmol/gram wet weight, means ± S.E.M with n indicated in brackets. ^a - Significantly different from the corresponding control value, P < 0.05; ^b - P < 0.01.

CHAPTER FOUR

GLUTATHIONE FUNCTION, GLUTATHIONE-RELATED ENZYMES AND ANOXIA TOLERANCE IN A FRESHWATER TURTLE, TRACHEMYS SCRIPTA ELEGANS

INTRODUCTION

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the ability to defend against, absorb or repair the damage that radicals cause (Halliwell and Gutteridge 1989). When the balance is tipped in favour of oxidative stress, the intracellular over-generation of such radicals may cause damage to structural proteins and enzymes, membrane lipids, and nucleic acids and thus, affect cell function in numerous ways (Jones 1985). Since ROS originate with oxygen as a reactant, a decrease in their production is expected under anoxic conditions.

Recent studies, however, have focused on production of ROS during anoxia (Rifkind et al. 1993). ROS formed under reduced oxygen pressures may contribute to anoxic cellular damage and such damage may prime the cell for the subsequent formation of ROS upon perfusion with reoxygenated blood. ROS are produced during anoxia from redox cycling, the autoxidation of proteins, and the leakage of radicals from the electron transport chain (Dawson et al. 1993, Rifkind et al. 1993).

Mammalian tissues are notoriously sensitive to anoxia and acidosis. Freshwater turtles, however, are remarkably tolerant of these stresses both in vitro and in vivo. The red-eared slider (T. s. elegans) is one of the best facultative anaerobes among freshwater turtles; second only to Chrysemys species (Ultsch 1985). Turtles submerged in oxygen-depleted water can endure several days of severe anoxia at elevated temperatures and as long as 3 months during winter hibernation at low temperatures (0-5°C). A decrease in ATP production in anoxia is matched by decreased ATP utilization (total ATP output is maintained) as the result of a profound metabolic rate depression (Storey and Storey 1990). The cytochromes in Complex IV of the mitochondrial electron transport chain become highly reduced (Lutz et al. 1984). Electrons are, therefore, readily available for the formation of ROS. Turtles must have mechanisms which prevent the formation of reactive oxygen species or which minimize oxygen free radical damage during oxygen

reperfusion in the recovery period. Such mechanisms could involve the use of both enzymatic and nonenzymatic antioxidant species. The most important of the nonenzymatic antioxidants is glutathione.

Reduced glutathione (GSH) is a hydroxyl radical and singlet oxygen scavenger (Halliwell and Gutteridge 1989, Meister and Anderson 1983). It is used in the metabolism of H_2O_2 and lipid hydroperoxides (formed as a result of oxygen radical generation) in a glutathione peroxidase-catalyzed reaction. Since GSH is present in high intracellular concentrations, there is a high probability that any reactive oxygen species will be immediately quenched upon formation (Jones et al. 1981). Oxidized glutathione (GSSG) is formed but is subsequently enzymatically reduced by glutathione reductase to restore GSH. When ROS are present in large amounts, GSSG formation can exceed its clearance and the ratio of reduced to oxidized glutathione (GSH/GSSG) decreases. This ratio is frequently used as an indicator of the level of oxidative stress in cells. GSSG itself can exert deleterious effects by reacting with the free sulfhydryl groups of proteins to form mixed disulfides. This may lead to the inactivation of enzymes with sulfhydryl groups at their active sites (Halliwell and Gutteridge 1989). The maintenance of GSH levels, and thereby the reducing environment of the cell, is crucial to organisms which periodically undergo oxidative stress.

To investigate the role of glutathione in vertebrate anoxia tolerance, we chose to study the effects of anoxia exposure and aerobic recovery on the activities of proteins which catalyze reactions involving GSH and the status of glutathione in the red-eared slider turtle, T. s. elegans. We were particularly interested in any adjustments to the glutathione-related enzymes that are initiated as early responses to anoxic submergence and could, therefore, contribute to a reorganization or suppression of glutathione metabolism as one part of the overall metabolic readjustments that support long term anaerobiosis. The present study shows that constitutively high glutathione levels in turtle organs would prevent the formation of or minimize damage by reactive oxygen species

during transitions between anoxic and aerobic states. As well, enzymatic degradation of glutathione was greatly reduced during anoxia in order to maintain the glutathione ratio for use in subsequent recovery from anoxia.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO or Boehringer Mannheim Corp., Montreal, Quebec. Adult red-eared slider turtles (*T. s. elegans*) were obtained from Wards Natural Science, Mississauga, Ont. and were maintained in large tanks of dechlorinated water at 7°C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed ad libitum on a diet of trout food pellets, lettuce and egg shells.

Control (normoxic) turtles were sampled directly from the tank. Anoxia was imposed by submerging turtles for 20 h at 5°C in deoxygenated sealed tanks of water that had been previously bubbled with 100 % nitrogen gas for 1 h. At low temperature this length of anoxia exposure is a relatively mild stress (Ultsch 1985) but our aim here was to determine what metabolic responses are put in place within the early hours of the anoxia stress in order that the animal can endure prolonged stress. Some turtles were sampled immediately at the end of anoxia treatment whereas others were returned to the aerobic holding tank and allowed to recover for 24 h before sampling. All animals were killed by decapitation and organ samples were quickly removed, frozen in liquid nitrogen and then transferred to -80°C for storage.

Glutathione S-transferase (GST; E.C. 2.5.1.18)

GST was assayed by monitoring the formation of the thioether product of the reaction between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) (ϵ

= 9.6 mMolar⁻¹) at 340 nm (Habig and Jakoby 1981). Frozen tissue samples were quickly weighed and homogenized 1:5 (w/v) in ice-cold 50 mM potassium phosphate buffer (pH 7.5, containing 1 mM EDTA) and with phenylmethylsulfonyl fluoride (1 mg/ml) added immediately before homogenizing using an Ultra-Turrax (Tekmar) tissue homogenizer. Homogenates were then sonicated for 10 s on ice with a Kontes micro-ultrasonic cell disrupter and centrifuged at 16,000 x g for 15 min at 4°C using an Eppendorf microcentrifuge. Supernatants were removed and desalted by passage through a small column (1 x 5 cm) of Sephadex G-25 (equilibrated in homogenizing buffer) with centrifugation for 1 min in an IEC benchtop centrifuge at full speed (Helmerhorst and Stokes 1980). One ml of reaction medium contained 50 mM potassium phosphate (KPi) buffer (pH 6.5), 1 mM EDTA, 6 mM GSH and 1 mM CDNB. The reaction was initiated by the addition of 5 µl of a 1:5 v/v dilution of enzyme extract. Blanks were run in the absence of either GSH or enzyme. One unit of activity is defined as the amount of enzyme that formed 1 µmol of product per min at 21°C.

Glutathione reductase (GR; E.C. 1.6.4.2)

GR was assayed according to Dillio et al. (1983) by following the oxidation of 0.2 mM NADPH at 340 nm in media containing 50 mM KPi (pH 7.4), 1 mM EDTA and 0.1 mM oxidized glutathione (GSSG). Enzyme extracts were prepared as described for GST and the reaction was initiated by the addition of 10 µl of extract. Blanks were run in the absence of either GSSG or enzyme. One unit is defined as the amount of enzyme that oxidized 1 µmol of NADPH ($\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$) per min at 21°C.

Glutathione synthetase (GS; E.C. 6.3.2.3)

The synthesis of glutathione by glutathione synthetase (GS) was measured by monitoring reduced glutathione conjugation to 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (Tanaka et al. 1992). Frozen tissue samples were homogenized 1:5 (w/v) in ice-cold 100

mM Tris-HCl (pH 8) with 1 mg/ml phenylmethylsulfonyl fluoride added using an Ultra-Turrax (Tekmar) tissue homogenizer. Centrifugation and desalting were as described for GST except that the Tris buffer was used for the Sephadex column. Fifty μ l of enzyme extract was incubated with 300 μ l of a reaction mixture containing 100 mM Tris-HCl (pH 8), 5 mM γ -L-glutamyl-L-cysteine (Kohjin Co. Ltd, Tokyo, Japan), 15 mM glycine, 10 mM ATP, and 10 mM MgSO_4 for 24 h at 21°C. The reaction was stopped by immersion in boiling water for 3 min. Samples were then centrifuged (15,000 rpm, 4°C, 5 min) in an Eppendorf microcentrifuge. Two hundred μ l of the supernatant was then assayed for reduced glutathione as described below. One unit of enzyme is defined as that which produces 1 μ mol glutathione per hour at 21°C.

γ -Glutamyl transpeptidase (γ -GTPase; E.C. 2.3.2.2)

γ -GTPase was assayed according to Tate (1985). Enzyme extracts were prepared as described for GS and aliquots (200 μ l liver, 10 μ l kidney, 800 μ l red muscle, white muscle, heart, 500 μ l brain) were added to cuvettes containing 100 mM Tris-HCl (pH 8), 1 mM L- γ -glutamyl-p-nitroanilide, and 20 mM glycylglycine. The release of p-nitroanilide was monitored at 410 nm ($\epsilon = 8800 \text{ M}^{-1}\text{cm}^{-1}$). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μ mol of p-nitroanilide per minute at 21°C.

Glutaredoxin (GRN)

Glutaredoxin catalyses reduction of certain disulfides by GSH and thus has GSH-disulfide-transhydrogenase or oxidoreductase activity. The method of assaying the protein's activity measures the interaction of GSH with hydroxyethyl disulfide (HED; Aldrich 98%) in a spectrophotometric assay (Holmgren 1985). Enzyme extracts were prepared as described for GS except for the further addition of 2 mM EDTA to the homogenization buffer. In two semimicro quartz cuvettes, 500 μ l of the following

mixture was prepared in an icebath: 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.4 mM NADPH, 0.1 mg/ml bovine serum albumin, 1 mM reduced glutathione and 1 Unit of glutathione reductase. Twenty five μ l of 15 mM HED (0.7 mM final concentration) was added to each cuvette to initiate the reaction. The cuvettes were placed in the sample and reference holders of the spectrophotometer and the absorbance at 340 nm was recorded for 2 min to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between GSH and HED. Fifty μ l of supernatant was then added to the sample cuvette (and an equal amount of homogenization buffer in the reference cuvette) and the decrease in absorbance was measured over the next 8 min. The change in absorbance was calculated and one unit of activity was defined as the amount that utilized 1 μ mol of NADPH ($\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$) per min at 21°C.

Reduced and oxidized glutathione (GSH/GSSG)

The assay for glutathione is based on the reaction of glutathione with DTNB (Tietze 1969). Tissue samples were homogenized 1:5 (w/v) in ice-cold sulfosalicylic acid (previously bubbled with N₂ gas for 10 min), then bubbled with N₂ gas for 10 s and centrifuged at 16,000 x g in an Eppendorf microcentrifuge for 5 min. Aliquots of supernatant were diluted 1:20 (v/v) with water and used immediately for the measurement of total glutathione equivalents ($\text{GSH}_{\text{eq}} = \text{GSH} + 2 \text{ GSSG}$). GSH equivalents were determined by following the rate of reduction of DTNB by GSH at 412 nm and comparing this to a GSH standard curve (0-0.8 μ molar GSH). Samples were added to an assay medium containing 100 mM sodium phosphate (NaPi) buffer (pH 7.5), 5 mM sodium EDTA, 0.21 mM NADPH and 0.6 mM DTNB and then the reaction was started with the addition of 0.5 units/ml GR. GSSG alone was measured in another aliquot of sample after first derivatizing GSH in the sample with 2-vinylpyridine (Griffith 1980). Tissue extracts (undiluted; 0.5 ml) were mixed with 100 μ l of 0.5 M 2-vinylpyridine and

1 ml of 0.5 M KPi, pH 7.0. The pH did not deviate from 7.0. After derivatization for 1 h at room temperature, GSSG was measured (as 2 GSH) using the assay above.

Protein

Soluble protein in tissue extracts was measured by the Coomassie blue dye-binding method (Bradford 1976) using the Bio-Rad prepared reagent and the micro assay procedure with bovine serum albumin as the standard.

Statistics

Values are reported as means $\pm$ S.E.M. Statistical testing used one-way analysis of variance followed by the Dunnett's two-tailed test to analyze the data. Calculations were performed using a statistical program for the analysis of enzyme kinetics (Brooks 1992).

RESULTS

Figure 9 shows the activity of GST in six organs of T. s. elegans under 3 metabolic conditions: aerobic controls, 20 h anoxia exposure, and 24 h aerobic recovery after anoxia. Highest GST activities were detected in kidney and liver, values for control organs being 4.1 ± 0.2 and 2.1 ± 0.3 units/mg protein whereas GST activity in other organs was less than 0.4 U/mg in all cases. In vivo anoxia exposure affected GST activity in liver and heart; activity in both organs decreased, by 25 and 42 % respectively, in organs of anoxic, compared with control, turtles. By contrast, GST activity changed during recovery from anoxia in other organs; compared with controls, activity decreased by 72, 56 and 39 %, respectively, in red skeletal muscle, kidney, and brain.

Figure 10 shows the effects of anoxia exposure and aerobic recovery on GR activities in turtle organs. GR activity was highest in kidney, 231-320 mU/mg, but ranged from 7-82 mU/mg in other organs. Anoxia exposure stimulated an increase in GR activity

in liver (by 52%) and red muscle (by 80%) but activity decreased by 67% in anoxic heart. During aerobic recovery, GR activity in liver and red muscle was again reduced whereas GR in heart rebounded to a value significantly greater (by 64%) than the initial control value. GR activities in white skeletal muscle, kidney and brain were unaffected by experimental state.

Activities of GS in turtle organs are shown in Figure 11. Low rates of catalytic activity required long incubation periods in order to produce measurable product. Activities were highest in heart and brain. Anoxia resulted in a 3 fold increase in activity over control values in white muscle. GS activity increased 2 fold during aerobic recovery compared to controls in heart and brain. GS activity remained unchanged during experimental treatment in liver, red muscle, and kidney.

Figure 12 shows the levels of γ -GTPase activity in turtle tissues. Kidney showed the highest activity, at least 10 fold greater than that of other tissues. Kidney γ -GTPase activity decreased dramatically during anoxia from 4.64 to 0.07 mUnits/mg protein. However, γ -GTPase activity returned to control values after 24 h recovery from anoxia. Liver also showed a 71% decrease in γ -GTPase activity during anoxia but this rebounded again during recovery. Brain activity increased to a value 63% above the control during recovery from anoxia. Red muscle, white muscle and heart activities did not change during experimental treatment.

GRN activity was highest in white muscle and heart of turtles (Figure 13). Activity was unchanged by anoxia or aerobic recovery in five tissues (liver, kidney, heart, spleen and brain). However, GRN decreased by 56% compared with controls during anoxia in red muscle and remained low during the recovery period. By contrast, activity in white muscle increased 4 fold during anoxia but returned to control levels during aerobic recovery.

With the exception of liver, the glutathione status indicated little or no oxidative stress in T. s. elegans during anoxia or recovery from anoxia. Glutathione status is

commonly used as an indicator of oxidative stress (Halliwell and Gutteridge 1989, Ji 1995) and this was largely unchanged in *T. s. elegans* tissues during anoxia or recovery from anoxia. Levels of total and oxidized glutathione are shown in Table 5 along with the GSH/GSSG ratio. Total glutathione content decreased significantly during anoxia in four organs, dropping to 49, 67, 54, and 59 % of control values in liver, heart, white muscle, and kidney, respectively. In all four organs, total glutathione also remained lower than control values during the aerobic recovery period. However, total glutathione content was unaffected by experimental state in red muscle and brain. GSSG content was also affected by experimental state. In white muscle and kidney GSSG fell along with the total glutathione content although in kidney the more pronounced change in GSSG resulted in a 6-fold increase in the GSH/GSSG ratio in anoxia that was sustained after 24 h of recovery. GSSG also decreased in brain, leading to a 3-fold increase in the GSH/GSSG ratio in anoxia (although this was not a significant change) and a 10-fold increase in the ratio during recovery. GSSG content rose in liver during anoxia and coupled with the decrease in total glutathione in the organ this led to a sharp decrease in the GSH/GSSG ratio in anoxia. GSSG remained reduced in white muscle, kidney and brain after 24 h aerobic recovery but in liver GSSG returned to control values. Heart showed a different pattern with GSSG dropping only during recovery, leading to a 5-fold increase in the GSH/GSSG ratio in the organ.

DISCUSSION

The maintenance of the GSH/GSSG ratio is of prime importance to the redox status of the cell; therefore, the ratio of GSH/GSSG is normally maintained at a very high value (Halliwell and Gutteridge 1989). If tissues are exposed to large fluxes of ROS, this value decreases and GSSG accumulates. Low molecular weight thiols, by forming disulfide bonds amongst themselves, act as a natural reservoir of reducing power which

can be rapidly mobilized as a cellular defense against oxidative stress (Del Corso et al. 1994). However, glutathione disulfides are not the only disulfides formed under oxidizing conditions. Mixed disulfides formed between GSH and proteins or GSH and small molecular weight compounds (such as coenzyme A) accumulate in tissues subjected to oxidative stress. Mixed disulfide formation can inactivate a number of enzymes and may also inhibit protein synthesis. Not only is protein synthesis affected, but proteins which form mixed disulfides are rendered more susceptible to proteolytic degradation by exposing allosteric regions recognized by many proteases (McKay et al. 1985, Offermann et al. 1984). Therefore, it is essential to maintain cellular glutathione redox status to prevent metabolic disruption.

Turtles maintain an enhanced antioxidant capacity to deal with potential ROS formation upon aerobic recovery after diving or underwater hibernation. When hibernating, pO_2 reaches a critical low. The cytochromes of the mitochondrial respiratory chain are rapidly reduced, aerobic ATP production is suppressed, and glycolytic ATP production is activated (Storey and Storey 1990). Below a critical pO_2 , however, an overall metabolic rate depression is initiated that conserves energy expenditures and lowers ATP demands to a rate that can be maintained over the long term by glycolytic ATP output alone; indeed, overall metabolic suppression can be so great that glycolytic rate also falls (Kelly and Storey 1988). Upon recovery, electrons are readily available from Complex IV for superoxide formation. As mentioned above, an accumulation of GSSG is the main result of such oxyradical formation. Turtles must therefore maintain a high ratio of GSH/GSSG to prevent the damaging effects of oxidative stress. Controlled synthesis and degradation of glutathione by GS and γ -GTPase, respectively, may maintain this ratio.

With the exception of GS and GRN, highest activities of the glutathione-related enzymes in control tissues were found in the kidney. The kidney is the major organ responsible for glutathione removal from blood circulation and at least 50 to 65 % of the

net plasma glutathione turnover occurs there (Griffith and Meister 1979, Häberle et al. 1979). GST activity decreased by at least half, compared with controls, in kidney during recovery from anoxia. Decreased GST activity during aerobic recovery may be an attempt to conserve glutathione levels when GSH is required for redox status. GR remained unchanged with experimental treatment but its activity in kidney was at least twice that in other tissues. The most dramatic change in kidney, however, was the almost total disappearance of γ -GTPase activity during anoxia, the consequence of which would be a nearly complete shutdown of the degradation of glutathione during anoxia. This is clearly a critical early response to the initiation of anoxic conditions that probably functions to readjust glutathione metabolism for long term anoxia survival. Thus, enzyme activity was suppressed to just 2 % of control values within 20 h of anoxia exposure at 5°C although this species can endure 2-3 months under the same conditions. Targeting this enzyme may help to maintain the GSH/GSSG ratio during anoxia in preparation for subsequent recovery from anoxia or this may be a key part of the mechanism by which net glutathione turnover could be strongly suppressed as one facet of overall anoxia-induced metabolic rate depression. Both total and reduced glutathione pools in kidney were decreased during anoxia and remained low during aerobic recovery. The reason for the overall net decrease in glutathione levels is not yet known. In their detoxification role, GSTs conjugate reduced glutathione to electrophilic substrates including xenobiotics, lipid hydroperoxides (including prostaglandins), and nucleotide hydroperoxides. A gradual accumulation of damage products during anoxia may possibly be controlled in this manner. Since no evidence of oxidative stress was seen (Willmore and Storey 1997a,b), it is unlikely that glutathione is being conjugated to damage products. The ratio of GSH/GSSG itself actually increased over controls indicating that oxidative stress was not occurring. Thus, anoxia tolerant turtles do not attempt to maintain total glutathione levels but control the breakdown of GSH in order to maintain a high GSH/GSSG ratio.

In all other organs, activities of the glutathione-related enzymes were lower. GST activities decreased during aerobic recovery in red muscle and brain, probably for the same reasons they did in kidney. An increased catalytic activity of GR during anoxia in liver suggests a need to reduce the higher amounts of GSSG occurring in this tissue during stress. GR activity decreased sharply in turtle heart during anoxia. This result was surprising in that GR was previously reported not to change during ischemia-reperfusion in rabbit heart (Curello et al. 1985). GS increased in heart and brain during recovery from anoxia in order to replenish total glutathione pools in these key tissues. GRN increased dramatically in white muscle during anoxia. This protein is used in the reduction of ribonucleotides to their corresponding deoxyribonucleotides, required for DNA synthesis, by ribonucleoside diphosphate reductase (Holmgren 1985). GRN couples the oxidation of GSH to the reduction of ribonucleotides. An increase in white muscle GRN activity may reflect an increased need for reduced ribonucleotides for DNA synthesis during anoxia. This need is reflected in an increase in translatable mRNA present in white muscle during anoxia (Douglas et al. 1994) that is not found in other organs. The reasons for an increased requirement for nucleic acids in this organ during anoxia remains speculative as increases in mRNA levels did not result in an overall increased protein synthesis or the appearance of new proteins (Douglas et al. 1994). Decreases in total glutathione in white muscle during anoxia may be the result of increased utilization of GSH by GRN.

A general pattern of response by organ glutathione levels to anoxia exposure and subsequent aerobic recovery was seen. Total glutathione (GSH + GSSG) fell by up to 50 % in heart and kidney along with a proportionally greater decrease in GSSG, such that the ratio GSH/GSSG actually increased. Total glutathione did not decrease during anoxia or normoxic recovery in the brain, however, but a drop in GSSG levels resulted in an increase in the GSH/GSSG ratio. The status of the GSH/GSSG ratio during anoxia is consistent with the more reduced state of many other cellular components (e.g. cytochromes, NADH) when oxygen is depleted (Lutz et al. 1984). This ratio appeared to

be maintained by the enzymes of glutathione metabolism. γ -GTPase activity decreased during anoxia in kidney; possibly to limit the breakdown of GSH. GS activity increased in key tissues during long term recovery in an attempt to replenish depleted GSH stores. Transferase activity decreased during recovery in red muscle, kidney and brain; possibly to restrict the amount of GSH conjugated to oxidative damage products. Increased reductase activity occurred in some tissues during the anoxic and recovery periods. A decrease in total peroxidase activity in some tissues during recovery from anoxia would also limit the amount of GSH utilized for reduction of hydrogen peroxide and lipid hydroperoxides (since these damage products do not accumulate during recovery from anoxia; see Willmore and Storey 1997a). These changes indicate a need to maintain the reduced status of glutathione as opposed to the maintenance of the levels of total glutathione within the cell.

The upkeep of constant, high antioxidant defenses by turtle organs is illustrated by organ levels of glutathione which were high compared with other ectothermic vertebrates. Total glutathione in liver of control turtles was 3.4 $\mu\text{mol/gww}$ compared with 0.9-2.2 $\mu\text{mol/gww}$ reported for liver glutathione in fish and amphibian species (Lopez-Torres et al. 1993, Joannis and Storey 1996a). Glutathione levels in turtle liver were closer to the levels in avian liver (2.5-3.7 $\mu\text{mol/gww}$) although lower than values reported for mammalian liver (6-8 $\mu\text{mol/gww}$) (Lopez-Torres et al. 1993). The glutathione contents of other turtle organs were very similar to values reported for the comparable organs in rats (Halliwell and Gutteridge 1989). High redox buffer capacity, attributed to augmented GSH levels, has been postulated as a mechanism preventing reperfusion injury in the hypoxia resistant turtle Phrynosoma hilarii (Reischl 1986) and our results support this idea.

In conclusion, anoxia tolerant turtles reduce the activities of enzymes involved in the utilization/degradation and increase the activities of enzymes involved in the production of reduced glutathione in key tissues during anoxia. These changes are put in place as relatively early responses to anoxic stress (within 20 h in an animal that can

withstand several weeks of the same stress at 5°C) indicating that they constitute important adjustments to glutathione metabolism that contribute to anaerobiosis. Oxidative stress does not occur in diving turtles either during anoxia or during recovery from anoxia as revealed by the glutathione ratio in tissues. Anoxia tolerant turtles maintain high levels of reduced glutathione in comparison to other vertebrates. Further studies will determine the controlling signals that regulate the activities of the glutathione-related enzymes in these animals with respect to varying oxygen availability.

Fig. 9. Effect of anoxia exposure and aerobic recovery on glutathione S-transferase (GST) activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., n = 3-4, expressed as units per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia. ^a - Significantly different from the corresponding control value, $P < 0.05$; ^b - $P < 0.01$.

Fig. 10. Effect of anoxia exposure and aerobic recovery on glutathione reductase (GR) activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., n = 3-4, expressed as milliunits per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia. ^a - Significantly different from the corresponding control value, $P < 0.05$; ^b - $P < 0.01$.

Fig. 11. Effect of anoxia exposure and aerobic recovery on glutathione synthetase activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., $n = 3-4$, expressed as milliunits per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia. ^a - Significantly different from the corresponding control value, $P < 0.05$; ^b - $P < 0.01$.

Fig. 12. Effect of anoxia exposure and aerobic recovery on γ -glutamyltranspeptidase activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., n = 3-4, expressed as milliunits per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia. ^a - Significantly different from the corresponding control value, $P < 0.05$; ^b - $P < 0.01$.

Fig. 13. Effect of anoxia exposure and aerobic recovery on glutaredoxin activity in Trachemys scripta elegans tissues. Values are means $\pm$ S.E.M., n = 3-4, expressed as milliunits per mg protein. Bars are: open, control; hatched, 20 h anoxia exposure, and filled, 24 h aerobic recovery after anoxia. ^a - Significantly different from the corresponding control value, $P < 0.05$; ^b - $P < 0.01$.

Table 5. Total (GSH + GSSG) and oxidized (GSSG) glutathione (nmol/gram wet weight) levels in control, 20 h anoxia exposed and 24 h aerobic recovered *Trachemys scripta elegans*.

Organ	Condition	Total Glutathione	GSSG	GSH/GSSG ratio
Liver	control	3360 ± 265 (5)	10.4 ± 3.0 (5)	454 ± 98 (5)
	anoxic	1666 ± 106 ^b	21.0 ± 1.8 ^b	80 ± 5 ^b
	recovered	2328 ± 177 ^b	9.0 ± 1.7	321 ± 74
Heart	control	1563 ± 112	19.5 ± 4.9	95 ± 20
	anoxic	1051 ± 127 ^b	12.2 ± 3.9	158 ± 59
	recovered	1222 ± 59 ^a	2.6 ± 0.1 ^b	483 ± 39 ^b
Red muscle	control	946 ± 195	5.2 ± 1.4	195 ± 21
	anoxic	634 ± 69	3.0 ± 1.1	290 ± 73
	recovered	847 ± 133	2.5 ± 0.5	327 ± 72
White muscle	control	1541 ± 196	6.0 ± 0.9	284 ± 57
	anoxic	835 ± 125 ^b	1.9 ± 0.2 ^b	460 ± 84
	recovered	732 ± 123 ^b	2.2 ± 0.4 ^b	336 ± 46
Kidney	control	3120 ± 229 (3)	42 ± 6.2 (4)	65 ± 4 (3)
	anoxic	1836 ± 94 (3) ^a	5.0 ± 0.6 (4) ^b	410 ± 34 (3) ^a
	recovered	1383 ± 380 (3) ^b	3.6 ± 0.6 (4) ^b	441 ± 89 ^a
Brain	control	1490 ± 372 (4)	84 ± 17 (3)	23 ± 7 (3)
	anoxic	1527 ± 65 (4)	20 ± 8 (4) ^b	83 ± 25 (4)
	recovered	1685 ± 200 (4)	6.9 ± 0.7 (4) ^b	247 ± 29 (4) ^b

Values are means ± S.E.M. with n = 6 unless otherwise indicated in brackets. a - Significantly different from the corresponding control value, $P < 0.05$; b - $P < 0.01$.

CHAPTER FIVE

PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S- TRANSFERASES FROM LIVER OF TRACHEMYS SCRIPTA ELEGANS

INTRODUCTION

The glutathione S-transferases (GSTs) belong to a multigene enzyme superfamily which catalyze the nucleophilic addition of the thiol of reduced glutathione (GSH) to a variety of electrophiles (Habig and Jacoby 1981, Ketterer et al. 1988, Mannervik and Danielson 1988, Morgenstern and DePierre 1988, Pickett and Lu 1989, Coles and Ketterer 1990, Rushmore and Pickett 1993). Thus they provide protection, not only against electrophiles which tend to be toxic to the cell, but also oxidants which they reduce.

The GSTs are homodimers or heterodimers comprised of seven different subunits (Pickett and Lu 1989). Five main classes of GSTs exist, each containing more than one isozyme based on substrate affinity, inhibitor properties. The cytosolic classes have been named Alpha, Mu, Pi and Theta and depend upon subunit composition. A fifth class encodes for a microsomal form of the enzyme. Specific GST subunits are induced by various xenobiotics and are expressed in a tissue specific manner (Daniel 1993). The enzymes contain two active sites, a G-site for the binding of GSH and an H-site for the binding of electrophile. Electrophiles have a slow spontaneous rate of reaction with GSH which can be greatly enhanced in the presence of GST.

Electrophilic substrates for GST include xenobiotics such as carcinogens and their metabolites, herbicides and mutagens. As well, the GSTs bind with varying affinities to a variety of hydrophobic compounds such as heme, bilirubin, polycyclic aromatic hydrocarbons and dexamethasone (Rushmore and Pickett 1993). Endogenous second substrates for GST are toxic products generated from tissue damage. These include the

compounds resulting from lipid peroxidation of biological membranes such as reactive alkenes, epoxides, hydroperoxides and aldehydes. These may be the primary substrates of the microsomal or membrane-bound GST in the same way as they are the substrates for Se-dependent glutathione peroxidases (the “classical” and a more recently discovered phospholipid hydroperoxide glutathione peroxidase) (Andersson et al. 1993). Most resulting conjugated products are cytotoxic and therefore must be eliminated.

Glutathione S-conjugated products are exported from the cell (in particular, liver cells where such cytotoxins are transported) via membrane ATP-dependent pump systems (known as the glutathione S-conjugate export pump) (Ishikawa 1992, Ishikawa 1993), converted to mercapturic acid in the kidney and epithelial cells and excreted in the urine.

A decrease in turtle liver and heart GST maximal activities during 20 hours of anoxia (Willmore and Storey 1997b, Chapter 4) indicates that a change in the enzyme(s) occur during stress. This change could be in the form of expression of a different GST isozyme within the same or different class during anoxia. Stress-related changes in the maximal activities of the GSTs have been shown to occur in many stress-tolerant organisms. The maximal activities of these enzymes increase during anoxia exposure in brain of the leopard frog Rana pipiens (Hermes-Lima and Storey 1996) but decrease during freezing in kidney and heart of the wood frog Rana sylvatica (Joannis and Storey 1996a). A decrease in GST(s) activities are seen during overwintering in the freeze-tolerant fly Eurosta solidaginis while an increase in activities is seen in the freeze-avoiding moth Epiblema scudderiana (Joannis and Storey 1996b). A decrease in maximal GST activity occurs during estivation in liver, heart, kidney, lung and gut of the spadefoot toad Scaphiopus couchi (Grundy 1996). As one of the physiological changes

occurring as turtles enter anoxia, turtles may express distinct form(s) of GST. These GST(s) would be better suited for the challenges imposed by the anoxic state.

In the present study, GSTs were purified from liver of the anoxia tolerant turtle T. s. elegans. Kinetic and inhibitory parameters were examined to determine if alternate forms of the enzyme were present. Differences in kinetic properties may explain differences in maximal activities found during anoxia in this tissue.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO., U.S.A.) or from Boehringer Mannheim Corporation (Montreal, Quebec) and were of the highest purity available. Winter acclimated adult red-eared sliders (T. s. elegans) were obtained from Wards Natural Science, Mississauga, Ontario and were maintained in large tanks of dechlorinated water at 7 °C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed ad libitum on a diet of trout pellets, lettuce and egg shells.

Control (normoxic) turtles were sampled directly from the tank. Anoxia was imposed by submerging turtles for 20 h at 5 °C in sealed tanks of 60 L deoxygenated water that had been previously bubbled with 100 % nitrogen gas for 1 h.. A wire mesh placed 20 cm below the surface of the water prevented turtles from surfacing. Some turtles were sampled immediately at the end of anoxia treatment whereas others were returned to aerobic conditions in a 5°C incubator and allowed to recover for 24 h before sampling. All animals were killed by decapitation and organ samples were quickly removed, frozen in liquid nitrogen and then transferred to -80 °C for storage.

GST assay

GST activity was assayed as described in Chapter Four. Optimal substrate concentrations which gave maximal enzyme activity in turtle liver were essentially the same as described previously. Enzymatic analysis were conducted at room temperature (21°C). One unit of activity is defined as the amount of enzyme that formed 1 μ mol of product per min at 21°C.

Preparation of tissue extracts

Frozen tissue samples were quickly weighed and homogenized 1:5 (w:v) in ice-cold buffer containing 5 mM potassium phosphate (pH 6.0), 1 mM EDTA, and a few crystals of PMSF using an Ultra-Turrax (Tekmar) tissue homogenizer. Homogenates were centrifuged at 15,000 rpm for 20 min at 4°C using an Sorvall refrigerated centrifuge. Supernatants were removed and desalted by passage through a small column (1 x 5 cm) of Sephadex G-25 (equilibrated in homogenizing buffer) with centrifugation for 45 seconds in a benchtop centrifuge (International Equipment Company) at a setting of 6 (Helmerhorst and Stokes 1980).

Turtle liver GST purification

The initial purification procedure was optimized for T. s. elegans liver and was used in subsequent purifications of GSTs. In the initial tests, aliquots of liver supernatant were loaded onto columns of DE-52 (pH 7.0, 8.0), DEAE Sephadex (pH 7.0, 8.0, 8.5), CM Sephadex (pH 6.0, 7.0), hydroxylapatite (pH 6.0, 7.0), light Blue Dextran (pH 6.0), dark Blue Dextran (pH 6.0), Matrix Red (pH 6.0), phosphocellulose (pH 6.0, 7.0, 8.0), and phenylsepharose (pH 6.0) equilibrated in 10 mM KPi, 1 mM EDTA, at the proper

pH. After enzyme loading, columns were washed in equilibration buffer and eluted using salt bumps of 0.1, 0.25, 0.5, 1 and 2 M KCl in equilibration buffer in that order (1, 0.5, 0.2, 0.1 and 0 M KCl in that order for phenylsepharose). All washes and bumps were assayed for GST activity as described above. From this battery of tests, it was determined that Matrix Red (pH 6.0) and hydroxyapatite (pH 6.0) were the chromatographic agents of choice. As well, S-hexylglutathione-agarose (pH 6.0) and sulfobromophthalein-S-glutathione-agarose (pH 6.0) were tested using bumps of 25 mM GSH. Both affinity matrices resulted in low yields of enzyme recovery. The usefulness of polyethylene glycol (PEG) for fractionation was also tested using PEG concentrations ranging from 0 to 50 %. GST precipitated at about 20 % w/v PEG (50 % enzyme in pellet at 16 % PEG). The majority of the proteins in crude precipitated at this concentration and therefore a PEG cut could not be used as a purification step.

Four ml of crude extract supernatant was applied to a Matrix Red column (2 cm length X 2.8 cm diameter) equilibrated in homogenization buffer. A 30 ml void volume was collected and assayed for GST activity. A KCl gradient (0 to 1 M) was run and 37 fractions of 1 ml each (51 drops/fraction) were collected. Ten μ l of each fraction was assayed for GST activity. Peak fractions were pooled and concentrated by placing in dialysis tubing (Spectra/Por molecularporous membrane tubing, Spectrum Medical Industries, Inc., Houston, Texas; molecular weight cut off = 12-14,000) and letting sit in solid PEG. The entire concentrated pooled enzyme was then applied to an Sephacryl S-200 gel filtration column (45 cm length X 1.8 cm diameter) equilibrated in homogenization buffer (pH 6.0). For native molecular weight determination, the same Sephacryl S-200 column used for purification was calibrated using Blue Dextran (void

volume), phosphofructokinase (360,000 Da), pyruvate kinase (238,000 Da), aldolase (150,000 Da), hexokinase (102,000 Da), hemoglobin (64,500 Da), and cytochrome c (13,700 Da). The concentrate was allowed to sink into the column bed and 2 further volumes of homogenization buffer wash was applied and allowed to sink into the column bed. The column was then eluted with homogenization buffer; a 34 ml void volume was collected and forty 1 ml fractions (51 drops/fraction) were gathered and assayed for GST activity. Peak fractions were pooled and concentrated as above and the entire concentrated dialysate was applied to a hydroxyapatite column (2 cm length X 1.8 cm diameter) equilibrated in homogenization buffer (pH 6.0). One ml of homogenization buffer was placed on the column bed after the dialysate had sunk in. A 3 mL void volume was collected and a gradient of 0 to 250 mM KPi was run on the column. Forty five fractions of 1 ml each (51 drops/fraction) were collected and assayed for GST activity. Stability tests revealed that the pure enzyme retained 27-64 % activity after 8 days at 4°C (or 2 days of freezing at -80°C).

Isoelectric focusing of turtle liver GSTs

Crude supernatant was subjected to isoelectric focusing (Vesterberg 1971) using an LKB 8101 isoelectric focusing column (110 ml) in a sucrose gradient containing ampholines of the pH range 3.5-10. The column was run for 14-18 hours at 450 V constant voltage at 5°C. After focusing, the column was drained into 2 ml fractions and the elution profile of enzyme activity and the pH gradient were measured. Peak fractions

were tested for isozyme activity using either CDNB or cumene hydroperoxide (which tests for Se-independent glutathione peroxidase activity which is catalyzed by GST).

SDS-PAGE of turtle liver GSTs

Peak fractions off of hydroxyapatite were subjected to discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Purified GSTs were mixed 1:1 (v:v) with 2 X SDS-PAGE loading buffer (100 mM Tris-HCl (pH 6.8), 4 % w:v SDS, 20 % v:v glycerol, 0.2 % w:v bromophenol blue) and boiled for 5 minutes. The enzyme preparation was then loaded on the gel (0.75 mm thick) and run adjacent to molecular weight standards (Bio-Rad broad range standards, Hercules California) and horse liver GST (Sigma, Oakville, Ontario) in 1 X Tris-Glycine (Laemmli) running buffer (3.02 g/L Tris-base, 18.8 g/L glycine, 0.1 % w:v SDS). The stacking gel was 5 % w:v acrylamide (30:0.8 w:w acrylamide:bisacrylamide) and the separating or resolving gel was 15 % w:v acrylamide. The gel was run at 200 V for 1 hour and then removed to a fixing solution of 30 % v:v methanol, 10 % v:v acetic acid. The gel was fixed for 1 hour at room temperature on a rotary shaker, and stained for 2 hours in 0.25 % Coomassie brilliant blue R, 50 % v:v methanol, and 7.5 % v:v acetic acid. The gel was then destained overnight in 30 % methanol, 10 % v:v acetic acid and photographed using a Polaroid DS34 Direct Screen Instant Camera (Bio/Can Scientific, Mississauga, Ontario). Protein standards included myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da). The log molecular weight of standards was plotted

against their R_f in the gel to create the standard curve. The R_f of the single bands representing turtle liver GST subunits was compared to the standard curve to determine the molecular weight of individual subunit monomers.

Kinetic and inhibition characteristics of turtle liver GSTs

Substrate affinity constants (K_m) of GSH, CDNB and cumene hydroperoxide were determined for Peak 1 enzyme and Peak 2 enzyme off of hydroxyapatite. As well, the I_{50} values (the concentration of inhibitor that reduces activity by 50 %) were determined for Peak 1 enzyme and Peak 2 enzyme off of hydroxyapatite for known inhibitors of GST (Cibacron Blue, Rose Bengal, GSSG, iodoacetamide, hexylglutathione, and various salts such as KCl, NaCl, Na_2SO_4 , NH_4Cl and Na acetate). I_{50} determinations were performed at optimal GSH and CDNB concentrations. Substrates for known classes of GSTs that were tested for activity included 1 mM of each of the following: ethacrynic acid, trans-4-phenyl-3-buten-2-one, 1,2-epoxy-3-(p-nitrophenoxy)propane, 1,2-dichloro-4-nitrobenzene, p-nitrobenzylchloride, and p-nitrophenylacetate.

Temperature and pH dependence of turtle liver GSTs

Both temperature and pH dependence were measured in KPi buffer under V_{max} substrate concentrations. Temperature dependence of GST activity in Peak 1 and Peak 2 fractions off of hydroxyapatite were assessed over the range from 5°C to 40°C using increments of 3°C. Temperature effects were analyzed using an Arrhenius plot of log V versus $1/^\circ K$. For pH curves, the initial buffer pH was obtained by mixing the appropriate amounts of KH_2PO_4 and K_2HPO_4 . At low and high pH values, however, phosphate is not

a suitable buffer, so pH was further adjusted using HCl or KOH. pH ranging from 3 to 8 were tested. Assay pH in the cuvette was measured prior and immediately following the enzyme assay.

Protein determinations

Soluble protein concentration was determined by Bio-Rad assay kit (Coomassie blue G-250 binding method; Bradford 1976) with bovine serum albumin as the standard. Spectrophotometric quantification was performed at 595 nm using a Dynatech MR-5000 microplate reader to a final well volume of 310 μ l.

Statistical analyses

Kinetic constants, E_a from Arrhenius plots, and statistical analyses were done using programs provided by Dr. S.P.J. Brooks (Brooks 1992).

RESULTS

GST Purification

A series purification of turtle liver GSTs was performed on Matrix Red (dye ligand column), Sephacryl S-200 (a MW gel filtration column) and hydroxyapatite (ion exchange column). Initial tests determined that these were the columns of choice. Salt gradients on Matrix Red and hydroxyapatite were modified to provide the best resolution on these columns. A typical elution profile for GST from Matrix Red is shown in Figure 14. Control and anoxic profiles were similar with a peak of enzyme activity eluting at

approximately 440 mM KCl. Elution from Matrix Red resulted in a 3.67-fold purification with an 86% yield of enzyme activity for control liver. With the anoxic enzyme, however, this column gave no purification (specific activity unchanged) but it was used anyway so that both enzymes were treated the same (Table 6). Typical elution profiles for control and anoxic turtle liver GSTs from Sephacryl S-200 are shown in Figure 15. Again, there was little difference between enzyme elution profiles with the approximate MW of GSTs being between 59.75 ± 3.25 kDa. Elution from Sephacryl S-200 resulted in an activation of the enzyme (125 and 118 % yield off of the column from the Matrix red pool with the final yield being 108 and 113 % for control and anoxic enzymes respectively) and a 36.4-fold (control) or 145-fold (anoxic) purification. A typical elution profile for control and anoxic turtle liver GSTs off of hydroxyapatite is shown in Figure 16. Two peaks eluted from the column at approximately 98 and 131 mM KPi. The ratio of the relative activities of Peak2/total Peak areas increased during anoxia. This is reflected in the ratio of GST activities (Units) of the Peak 1 and Peak 2 pools using CDNB as a substrate when the same volume was assayed for control and anoxic isolations; the Peak 1/Peak 2 ratio being 1.46:1 for control isolations and only 1.03:1 for anoxic. The total combined yield of enzyme activity of the two peaks was 96 (control) or 103 (anoxic) % off of the column from the Sephacryl S-200 pool with the final yield being 103 and 117 % for control and anoxic enzymes respectively (Table 6). No activity with H_2O_2 was detected in either of the CDNB-utilizing GST peaks eluted from the hydroxyapatite column indicating that this column had separated Se-dependent GPOX activity from GST. No new peaks were found from anoxic isolations of GST compared to controls, off of any column. It was therefore concluded that no new

isozymes were formed during anoxia. Further kinetics were only performed on control hydroxyapatite Peak 1 and Peak 2 isolations to characterize the two isozymes separated by this column.

Isoelectric focusing

The profiles of isoelectric focusing of control and anoxic turtle liver GSTs are shown in Figures 17 and 18. Isoelectric focusing of control and anoxic turtle liver GSTs resulted in two peaks with the first peak displaying the largest activity using CDNB as a substrate. The pI points were 8.52 to 8.68 and 6.11 to 6.82 for Peaks 1 and 2 respectively and did not differ significantly between control and anoxic isolations. Cumene hydroperoxide activity composed from 27 to 28 and from 37 to 45 % of the total (CDNB + cumene hydroperoxide per 50 μ L assay) activity for Peaks 1 and 2 respectively in both control and anoxic situations. No H_2O_2 activity was detected in either peak.

SDS-PAGE

The results of SDS-PAGE of purified proteins from Peak 1 (Lane 3) and Peak 2 (Lane 4) pools off of hydroxyapatite are shown in Figure 19. Equine liver GST (Sigma) (Lane 2) as well as MW standards (Lane 1) were run for comparison. A standard curve created from the MW standards is shown in Figure 20. Peak 1 contained a single subunit of MW 34.0 kDa ($n = 1$) (Table 7). Peak 2 contained 2 subunits of MWs 36.8 and 32.6 kDa ($n = 1$). All turtle liver subunits were larger than the 2 equine liver subunits (28.9 kDa and 21.1 kDa). It was concluded that the GST of Peak 1 off of hydroxyapatite is a homodimer with an approximate MW of 68 kDa and the GST of Peak 2 is a heterodimer

with an approximate MW of 69.4 kDa; both larger than equine liver GST which is a heterodimer of 50.0 kDa.

Kinetic and inhibition characteristics

The results of kinetic and inhibition characterization of the two GST isozymes from control liver off of hydroxyapatite are shown in Table 8. Peak 1 GST had the greater affinity of the two isozymes for GSH, displaying a K_m that was only 63 % of that of Peak 2. Peak 2 had the larger affinity of the two isozymes for CDNB with a K_m that was 67 % of that of Peak 1. Peak 2 GST could not use cumene hydroperoxide as a substrate. No H_2O_2 activity (tests for Se-dependent GPOX activity) was detected for either peak. Cibacron Blue and iodoacetamide inhibited turtle liver GSTs in a similar fashion with the I_{50} for Peak 1 being between 17.5 and 21.7 % that of Peak 2 for Cibacron Blue and iodoacetamide respectively. Salts containing the Cl^- ion also inhibited turtle liver GSTs in a similar fashion whereas acetate did not inhibit either enzyme and SO_4 inhibited only the Peak 2 enzyme. The I_{50} s for Peak 2 were 53.8, 51.8 and 60.5 % of that of Peak 2 for KCl, NaCl and NH_4Cl respectively. The NH_4^+ ion may add to the Cl^- inhibition of turtle liver GSTs when using NH_4Cl . Rose Bengal, GSSG, and hexylglutathione inhibited turtle liver GST isozymes to the same extent for both Peaks 1 and 2. Of other substrates tested for catalytic activity, no activity occurred with either Peak GST using ethacrynic acid, trans-4-phenyl-3-buten-2-one, or 1,2-epoxy-3-(p-nitrophenoxy)propane. Peak 2 GST showed 2.56-, 1.56-, and 7.93-fold greater activity than Peak 1 GST using 1,2-dichloro-4-nitrobenzene, p-nitrobenzylchloride, and p-nitrophenylacetate respectively.

Temperature and pH dependence

Arrhenius plots for hydroxyapatite Peak 1 and Peak 2 turtle liver GSTs are shown in Figure 21. No breaks in Arrhenius plot slopes occurred for either Peak GST. The activation energies for Peaks 1 and 2 were not significantly different (Table 7).

The pH curves for hydroxyapatite Peak 1 and Peak 2 turtle liver GSTs are shown in Figure 22. GSTs showed gradually increasing activity with increasing pH to a pH optimum of 7.2 (Table 7), after which there was a sharp decline in activity at the higher pHs.

DISCUSSION

Maximal GST activity in turtle liver had previously been found to decrease by 25 % during 20 hours of anoxia exposure and this suggested a possible adaptive role for changes in GST levels in support of anaerobiosis (Willmore and Storey 1997b, Chapter 4). As outlined in the Introduction, the mechanism of GST modulation in anoxia could take one of several forms and, hence, an assessment of the isozymic forms, specific activities and kinetic properties of turtle liver GST seemed appropriate. The effect of anoxia exposure on liver GST was even more pronounced when the purified enzymes were examined; the specific CDNB activities of purified GSTs from anoxic liver being only 38 and 11 % of its corresponding purified control activities for Peaks 1 and 2 respectively (Table 7). The decrease in maximal activity could be the result of a) a reduction of the total amount of enzyme present in tissues, due to either decreased

synthesis or increased degradation (by proteolysis) during anoxia, b) post-translational modification of GSTs resulting in decreased activity, or c) a change in GST isozymes, either expression of different GST subunits or a recombination of preexisting subunits to create a different GST isozyme; both with lower CDNB activity. Option (a) would be supported by identical kinetic parameters in both anoxic and control states but differing V_{max} levels, indicating that the enzyme varied in amount only between the two states. To date, there is no evidence in the literature that any of the known GSTs are posttranslationally modified. Most GSTs are regulated by a change in isozyme form and specific GST subunits are induced by various xenobiotics and are expressed in a tissue specific manner (Pickett and Lu 1989, Daniel 1993). The main purpose of the present study was to determine if a change in GST isozymes occurred during anoxia and to characterize the GSTs present in T. s. elegans liver.

In general, most forms of GST can bind to an affinity column which has GSH attached to the stationary phase, either S-hexylglutathione or sulfobromophthalein glutathione (Nishinaka et al. 1992). This was not the case for turtle liver GSTs and so the matrices could not be used as an affinity step in the purification scheme. Turtle liver GSTs either bound irreversibly to (even when eluting with very high concentrations of GSH) or were denatured by such matrices.

The purification scheme developed for turtle liver GSTs resulted in a final specific activity of 7.31 and 55.4 U/mg protein for hydroxyapatite Peaks 1 and 2 from control livers and 2.80 and 6.37 U/mg protein for hydroxyapatite Peaks 1 and 2 from anoxic livers using CDNB as a substrate (Table 6). Specific activities for both hydroxyapatite peak pools were in the range of values found for Alpha class GSTs in

hamster liver (8.0-8.1 U/mg protein) (Bogaards et al. 1992). With the exception of Peak 2 in control isolations, these values were lower than that reported for human liver (16-37 U/mg protein) (Habig and Jakoby 1981), various mammalian tissues (20-357 U/mg protein) (Addya et al. 1994), adult toad (Bufo bufo) liver (24-55 U/mg protein) (Aceto et al. 1993), and snail foot muscle (11 U/mg protein) (Pannunzio 1994) but SDS-PAGE of pooled peak fractions revealed that the enzymes were purified to homogeneity (Figure 19). The apparent lower fold purification of turtle liver GSTs, both control and anoxic, during the last step of purification (hydroxyapatite) was due to the separation into the two isoforms; the additive fold purifications of the two isozymes being slightly greater than the fold purification of the previous step (Sephacryl S-200) (Table 6).

Many cytosolic GSTs can be separated into one of the four classes (alpha, mu, pi, theta) based on their pI and kinetic characteristics (Mannervik et al. 1992). Alpha, Mu, and Pi GSTs have basic, near-neutral, and acidic pIs respectively. Isoelectric focusing separated turtle liver GSTs into one major and at least one minor isozyme acting upon CDNB. Subsequent characterization of the peaks revealed that each had some cumene hydroperoxide activity, suggesting that both isozymes belong to class Alpha. However, some Mu and Pi classes also have a small amount of cumene hydroperoxide activity (although this is markedly lower), and peaks with near neutrality pIs (pIs 6.60 and 6.83 for control and 6.45 for anoxic) are probably of the Mu class. Basic peaks (pIs 8.63 and 8.91 for control and 8.48 for anoxic) are probably of Alpha class.

Turtle liver GSTs showed a higher MW than most known GSTs. SDS-PAGE showed that turtle GST subunits were higher in MW than those of horse liver GST (Figure 19). Peak 1 off of hydroxyapatite contained a homodimer of a single subunit and

Peak 2 contained a heterodimer with 2 subunits of unequal MW. The reason for the discrepancy between the MWs found off of the Sephacryl S-200 column and SDS-PAGE may be due to the lack of resolving power of the gel filtration column; elution of peak enzyme activity from the column ½ mL earlier would have resulted in the same MW range for turtle liver GSTs as that found on SDS-PAGE. The native MW of these isozymes, according to subunit composition, would be between 60 and 70 kDa. This is much higher than the MWs of between 37 and 50 kDa that have been reported from various sources such as flatworms (37 kDa; Clark 1989), molluscs (40 kDa; Balabaskaran et al. 1986), snail hepatopancreas (44 kDa; Ajele and Afolayan 1992), rabbit hepatic tissue (45 kDa; Primiano and Novak 1993), toad liver (Bufo bufo) (50 kDa; Aceto et al. 1993) and rat and human liver (45-49 kDa; Habig and Jakoby 1981). Elution from Sephacryl S-200 again placed the MW of turtle liver GSTs around 60 kDa, although the gel filtration column had less resolving power than SDS-PAGE. The larger MW of turtle liver GSTs may be the result of an increased need for secondary structure in the turtle enzymes to maintain the active sites of the enzyme in proper conformation.

GSTs are also grouped into classes according to their kinetic properties using various substrates and their reactions with various inhibitors. For example, Alpha class GSTs have high peroxidase activity using cumene hydroperoxide as a substrate. Mu and Pi classes of GSTs have almost negligible activities using this as a substrate. Other class specific substrates include p-nitrobenzylchloride (Alpha 1 subunit specific but not Alpha 2 subunit specific in rat), ethacrynic acid (Pi class with small amounts of Alpha and Mu activities), and trans-4-phenyl-3-buten-2-one (Mu class) (Mannervik 1985). 1,2-Epoxy-3-(p-nitrophenoxy)propane reveals some activity with Mu and Pi classes and no activity

with Alpha class (Mannervik 1985). Nonspecific (general class) substrates include CDNB, 1,2-dichloro-4-nitrobenzene and p-nitrophenylacetate. Cibacron Blue shows highest to lowest inhibition (lowest to highest I_{50}) of Mu, Pi and Alpha isozymes respectively (Mannervik 1985). Rose Bengal specifically inhibits Pi class GSTs (Mannervik 1985) while iodoacetamide, a reagent directed against thiol groups, is a nonspecific inhibitor of all classes. S-hexylglutathione shows highest to lowest inhibition of Alpha, Mu and Pi GST isozymes respectively (Mannervik 1985). Despite having lower specific activities than most invertebrate and vertebrate purified GSTs, turtle liver GSTs had high affinity for GSH but a lower affinity for CDNB as seen by their K_m values (Table 8). K_m values for GSH were within the range of that found for blue crab hepatopancreas ($K_m = 0.4$ mM; Keeran and Lee 1987) and lower than that found in the mosquito ($K_m = 1.4$ - 1.8 mM; Grant et al. 1991). K_m values for CDNB were higher, however, than that found in blue crab hepatopancreas ($K_m = 0.3$ mM; Keeran and Lee 1987), mosquito ($K_m = 0.064$ - 0.24 mM; Grant et al. 1991) and human lung ($K_m = 0.033$ - 0.042 mM; Singhal et al. 1992). K_m values for CDNB were comparable to that found in the tapeworm ($K_m = 1.4$ mM; Brophy et al. 1989). Peak 1 off of hydroxyapatite had a significantly higher affinity for GSH than Peak 2 while Peak 2 had significantly higher affinity for CDNB. Peak 1 had high affinity for cumene hydroperoxide as a substrate which places the enzyme in this peak in the Alpha class. No detectable levels of cumene hydroperoxide activity was detected for Peak 2. Specific activities of Peak 1 enzyme using cumene hydroperoxide as a substrate were in the range of Alpha GSTs found in human lung (1.84 U/mg protein; Singhal et al. 1992) which play a protective role in lipid peroxidation. Specific activities using cumene hydroperoxide were not as high as Alpha

GSTs found in human liver (10.6 U/mg protein; Mannervik 1985) or hamster liver (2.65-3.39 U/mg protein; Bogaards et al. 1992). Of the other substrates tested for activity, no detectable activity was found for either isozyme for ethacrynic acid, trans-4-phenyl-3-buten-2-one, 1,2-epoxy-3-(p-nitrophenoxy)propane. These results would suggest that both isozymes belong to the Alpha class of GSTs. Activity was detected for both peaks using p-nitrobenzylchloride as a substrate which also suggests that both isozymes are Alpha class. The nonspecific substrates 1,2-dichloro-4-nitrobenzene and p-nitrophenylacetate had differing activities between the two peaks suggesting that these Alpha class isozymes do differ. This is also clearly supported by the fact that, on SDS-PAGE, one is a homo- and the other a heterodimer so they are at least 50 % different (i.e. A-A vs A-B) or they could be totally different but with similar MW (i.e. A-A vs B-C). Both enzymes were inhibited by GSSG, the oxidized form of GSH, with I_{50} values of 2-2.6 mM; however, this is about 100-fold higher than GSSG levels in vivo so inhibition this compound, which accumulates during oxidative stress, may not be a significant influence on enzyme activity in vivo. Cibacron Blue inhibition was low for both Peaks 1 and 2 but inhibition was significantly higher for Peak 2. Again this would suggest that Peak 1 belongs to Alpha class GSTs, and possibly Peak 2 enzyme as well. Rose Bengal inhibition of turtle liver GSTs was in the range of human Alpha and Mu class GSTs (Tahir et al. 1985) but did not differ significantly in their inhibition when comparing between peaks off of hydroxyapatite. Both turtle liver GST isozymes could be inhibited by GSSG to the same extent. Iodoacetamide was a poor inhibitor of Peak 1 enzyme but inhibited Peak 2 enzyme to an I_{50} value between that of snail ($I_{50} = 14.1$; Ajele and Afolayan 1992) and camel (I_{50} slightly over 2 mM; Hunaiti and Sarhan 1987). Inhibition

by S-hexylglutathione was the same for both Peak 1 and Peak 2 isozymes and was stronger than the inhibition of human Alpha GST (Tahir et al. 1985), embryonic toad Pi GSTs (Di Ilio et al. 1992), snail GST (Ajele and Afolayan 1992), or mosquito GSTs (Grant et al. 1991) by this compound. This large inhibition (high affinity binding) may be the reason high concentrations of GSH could not elute turtle liver GSTs from an S-hexylglutathione matrix. Peak 2 isozyme was inhibited by the chloride ion to a much greater extent than Peak 1 isozyme. Kinetic and inhibition studies show that the isozymes are distinct and suggest that Peak 1 isozyme is an Alpha class GST while Peak 2 may be an Alpha-like isozyme without peroxidase activity.

Activation energies for turtle liver GST isozymes differed significantly from one another while their pH optima were found to be the same. The activation energy for Peak 1 GST isozyme was significantly lower than the activation energy for Peak 2 isozyme. The activation energies of both isozymes were lower than that found for Mu or Pi GSTs purified from an insect cell line (49.1 kJ/mol) (Chang et al. 1994). The lack of a break in the Arrhenius relationship shows that, for both GSTs in turtle liver, enzyme structure, conformation, etc is not compromised over the range of temperatures tested. This range covers the physiological temperature range that the animal functions within normally. Peak 1 and Peak 2 GSTs both had a pH optimum of around 7.2. This pH optimum is on the acidic side of the pH optima of most known GSTs, including non-vertebrates (Clark 1989) and human Mu class enzymes (Comstock et al. 1994). The adaptive significance of this is that turtle GSTs may function normally during the acidosis which turtles encounter during anoxia.

In conclusion, the lower maximal activity of GST in turtle liver seen during anoxia possibly indicates that there is less enzyme in the tissue and suggests either decreased synthesis or increased degradation of one or more of the enzymes. No new isozymes are made during anoxia as seen by elution profiles from columns. In general, new isozymes would be seen as either a change in MW of activity peaks during gel filtration or a change in pI during I.E.F. During anoxia, activity using cumene hydroperoxide as a substrate becomes more prevalent with Peak 2 isozyme off of I.E.F., even in the face of decreased maximal activity of the enzyme in crude supernatant. This Alpha class isozyme, which may comprise part of the activity of Peak 1 isozyme off of hydroxyapatite, may play an important role in removing the products of lipid peroxidation during anoxia in this tissue (some oxidative stress may occur in liver during anoxia as indicated by the GSH/GSSG ratio; Chapter 4). Peak 1 isozyme off of hydroxyapatite likely belongs to class Alpha GSTs (a homodimer), based on kinetic and inhibitory properties, and Peak 2 isozyme is a class Alpha-like enzyme (a heterodimer) with undetectable peroxidase activity.

Fig. 14. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GST from Matrix Red. The enzyme were eluted with a linear gradient of KCl (0 to 1 M). Activities are expressed as relative activities with the peak fraction being 100 %. GST activity from control and anoxic turtle liver extracts eluted in one peak at the same KCl concentration (440 mM KCl). Cumene hydroperoxide activity was associated exactly with the peak of CDNB activity. Closed and open circles represent control and anoxic isolations respectively.

Fig. 15. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GST from Sephacryl S-200. Activities are expressed as relative activities with the peak fraction being 100 %. GST activity from control and anoxic turtle liver pools eluted in one peak at the same MW (between 56.5 and 63.0 kDa). MW standards used include Blue Dextran (BD; 2,000,000 Da), phosphofructokinase (PFK; 360,000 Da), pyruvate kinase (PK; 238,000 Da), aldolase (ALD; 150,000 Da), hexokinase (HK; 102,000 Da), hemoglobin (Hb; 64,500 Da), and cytochrome c (Cyt c; 13,700 Da). Closed and open circles represent control and anoxic isolations respectively.

Fig. 16. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GST from hydroxylapatite. Activities are expressed as relative activities with the peak fraction being 100 %. GST activity from control and anoxic turtle liver pools eluted in two peaks at the KPi concentrations (98 and 131 mM KPi for Peak 1 and Peak 2 respectively). The percentage of total GST activity present in peak 2 increased during anoxia. Closed and open circles represent control and anoxic isolations respectively.

Fig. 17. Typical profiles of control Trachemys scripta elegans liver GST from isoelectric focusing. Activities are expressed as relative activities with the peak fraction being 100 %. GST activity from control turtle liver focused into two peaks with pI points of 6.11 and 8.52 respectively. Cumene hydroperoxide activity (open squares) was associated exactly with the peak of CDNB activity (closed circles) in every peak. Open circles represent pH.

Fig. 18. Typical profiles of anoxic Trachemys scripta elegans liver GST from isoelectric focusing. Activities are expressed as relative activities with the peak fraction being 100 %. GST activity from anoxic turtle liver focused into two peaks with pI points of 6.82 and 8.68 respectively. Cumene hydroperoxide activity (open squares) was associated exactly with the peak of CDNB activity (closed circles) in every peak. Open circles represent pH.

Fig. 19. SDS-PAGE (15 % w/v acrylamide gel) of purified GSTs from control Trachemys scripta elegans liver stained with Coomassie blue. Lane 1, MW standards; Lane 2, horse liver GST (Sigma); Lane 3, turtle liver GST from Peak 1; Lane 4, turtle liver GST from Peak 2. The MW of standards (closed circles) used are as follows: myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da).

Fig. 20. Standard curve used to determine the subunit MW of Trachemys scripta elegans liver GSTs from SDS-PAGE. The MW of standards (closed circles) used are as follows: myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da). Turtle liver GST subunits are indicated with open circles.

Fig. 21. pH profiles of GSTs purified from control liver Trachemys scripta elegans.

Values are means of three trials performed on a single enzyme preparation, $\pm$ S.E.M.

Where error bars are not visible, they are contained within the symbol. Phosphate buffer was used for all assays and pH was checked immediately prior to and following the assay; the pHs reported are an average of these two values. Closed (with solid line) and open (with dotted line) circles represent Peak 1 and Peak 2 isolations from hydroxyapatite respectively. The pH optima were 7.20 and 7.21 for Peak 1 and Peak 2 isozymes respectively.

Fig. 22. Arrhenius plots of GSTs purified from control liver of Trachemys scripta

elegans. Values are means of three trials performed on a single enzyme preparation, $\pm$

S.E.M. Where error bars are not visible, they are contained within the symbol.

Phosphate buffer was used for all assays and cuvette temperature was checked

immediately prior to and following the assay; the temperatures used are an average of

these two values. Closed (with solid line) and open (with dotted line) circles represent

Peak 1 and Peak 2 isolations from hydroxyapatite respectively. The activation energies

(E_a) were 36.0 ± 2.19 and 46.4 ± 3.13 kJ/mol for Peak 1 and Peak 2 respectively and

were significantly different ($p < 0.1$). For both isozymes, denaturation was evident at 40

°C.

Table 6. Fold purifications and % yields of GST purified from liver of Trachemys scripta elegans using optimal CDNB and

GSH concentrations. Results are from one purification; further purifications yielded similar results.

Column	Control Turtle Liver			Anoxic Turtle Liver		
	Specific activity U/mg	Fold purification	% yield	Specific activity U/mg	Fold purification	% yield
supernatant	0.319	1.00	100	0.0408	1.00	100
Matrix Red	1.17	3.67	86.0	0.0335	0.821	95.9
Sephacryl S-200	11.6	36.4	108	5.91	145	113
hydroxyapatite (Peak 1)	7.31	22.9	103	2.80	68.6	117
hydroxyapatite (Peak 2)	55.4	174		6.37	156	

Table 7. General characteristics of GSTs purified from *Trachemys scripta elegans* liver. Results with $\pm$ S.E.M.s are means of 3

determinations. Two-tailed Students t-tests: ^a: significantly different from Peak 1 values $p < 0.005$; ^b: $p < 0.001$; ^c: significantly different from control values $p < 0.005$; ^d: $p < 0.001$.

	hydroxyapatite (Peak 1) hydroxyapatite (Peak 2)	
Arrhenius plots		
Ea (kJ/mol)	36.0 $\pm$ 2.19	46.4 $\pm$ 3.13
pH curves		
pH optimum	7.20	7.21
SDS-PAGE		
MW (Da)	34,000	36,800 (subunit 1) 32,600 (subunit 2)
Specific activity using CDNB (U/mg protein)		
Control	7.31 $\pm$ 0.380	55.4 $\pm$ 5.77 ^a
Anoxic	2.80 $\pm$ 0.0512 ^d	6.37 $\pm$ 0.0984 ^{b,c}
Specific activity using cumene hydroperoxide (U/mg protein)		
Control	1.78 $\pm$ 0.0536	0
Anoxic	0.943 $\pm$ 0.00863 ^d	0

Table 8. Kinetic parameters of GST isozymes purified from *Trachemys scripta elegans* liver. Results are means of 3

determinations $\pm$ S.E.M. Two-tailed Students t-test: ^a: significantly different from control values $p < 0.1$; ^b: $p < 0.05$; ^c: $p < 0.025$; ^d: $p < 0.001$.

Effector	hydroxyapatite (Peak 1)	hydroxyapatite (Peak 2)
K_m GSH (mM)	0.381 ± 0.0191	0.601 ± 0.0837^a
K_m CDNB (mM)	1.70 ± 0.152	1.14 ± 0.0403^c
K_m cumene hydroperoxide (mM)	0.111 ± 0.0213	no activity detected
I_{50} GSSG (mM)	2.03 ± 0.186	2.58 ± 0.572
I_{50} Cibacron Blue (μ M)	48.0 ± 0.972	8.42 ± 0.380^d
I_{50} Rose Bengal (μ M)	0.313 ± 0.0160	0.470 ± 0.0849
I_{50} S-hexylglutathione (μ M)	0.314 ± 0.0355	0.387 ± 0.189
I_{50} iodoacetamide (mM)	40.2 ± 0.457	8.72 ± 0.327^d
I_{50} KCl (M)	0.325 ± 0.0392	0.175 ± 0.0200^b
I_{50} NaCl (M)	0.332 ± 0.0341	0.172 ± 0.0118^c
I_{50} Na ₂ SO ₄ (M)	no inhibition	0.187 ± 0.0453
I_{50} NH ₄ Cl (M)	0.195 ± 0.0250	0.118 ± 0.0133^a
I_{50} Na acetate (M)	no inhibition	no inhibition
Other substrates tested (specific activity in U/mg; n = 1 determination)		
1,2-dichloro-4-nitrobenzene (1mM)	0.00353	0.00902
p-nitrobenzylchloride (1mM)	0.178	0.277
p-nitrophenylacetate (1mM)	0.145	1.15

Table 9. Isoelectric focusing parameters of control and anoxic Trachemys scripta**elegans liver GSTs.** Units are corrected for the volumes assayed. Total activity =CDNB activity + cumene hydroperoxide activity. Values are means $\pm$ S.E.M. for 3

determinations; otherwise n = 1.

	Control	Anoxic
pI		
Peak 1	8.52 $\pm$ 0.240*	8.68 $\pm$ 0.101*
Peak 2	6.11 $\pm$ 0.262	6.82 $\pm$ 0.256
* indicates major peak activity using CDNB		
CDNB activity (U/mL of peak fraction assayed)		
Peak 1	1.875	1.674
Peak 2	0.4566	0.2604
cumene hydroperoxide activity (U/mL of peak fraction assayed)		
Peak 1	0.7241	0.6116
Peak 2	0.2633	0.2097
% cumene hydroperoxide activity of total activity		
Peak 1	27.9	26.8
Peak 2	36.6	44.6

CHAPTER SIX

PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE REDUCTASE FROM LIVER OF TRACHEMYS SCRIPTA ELEGANS

INTRODUCTION

Glutathione reductase (E.C. 1.6.4.2) is a ubiquitous FAD-enzyme which catalyzes the flow of reducing equivalents from the pyridine nucleotide redox system to the thiol-disulfide system of glutathione (Williams 1976, Holmgren 1980, Schirmer et al. 1989) in the following reaction:

Each monomer of the dimeric enzyme contains one FAD molecule and an active disulfide bridge which separates the distinct binding sites for GSSG and NADPH. The binding site for GSSG resides between the cleft formed at the interface of the two identical subunits of this enzyme (Scrutton et al. 1992). In the catalytic reaction, NADPH binds to the enzyme and releases its reducing equivalents while being converted to NADP⁺ (Schirmer et al. 1989). Reducing equivalents flow as electrons via a charge-transfer complex between the nicotinamide ring of NADPH and the flavin ring system of FAD. Electrons proceed to the active disulfide bridge and open it, reducing the enzyme. Nucleophilic attack of the newly formed thiol upon one of the sulfurs of GSSG leads, by means of a thiol disulfide interchange, to the formation of a mixed disulfide between one of the glutathiones of GSSG and the enzyme. Protons are picked up by GSSG from the active thiol and a nearby histidine and reduced GSH equivalents are released, leading to the regeneration of the oxidized form of the enzyme.

The GSH supplied by the reaction is important in 1) maintenance of the redox state of catalytic and structural proteins by thioltransferases, 2) directly scavenging oxygen radicals, and 3) acting as a substrate for antioxidant enzymes including GPOX

and GST. In the reduction of GSSG, GR is highly dependent upon the NADPH-generating enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). Maintenance of a high GSH/GSSG ratio is important for the cellular redox state; a lowering of this ratio is frequently used as an indicator of oxidative stress. GR plays an important role in the glutathione redox cycle and thus is involved in the protection of nucleic acids and membrane lipids from peroxidation. The high GSH/GSSG ratio which occurs in cells (ranging from 20 to 1000) is maintained by GR due to its high affinity for GSSG (Schirmer et al. 1989).

Possible mechanisms of regulation of GR in vivo have been suggested (Schirmer et al. 1989). These include 1) inactivation by redox interconversion involving an erroneous intramolecular disulfide bridge between a cysteine near the N terminal of one of the subunits and an active site cysteine, 2) reduction of the intersubunit disulfide bridge (not the active disulfide bridge) by an intracellular thiol promoting dissociation of the enzyme into inactive monomers, and 3) covalent crosslinking with haemin which targets GR for proteolytic degradation (Schirmer et al. 1989). The latter process requires both NADPH and O₂, which indicates the involvement of a reactive oxygen intermediate. All regulatory mechanisms postulated depend upon a change in redox state. One study has shown de novo expression of different GR isoforms during stress situations (Tanaka et al. 1994).

An 52 % increase in GR maximal activity occurred during anoxia in T. s. elegans liver (Chapter 4; Willmore and Storey 1997b). Despite this, GSSG content increased whereas total glutathione decreased over 20 hours of anoxia exposure in turtles. The overall GSH/GSSG ratio was reduced in anoxic turtles, indicating possible oxidative

stress occurring during anoxia. While counterintuitive, the possibility of this occurring is discussed at the end of this chapter. An increase in GR activity in liver during anoxia may be an attempt to return the GSH/GSSG ratio to normal (aerobic) values.

The purpose of the present study was to purify and to characterize T. s. elegans liver GR and to determine if the increase in maximal activity seen during anoxia was due to the synthesis of new isoforms of the enzyme. The role of GR in turtle anoxic survival and cell redox homeostasis is discussed.

MATERIALS AND METHODS

Chemicals and animals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO., U.S.A.) or from Boehringer Mannheim Corporation (Montreal, Quebec) and were of the highest purity available. Winter acclimated adult red-eared sliders (Trachemys scripta elegans) were obtained from Wards Natural Science, Mississauga, Ontario and were maintained in large tanks of dechlorinated water at 7 °C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed ad libitum on a diet of trout pellets, lettuce and egg shells.

Control (normoxic) turtles were sampled directly from the tank. Anoxia was imposed by submerging turtles for 20 h at 5 °C in sealed tanks of 60 L deoxygenated water that had been previously bubbled with 100 % nitrogen gas for 1 h.. A wire mesh placed 20 cm below the surface of the water prevented turtles from surfacing. Some turtles were sampled immediately at the end of anoxia treatment whereas others were

returned to aerobic conditions in a 5°C incubator and allowed to recover for 24 h before sampling. All animals were killed by decapitation and organ samples were quickly removed, frozen in liquid nitrogen and then transferred to -80 °C for storage.

GR assay

GR activity was assayed as described in Chapter Four. Optimal substrate concentrations which gave maximal enzyme activity in turtle liver were essentially the same as described previously. Enzyme assays were conducted at 21°C and one unit is defined as the amount of enzyme that oxidizes 1 µmol of NADPH per min at 21°C.

Preparation of tissue extracts

Frozen tissue samples were quickly weighed and homogenized 1:5 (w:v) in ice-cold buffer containing 5 mM potassium phosphate (pH 6.0), 1 mM EDTA, and a few crystals of PMSF using an Ultra-Turrax (Tekmar) tissue homogenizer. Homogenates were centrifuged at 15,000 rpm for 20 min at 4°C using an Sorvall refrigerated centrifuge. Supernatants were removed and desalted by passage through a small column (1 x 5 cm) of Sephadex G-25 (equilibrated in homogenizing buffer) with centrifugation for 45 seconds in a benchtop centrifuge (International Equipment Company) at full speed (Helmerhorst and Stokes 1980).

Purification of turtle liver GR

The purification procedure was optimized for T. s. elegans liver GR and was used in all subsequent purifications of the enzyme from this tissue. In initial tests, aliquots of liver supernatant were loaded onto columns of DE-52 (pH 7.0, 8.0), DEAE Sephadex (pH

7.0, 8.0, 8.5), CM Sephadex (pH 6.0, 7.0), hydroxylapatite (pH 6.0, 7.0), light Blue Dextran (pH 6.0), Matrix Red (pH 6.0), phenyl-Sepharose (pH 6.0, 7.0), butylagarose (pH 6.0) and Concanavalin A (pH 6.0) equilibrated in 10 mM KPi, 1 mM EDTA, at the proper pH. Tissues were homogenized in column equilibration buffer that contained 1 M KCl for loading onto phenyl-Sepharose and butylagarose (equilibrated in the same high salt buffer). After enzyme loading, columns were washed in equilibration buffer and eluted using salt bumps of increasing concentrations (0.1, 0.25, 0.5, 1, 2 M KCl) for most columns or decreasing KCl (1, 0.5, 0.2, 0.1, 0 M) for phenyl-Sepharose and butylagarose. Bumps of 0, 0.5, 1 and 2 M glucose were used for Concanavalin A. All washes and bumps were assayed for GR activity as described above. From this battery of tests, it was determined that hydroxylapatite (pH 6.0) and Matrix Red (pH 6.0) were the chromatographic agents of choice. Assessment of polyethylene glycol (PEG) cuts as a purification tool showed that GR was pelleted at about 16 % PEG (97 % of enzyme was in the pellet at 16 % PEG) which is the concentration at which most proteins precipitate. Therefore, a PEG cut could not be used as a purification step.

For enzyme purification, 36 ml of crude supernatant was applied to a scaled-up hydroxylapatite column (2 cm length X 2.8 cm diameter) equilibrated in homogenization buffer (pH 6.0). A 50 ml void volume was collected and then a KPi gradient (0 to 1 M) was run with 76 fractions of 1 ml each (51 drops/fraction) collected. Ten μ l of each fraction was assayed for GR activity. Peak fractions were pooled and then applied to an I.E.F. column (Vesterberg 1971) using an LKB 8101 I.E.F. column (110 ml) in a sucrose gradient containing ampholines of the pH range 3.5-10. The column was run for 14-18 hours at 450 V constant voltage at 5°C. After focusing, the column was drained into 2 ml

fractions and 10 μ L of each fraction was assayed for GR activity. The pH of each fraction was then determined. Peak fractions from I.E.F. were pooled, dialyzed against 2 L of low salt (homogenization) buffer for 3 hours, concentrated to 1-1.5 mL against solid PEG, and then applied to a Sephacryl S-300 column (51 cm length X 1.8 cm diameter) equilibrated in homogenization buffer (pH 6.0). For native molecular weight determination, the same Sephacryl S-300 column as was used for purification was calibrated using Blue Dextran (void volume), phosphofructokinase (360,000 Da), aldolase (150,000 Da), hexokinase (102,000 Da), hemoglobin (64,500 Da), and cytochrome c (13,700 Da). The concentrate was allowed to sink into the column bed and then 2 X column buffer wash was applied and allowed to sink into the column bed. A 34 ml void volume was collected and forty 1 ml fractions (51 drops/fraction) were gathered and assayed for GR activity. Peak fractions were pooled and then applied to a Matrix Red column (2 cm length X 1.8 cm diameter) equilibrated in homogenization buffer (pH 6.0). Four mL of homogenization buffer were placed on the column bed after the dialysate had sunk in. A 4 mL void volume was collected and then a gradient of 0 to 2 M KCl was run; forty one fractions of 1 ml each (51 drops/fraction) were collected and assayed for GR activity. Peak fractions were pooled and stored at 4° C until use for kinetic analysis. Stability tests revealed that the pure enzyme retained 75 % activity after 8 days at 4° C.

SDS-PAGE of turtle liver GR

A sample of the Matrix Red pool was subjected to discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Purified GR

was mixed 1:1 (v:v) with 2 X SDS-PAGE loading buffer (100 mM Tris-HCl, pH 6.8, 4 % w:v SDS, 20 % v:v glycerol, 0.2 % w:v bromophenol blue) and boiled for 5 minutes. The enzyme preparation was then loaded on the gel (0.75 mm thick) and run adjacent to molecular weight standards (Bio-Rad broad range standards, Hercules California) and Baker's Yeast GR (Sigma, Oakville, Ontario) in 1 X Tris-Glycine (Laemmli) running buffer (3.02 g/L Tris-base, 18.8 g/L glycine, 0.1 % w:v SDS). The stacking gel was 5 % w:v acrylamide (30:0.8 w:w acrylamide:bisacrylamide) and the separating or resolving gel was 15 % w:v acrylamide. The gel was run at 200 V for 1 hour and then removed to a fixing solution of 30 % v:v methanol, 10 % v:v acetic acid. The gel was fixed for 1 hour at room temperature on a rotary shaker, and stained for 2 hours in 0.25 % Coomassie brilliant blue R, 50 % v:v methanol, and 7.5 % v:v acetic acid. The gel was then destained overnight in 30 % methanol, 10 % v:v acetic acid and photographed using a Polaroid DS34 Direct Screen Instant Camera (Bio/Can Scientific, Mississauga, Ontario). Protein standards included myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). The log molecular weight of standards was plotted against their R_f in the gel to create the standard curve. The R_f of the single bands representing turtle liver GR subunits were compared to the standard curve to determine the molecular weight of individual subunit monomers.

Reverse phase HPLC

Purified turtle liver GR (3.75 μ g) was injected into a Zorbox 300 SB-C8 column (2.1 X 15 cm) using two Beckman 110B pumps with a Beckman 163 Variable Wavelength Detector. The enzyme was eluted with a gradient of 0 to 65 % acetonitrile over 50 min at a flow rate of 0.2 mL/min. Baker's Yeast GR (Sigma) was used as a standard. This method was employed to determine the purity of the sample as well as search for the presence of isozymes.

Kinetic and inhibition characteristics of turtle liver GR

Substrate affinity constants (K_m) for GSSG and NADPH were determined for purified turtle liver GR. I_{50} values (the concentration of inhibitor that reduces activity by 50 %) were also determined for known inhibitors of GR including GSH, $NADP^+$, and various salts such as KCl, NaCl, Na_2SO_4 , NH_4Cl and Na acetate. I_{50} determinations used optimal GSSG and NADPH concentrations.

Temperature and pH dependence of turtle liver GR

Both temperature and pH dependence were measured in KPi buffer under V_{max} substrate concentrations. Temperature dependence of purified GR was assessed over the range from 5°C to 40°C using increments of 3°C. The initial and final temperatures of cuvettes were measured and an average of these two temperatures was used for Arrhenius plots of log V versus $1/^\circ K$. Effects of pH on enzyme activity were tested over the range pH 3 to 8. For pH curves, the initial buffer pH was obtained by mixing the appropriate amounts of KH_2PO_4 and K_2HPO_4 . At low and high pH values, however, phosphate is not

a suitable buffer, so pH was further adjusted using HCl or KOH. The pH in the cuvette was measured prior and immediately following the enzyme assay.

Protein determinations

Soluble protein concentration was determined by Bio-Rad assay kit (Coomassie blue G-250 binding method; Bradford 1976) with bovine serum albumin as the standard. Spectrophotometric quantification was performed at 595 nm using a Dynatech MR-5000 microplate reader with a final well volume of 310 μ l.

Statistical analyses

Kinetic constants, activation energies (E_a) from Arrhenius plots, and statistical analyses were done using programs provided by Dr. S.P.J. Brooks (Brooks 1992).

RESULTS

GR purification

Purification of turtle liver GR used four steps: ion exchange chromatography on hydroxylapatite, isoelectric focusing, Sephacryl S-300 gel filtration, and dye ligand chromatography on Matrix Red. Initial tests determined that these were the columns of choice. As with the purification of turtle liver GSTs, salt gradients on hydroxylapatite and Matrix Red were modified to provide the best resolution of proteins. A typical elution profile for GR off hydroxylapatite is shown in Figure 23 for liver extracts from both control and anoxic turtles. Control and anoxic profiles were similar (with enzyme

elution from anoxic samples occurring over a slightly broader range of salt concentrations); a single peak of GR activity eluted at approximately 250 mM KPi. Elution from hydroxylapatite resulted in an almost two-fold purification for anoxic liver but with a low yield of activity (86.7 %) for this tissue (Table 10). Hydroxylapatite did not increase the purity of control enzyme but full recovery occurred from the column (103 % yield). Isoelectric focusing profiles for GR from liver of control and anoxic turtles are shown in Figures 24 and 25, respectively. Turtle liver GR focused into a single peak with pI values of 7.2 to 7.32, that were not significantly different between control and anoxic situations (n = 3 runs of each) (Table 12). I.E.F. resulted in a slight activation of the control enzyme but resulted in a much lower yield of the anoxic enzyme (113.5 and 47.1 % yield off the column for an overall yield to this point in the purification of 117 and 38 % for control and anoxic enzymes, respectively). A 4.76- and 9.41-fold purification occurred from I.E.F. for control and anoxic isolations, respectively. Typical elution profiles for control and anoxic turtle liver GR on Sephacryl S-300 are shown in Figure 26. Elution profiles were virtually identical and gave estimated native MW values of between 70.1 and 78.6 kDa (Table 12). Elution from Sephacryl S-300 resulted in a large activation of both control and anoxic enzymes (162.2 and 141.5 % yield compared with activity in the dialyzed I.E.F. pool) and a 11.3-fold (control) or 16.5-fold (anoxic) purification. A typical elution profile for GR on Matrix Red is shown in Figure 27. A gradient of 0 to 2 M KCl resulted in broad overlapping peaks for both control and anoxic enzymes with the majority of the enzyme eluting around 1 M KCl. Elution from Matrix Red resulted in poor recovery for control GR but a large activation of the anoxic enzyme (57.8 and 188.5 % yield off the column compared with the

Sephacryl S-300 pool) and gave a final overall yield for the enzyme purification of 109 and 101 % for control and anoxic enzymes, respectively. The overall fold purification was 13.2-fold for control and 12.1-fold for anoxic GR with final specific activities of 5.5 and 1.44 U/mg protein, respectively. Congruent with the literature, no new isozymes of GR were found in extracts from anoxic liver and therefore further kinetic studies were performed on control enzyme only.

SDS-PAGE

The results of SDS-PAGE of the purified protein are shown in Figure 28. Baker's yeast GR (Sigma) (Lane 2) as well as MW standards (Lane 1) were run for comparison. A standard curve created from the MW markers is shown in Figure 29. The purified GR preparation after Matrix Red chromatography showed four protein bands at molecular weights of 28.1, 33.0, 79.5, and 82.7 kDa, respectively. The two heaviest bands may represent undissociated homodimer GR whereas the lighter two bands may represent monomeric subunit and an impurity (33.0 and 28.1 kDa respectively). The 33.0 kDa band is clearly the major band in partially purified preparations after the I.E.F. (Lane 5) or Sephacryl S-300 (Lane 6) steps. The monomer had the same MW as the heavier bands of the Baker's yeast GR.

Reverse phase HPLC

Reverse phase HPLC elution profiles for Baker's yeast (A) and purified turtle liver (B) GR are shown in Figure 30. Elution from reverse phase resulted in a single peak (representing the monomer) which overlapped for Baker's yeast and turtle liver GR. GR

had an elution time of approximately 42 minutes on a 0 to 65 % acetonitrile gradient (50 minutes total). This corresponded to an acetonitrile concentration of 54.6 %.

Kinetic and inhibition characteristics

The results of kinetic and inhibitor characterization of *T. s. elegans* GR are shown in Table 11. Turtle liver GR had high affinities for both substrates, GSSG and NADPH. No GR activity was detected when NADH was used as a substrate. GSH inhibited the enzyme at fairly high concentrations although the I_{50} value at 6 mM is not too far above the physiological range whereas NADP^+ did not inhibit GR. Five salts tested inhibited turtle liver GR, with I_{50} s ranging between 450 and 770 mM.

Temperature and pH dependence

An Arrhenius plot for turtle liver GR is shown in Figure 31. The plot was linear over the full range of temperatures tested. The activation energy (E_a) calculated from the slope of the relationship was 32.2 ± 2.64 kJ/mol (Table 12). Denaturation was evident at approximately 55 °C.

The effect of pH on turtle liver GR is shown in Figure 32. The enzyme showed gradually increasing activity with increasing pH to an optimum at pH 6.5 (Table 12), after which there was a gradual decline to no activity at pH 10. The enzyme retained about 50 % or more of maximal activity over the range between pH 6 and 7.5.

DISCUSSION

In recent studies, free radical formation under low oxygen tensions has been observed (for a review, see Rifkind et al. 1993). The majority of these studies have found superoxide production under reduced oxygen tensions in brain and erythrocytes. The mechanism proposed involves the leakage of radicals from the electron transport chain associated with the mitochondrial membrane when the oxygen pressure drops and cytochrome oxidase is only partially saturated with oxygen. Evidence for an uncoupling of oxidative phosphorylation and presumably enhanced leakage of oxyradicals has been provided by the determination of phosphorylation efficiency as a function of both substrate level and oxygen pressure. Purified human hemoglobin has been found to be oxidized at reduced oxygen pressures. Red cell lysis also increases at these reduced pressures. Erythrocyte degradation has been targeted to damage from increased oxyradical formation under these situations. Turtles, after 20 hours of anoxic treatment at 5 °C, may not be completely anoxic; instead tissues may still be at a severely hypoxic stage with oxygen tensions slightly above zero. Thus, the possibility that oxyradicals are formed after 20 hours of anoxia treatment at this temperature is present.

Turtle liver showed evidence that oxidative stress occurred after 20 hours of anoxia exposure (Chapter 4, Willmore and Storey 1997b). GSSG increased 2-fold during anoxia whereas total glutathione decreased by half. The overall GSH/GSSG ratio decreased to 17 % of control values during anoxic exposure in liver. In response, the maximal activity of GR increased by 52 % during anoxia. Similar results have been seen in other studies. An increase in GSSG (and thereby a decrease in the GSH/GSSG ratio) was seen in hepatopancreas and foot muscle of Otala lactea estivating for 30 days (a

hypoxic stress) (Hermes-Lima et al. 1997) but no increase in GR activity occurred. A decrease in liver GSH was seen during estivation of the desert toad Scaphiopus couchi, causing the GSH/GSSG ratio to decrease (Grundy 1996). No change occurred in the GSSG levels in this animal, however, but a decrease in GR activity occurred in liver. Similarly, no change in glutathione status or GR activity occurred in liver during freezing exposure in wood frogs, Rana sylvatica (Joannis and Storey 1996a). The frozen state in these frogs may be a completely anoxic state as circulation is interrupted by frozen blood plasma. The hypoxic transition to complete anoxia in this case may be too brief to generate sufficient radicals to cause oxidative stress.

Failure to maintain the intracellular GSH/GSSG ratio may lead to the disturbance of the oxidation-reduction state of the cell, formation of mixed disulfides, or inactivation of enzymes possessing sulfhydryl groups at their active sites (Halliwell and Gutteridge 1989). In the absence of GR, exposure to atmospheric oxygen would lead very quickly to a complete disappearance of intracellular thiols. Modification of GR activity or expression of novel GR isozymes has been correlated with tolerance of various environmental stresses such as heat (Nieto-Sotelo and Ho 1986), chilling (Schoner and Krause 1990, Hausladen and Alscher 1994), hyperoxia (Foster and Hess 1980), air pollution (Tanaka et al. 1988a), heavy metals (Grill et al. 1985), high light intensity and magnesium deficiency (Cakmak and Marschner 1992), and water deficiency (Smirnoff and Colombe 1988, Tanaka et al. 1990). An increase in turtle liver GR activity during anoxia thus merited further purification and characterization of the enzyme.

During purification of GR, it became evident that no new isoforms of the enzyme were formed during anoxia. No new peaks of enzyme activity were not observed in the

comparison between control and anoxic extracts by any of the separation criteria used (MW, pI, phosphate or salt concentrations). Characterization of the kinetic properties of the control enzyme was therefore undertaken on the assumption that the same isozyme was present under both aerobic and anoxia conditions.

The specific activities of the control and anoxic GR preparations purified from turtle liver (5.50 and 1.44 U/mg, respectively) were comparable to those of the partially purified enzyme from Scaphiopus couchi liver (0.07 to 3.67 U/mg) (Grundy 1996) or from hepatopancreas and foot muscle of Littorina littorea (2.14 and 1.20 U/mg, respectively) (Pannunzio 1994). Specific activities in turtle liver were also close to that reported for rat liver (9.5 U/mg) (Carlberg and Mannervik 1975) but was less than maize (30 U/mg) (Turner and Pollock 1993) or pea chloroplast, mouse liver, and horse liver (ca. 150-250 U/mg) (Connell and Mullet 1986, López-Barea and Lee 1979, Garcia-Alfonso et al. 1993).

The pI of turtle liver GR was higher than that found in mouse (López-Barea and Lee 1979), horse (Garcia-Alfonso et al. 1993), calf, and rat (Carlberg and Mannervik 1985) livers which tend to have a pI point of around 6.5. The significance of this higher pI point remains to be determined. Some structural differences (ie. changes in amino acid composition and hence net enzyme pI) would likely occur between a mammalian (homeotherm) and a reptile (poikilotherm) if only because the reptile protein typically functions over a different and more variable temperature range and hence may need greater flexibility in its structure.

The subunit MW of GR from turtle liver (ca. 33 kDa) was substantially smaller than that of the enzyme from a variety of mammalian sources: human erythrocyte (ca.

52.5 kDa) (Krauth-Siegel et al. 1982), horse liver (ca. 58 kDa) (Garcia-Alfonso et al. 1993), rat liver (ca. 60 kDa) (Carlberg and Mannervik 1975), and mouse liver (ca. 55 kDa) (López-Barea and Lee 1979). The native MW of the dimeric enzymes was also smaller (ca. 70-78 kDa) than the enzyme from other sources such as desert toads (ca. 106 kDa) (Grundy 1996) and snails (ca. 103 kDa) (Pannunzio 1994).

The K_m GSSG of turtle liver GR (44.6 μ M, Table 11) was much lower than that reported for mouse liver (107 μ M) (López-Barea and Lee 1979), pea chloroplast (62 μ M) (Connell and Mullet 1986), horse liver (59 μ M) (Carlberg and Mannervik 1975), rat liver (63 μ M) (Garcia-Alfonso et al. 1993), Littorina littorea hepatopancreas (50 μ M) (Pannunzio 1994), or Scaphiopus couchi liver (88 μ M) (Grundy 1996). The lower K_m reveals a much higher affinity for GSSG than most known GRs. However, the K_m value for NADPH (6.82 μ M, Table 11) was within the range for GR from most other sources (4.9-8.8 μ M). NADP⁺ did not inhibit GR activity. The I_{50} for GSH (6.02 mM, Table 11) was well above physiological concentrations of GSH (3.4 mM and 1.7 mM for control and anoxic liver, respectively) (Chapter 4, Willmore and Storey 1997b) indicating that the enzyme would not be inhibited by GSH in vivo.

The activation energy of turtle liver GR (32.2 kJ/mol, Table 12) was much lower than that of GR from the desert toad (79-86 kJ/mol), bovine intestinal mucosa (61.1 kJ/mol) (Grundy 1996) or Littorina littorea hepatopancreas (65.2 kJ/mol) and foot muscle (59.2 kJ/mol) (Pannunzio 1994).

The pH optimum for turtle liver GR (pH 6.5) was lower than that for the enzyme from most other sources including rat liver (pH 7.0) (Carlberg and Mannervik 1975), mouse liver (pH 7.25) (López-Barea and Lee 1979), desert toad liver (pH 6.75-7.5)

(Grundy 1996), Littorina littorea hepatopancreas and foot muscle (pH 7.0-7.5) (Pannunzio 1994), and spinach root (pH 7.2) and leaf (pH 7.8) (Tanaka et al. 1994). The pH range was also very broad, lying between pH 4 and 10. This may have adaptive significance as the buildup of lactate during anoxic hibernation in turtles causes body pH to drop almost one full pH unit. Organisms which periodically encounter lower body pHs will, in general, have enzymes which work optimally over lower pH ranges.

In summary, new isoforms of GR were not synthesized during anoxia in T. s. elegans as evidenced by pI, MW, and elution profiles from salt gradients. Since no reports of post-translational modification of this enzyme exist, the increased maximal activity of this enzyme that occurred in turtle liver during anoxia exposure (Chapter 4) is probably most likely ascribed to increased synthesis of the enzyme. The high affinity of the turtle enzyme for GSSG (and the low levels of GSSG found in this tissue, Chapter 4) indicate that turtles have a superior mechanism in place to maintain the GSH pool in the cell during oxidative stress, either during the hypoxic transition into the anoxic state or during recovery from anoxia. This GSH provides the redox buffering capacity to protect protein sulfhydryl groups from oxidative attack. One report (Reischl 1986) suggests another molecule, the sulfhydryl-rich hemoglobins of turtle erythrocytes, may form disulfide bridges amongst hemoglobin tetramers as well as forming mixed disulfides with GSSG and provide additional redox buffering capacity. High redox buffering capacity may be an integral part of the overall biochemical adaptation to anoxia in turtles.

Fig. 23. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GR from hydroxyapatite. The enzymes were eluted with a linear gradient of KPi (0 to 1 M). Activities are expressed relative to the peak fraction. GR activity from control and anoxic turtle liver extracts eluted in one peak at the same KPi concentration (between 200 and 263 mM KPi). Closed and open circles represent control and anoxic isolations, respectively.

Fig. 24. Typical profiles of control Trachemys scripta elegans liver GR from isoelectric focusing. Activities (closed circles) are expressed relative to the peak fraction. The isoelectric point (pI) was 7.2 ± 0.0635 (n = 3 determinations). Open circles show the pH gradient.

Fig. 25. Typical profiles of anoxic Trachemys scripta elegans liver GR from isoelectric focusing. Activities (closed circles) are expressed relative to the peak fraction. GR focused into a single peak with a pI of 7.32 ± 0.0493 (n = 3 determinations). Open circles show the pH gradient.

Fig. 26. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GR from Sephacryl S-300. Activities (control, closed circle; anoxic, open circle) are expressed relative to the peak fraction. GR activity from control and anoxic turtle liver pools eluted in one peak at the same MW (approximately 70,100 to 78,600 Da). MW standards (closed squares) used were phosphofructokinase (PFK; 360,000 Da), aldolase (ALD; 150,000 Da), hexokinase (HK; 102,000 Da), hemoglobin (Hb; 64,500 Da), and cytochrome c (Cyt c; 13,700 Da). Blue Dextran (BD; 2,000,000 Da) eluted at the same volume as PFK.

Fig. 27. Typical elution profiles of control and anoxic Trachemys scripta elegans liver GR from Matrix Red. The enzyme were eluted with a linear gradient of KCl (0 to 2 M). Activities are expressed relative to the peak fraction which was set to 100. GR activity from control and anoxic turtle liver extracts eluted in one peak at the same KCl concentration (between 0.85 and 1.00 M KCl). Closed and open circles represent control and anoxic isolations, respectively.

Fig. 28. SDS-PAGE (15 % w/v acrylamide gel) of purified GR from control Trachemys scripta elegans liver stained with Coomassie blue. Lane 1, MW standards; Lane 2, Baker's yeast GR (Sigma); Lane 3, turtle liver crude supernatant; Lane 4, pooled peak after hydroxylapatite chromatography; Lane 5, pooled, dialyzed peak after isoelectrofocusing; Lane 6, pooled peak after Sephacryl S-300; Lane 7, pooled peak after Matrix red showing the final partially purified enzyme. The MW of standards (closed circles) are: myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da).

Fig. 29. Standard curve used to determine the subunit MW of Trachemys scripta elegans liver GR from SDS-PAGE. The MW of standards (closed circles) are: myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). Turtle liver GR subunits are indicated with an open circle.

Fig 30. Reverse phase HPLC profile of GR purified from control liver of Trachemys scripta elegans. A: Baker's yeast GR; B: turtle liver GR. GR had an elution time of approximately 42 minutes on a 0 to 65 % acetonitrile gradient (50 minutes total). This corresponded to an acetonitrile concentration of 54.6 %.

Fig. 31. Arrhenius plots of GR purified from control liver of Trachemys scripta elegans. Values are means of three trials performed on a single enzyme preparation, $\pm$ S.E.M. Where error bars are not visible, they are contained within the symbol.

Phosphate buffer was used for all assays and cuvette temperature was checked immediately prior to and following the assay; the temperatures used are an average of these two values. The activation energy (E_a) was 32.2 ± 2.64 kJ/mol. Denaturation was evident at approximately 55 °C.

Fig. 32. pH profiles of GR purified from control liver Trachemys scripta elegans.

Values are means of three trials performed on a single enzyme preparation, $\pm$ S.E.M.

Where error bars are not visible, they are contained within the symbol. Phosphate buffer was used for all assays and pH was checked immediately prior to and following the assay; the pH values reported are an average of these. The pH optimum was 6.5.

Table 10. Fold purifications and % yields of GR purified from liver of Trachemys scripta elegans using optimal GSSG and NADPH concentrations. Results are from one purification; replicates yielded similar results. Enzyme was assayed using optimal CDNB and GSH concentrations.

Column	Control Turtle Liver			Anoxic Turtle Liver		
	Specific activity U/mg	Fold Purification	% yield	Specific activity U/mg	Fold purification	% yield
supernatant	0.418			0.119		
hydroxylapatite pool	0.343	0.821	103	0.231	1.94	80.7
I.E.F. pool (dialyzed)	1.99	4.76	117	1.12	9.41	38.0
Sephacryl S-300 pool	4.72	11.3	189	1.96	16.5	53.8
Matrix Red pool	5.50	13.2	109	1.44	12.1	101

Table 11. Kinetic parameters of GR purified from control Trachemys scripta**elegans liver.** Results are means of 3 determinations $\pm$ S.E.M.

K_m GSSG (μ M)	44.6 ± 2.29
K_m NADPH (μ M)	6.82 ± 0.427
I_{50} GSH (mM)	6.02 ± 0.260
I_{50} NADP ⁺ (mM)	no inhibition
I_{50} KCl (M)	0.506 ± 0.0539
I_{50} NaCl (M)	0.551 ± 0.0275
I_{50} Na ₂ SO ₄ (M)	0.452 ± 0.0391
I_{50} NH ₄ Cl (M)	0.615 ± 0.0155
I_{50} Na acetate (M)	0.772 ± 0.0313

Table 12. General characteristics of GR purified from Trachemys scripta elegans

liver. Results $\pm$ S.E.M. are means of 3 determinations; values for the enzyme from anoxic liver are not significantly different from control values.

	Control	Anoxic
I.E.F.		
pI	7.20 ± 0.0635	7.32 ± 0.0493
Sephacryl S-300		
MW (Da)	78,600	70,100
SDS-PAGE		
subunit MW (Da)	33,000	
Arrhenius plot		
Ea (kJ/mol)	32.2 ± 2.64	
pH curve		
pH optimum	6.50 ± 0.00	

CHAPTER SEVEN

MULTICATALYTIC PROTEINASE ACTIVITY IN TURTLE LIVER: RESPONSES TO ANOXIA STRESS AND RECOVERY

INTRODUCTION

The multicatalytic proteinase complex (MPC; E.C. 3.4.99.46) is a major extralysosomal proteolytic system that plays a key role in intracellular protein turnover (Orlowski 1990, Orlowski et al. 1991, Rivett 1993, Coux et al. 1996, Baumeister and Lupas 1997, Baumeister et al. 1997, Rivett et al. 1997). The proteinase associates with other factors to form a larger 26S complex that plays an essential role in the ATP/ubiquitin-dependent pathway of proteolysis in the cytosol (Etlinger and Goldberg 1977, Tanaka et al. 1988b, Matthews et al. 1989, McGuire and DeMartino 1989). The enzyme has an apparent molecular mass ranging from 700 to 900 kDa, a sedimentation coefficient of 16-20S and 10-14 electrophoretically distinct subunits in the 25-35 kDa range (Orlowski 1990). Electron microscopy reveals that the proteinase is organized as a stack of four rings; each ring consisting of six to eight subunits (Tanaka et al. 1988b, Kopp et al. 1986). An active and an inactive form of the complex exists (Falkenburg et al. 1988, McGuire et al 1989), the enzyme being activated by 0.04% SDS and polylysine. The proteinase degrades a variety of naturally occurring peptides in vitro such as bradykinin, angiotensin II, LH-RH, neurotensin and substance-P (Wilk and Orlowski 1980). The complex has been found in a wide variety of eukaryotic cells, from both vertebrates and invertebrates, and may constitute 0.5-1 % of total cellular protein (Orlowski 1990, Rivett 1989). The MPC exhibits three distinct endopeptidase activities, cleaving peptide bonds on the carboxyl side of hydrophobic (chymotrypsin-like), basic (trypsin-like), and acidic (peptidylglutamyl-peptide bond hydrolyzing) amino acids (Orlowski 1990, Wilk and Orlowski 1989). Studies of the effects of inhibitors and activators on the complex have shown that each of the three activities is associated with a separate component (Orlowski 1990). Examination of the effects of SDS on proteolysis indicates that the main component responsible for the degradation of proteins is the peptidylglutamyl-peptide hydrolyzing activity (Orlowski and Michaud 1989).

Protein metabolism, including synthesis, post-translational modification, and degradation, is a major energy expenditure for cells, one that must be closely regulated with respect to energy availability. Many animals have the ability to reversibly suppress metabolic rate, often to as little as 1-20 % of normal aerobic resting rate (Storey and Storey 1990). Escape to a hypometabolic or dormant state is typically a survival strategy for dealing with severe environmental stress and characterizes, for example, hibernation, estivation, diapause, and facultative anaerobiosis. Studies in recent years have identified molecular mechanisms that coordinate the suppression of glycolysis and other enzymes of intermediary metabolism, as well as membrane ion transport activity, during metabolic arrest (Storey and Storey 1990, Lutz 1992). Amongst vertebrates, the mechanisms of metabolic arrest have been extensively studied in anoxia-tolerant freshwater turtles (Storey and Storey 1990, Lutz 1992). During anoxic submergence turtles rapidly reduce metabolic rate to only 10-20 % of the corresponding aerobic rate at the same temperature and, by doing so, can survive without oxygen for several days at warm temperatures and as long as 3 months at 3°C (Herbert and Jackson 1985, Ultsch 1989).

The maintenance of long-term homeostasis in an anoxic, energy-restricted, hypometabolic state should, predictably, include suppression of the rate of protein turnover in cells involving coordinated changes in both protein synthesis and protein degradation. Changes in protein degradation rates may involve modulation of the MPC activity in organs. The present study analyzes the effects of in vivo anoxia exposure and subsequent aerobic recovery on the activities and substrate profiles of the MPC from liver of the anoxia-tolerant turtle T. s. elegans.

MATERIALS AND METHODS

Chemicals and animals

N-CBZ-LEU-LEU-GLU β -naphthylamide (Z-LLE-MNA), N-CBZ-ALA-ARG-ARG 4-methoxy β -naphthylamide (Z-ARR-MNA), N-succinyl-LEU-LEU-VAL-TYR 7-amido-4-methylcoumarin (Suc-LLVY-AMC), fluorescent end product standards (β -naphthylamine, 4-methoxy β -naphthylamine, and 7-amino-4-methylcoumarin), molecular weight markers and other biochemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Adult red-eared sliders (*T. s. elegans*) were obtained from Wards Natural Science, Mississauga, Ont. and were maintained in large tanks with dechlorinated water at 7°C for at least 3 weeks prior to experimentation. Turtles had access to deep water and a dry platform supplied with a heat lamp and were fed a diet of lettuce, egg shells and trout food pellets supplied ad libitum.

Control (normoxic) turtles were sampled directly from the holding tank. Anoxia was imposed by submerging turtles for 20 h at 5°C in sealed tanks of water that had been previously bubbled with 100% N₂ gas for 1 h. Anoxic turtles were sampled immediately after prolonged submergence whereas others were returned to an aerobic holding tank at 5°C and allowed to recover for 24 h before sampling. Animals were killed by decapitation and livers were quickly removed, frozen in liquid nitrogen and then transferred to -80°C for storage.

MPC (E.C. 3.4.99.46) activity assay

Liver samples were homogenized 1:3 (w:v) in Buffer A containing 20 mmol l⁻¹ imidazole (pH 8.0), 15 mmol l⁻¹ β -MeSH, 2 mmol l⁻¹ EDTA, 2 mmol l⁻¹ EGTA, 50 mmol l⁻¹ NaF, 20 % v/v glycerol and 0.04 % w/v sodium azide for 20 s using an Ultra-Turrax (Tekmar) tissue homogenizer and centrifuged at 13 000 x g for 10 min (Eppendorf

microcentrifuge) at 4°C. All three catalytic activities of the MPC were assayed. To measure peptidylglutamyl peptide bond hydrolyzing, trypsin-like or chymotrypsin-like activities, 10 µl of crude supernatant was incubated for 2 h at 21°C in a total volume of 300 µl of Buffer B (50 mmol l⁻¹ Tris-HCl, pH 8.0, 10 mmol l⁻¹ β-MeSH) containing either 67 µmol l⁻¹ Z-LLE-MNA, 67 µmol l⁻¹ Z-ARR-MNA or 6.7 µmol l⁻¹ Suc-LLVY-AMC, respectively. Reactions were stopped by the addition of 1.5 ml of 0.2 M sodium borate (pH 9.1) (Strack et al. 1992). Stock solutions (2 mg/ml) of peptides were made by dissolving them in 100 % DMSO and then adding them to Buffer B in the appropriate concentrations. Blanks contained the same components but the sodium borate solution was added to the reaction mixture prior to the addition of enzyme. Fluorescence of both blanks and samples was measured on a Perkin-Elmer LS50 spectrofluorometer (excitation 335 nm, emission 410 nm). One unit of enzyme activity was defined as the amount of enzyme that cleaves one µmol of substrate per hour.

Stock solutions (2 mg/ml) of standards were made by dissolving them in 100 % DMSO. Aliquots of standards were diluted in Buffer B to create standard curves ranging from 0-15 µmol l⁻¹ for β-naphthylamine and 4-methoxy β-naphthylamine, and 0-5 µmol l⁻¹ for 7-amino-4-methylcoumarin.

Preparation of semipurified MPC

Liver samples were homogenized and centrifuged as above. Supernatant (2 ml) was applied to a Sepharose CL-6B column (2.5 x 30 cm) (Pharmacia, Milwaukee, WI) equilibrated with Buffer A. A small volume of supernatant was reserved for crude assays. A 30 ml void volume was eluted and then one ml fractions were collected. A 10 µl sample of each fraction was incubated for 2 h at 21°C in a total volume of 300 µl of Buffer B containing 67 µmol l⁻¹ Z-LLE-MNA. Reactions were stopped by the addition of the sodium borate solution and fluorescence was measured. Blanks were prepared as described above. Column fractions containing greater than 0.075 nmol product produced

per h were pooled and used for further characterization of the enzyme. Assays of all three MPC catalytic activities in pooled peak fractions were conducted as described for the crude enzyme using 10 μ l of enzyme preparation. The molecular weight standards used to calibrate the Sepharose CL-6B column were: blue dextran, 2,000 kD; thyroglobulin 669 kD; apoferritin 443 kD; pyruvate kinase 237 kDa; and lactate dehydrogenase 140 kD.

Gel electrophoresis

After gel filtration, samples of fractions (2.3 to 5.3 μ g of MPC) were resolved by nondissociating PAGE on a 4.5% gel in 20 mM Tris-HCl buffer, pH 8.8 and stained for protein using Silver stain. A second gel run concurrently with the first was sliced into 6 mm sections from top (slice 1) to dye front (slice 10) and slices were incubated in 500 μ l of Buffer B containing 67 μ mol l⁻¹ Z-LLE-MNA for 36 h at room temperature. Proteolytic activities of each gel slice were then determined as above.

Protein

Protein concentrations were measured using the Coomassie blue dye-binding method (Bradford 1976) with bovine serum albumin as the standard and the prepared reagent from Bio-Rad.

Statistics

Data are reported as means $\pm$ S.E.M. Statistical analysis was performed using analysis of variance followed by the Dunnett's test (two-tailed).

RESULTS AND DISCUSSION

Partial purification of the MPC complex

Multicatalytic proteinase complex activity was readily detectable in turtle liver. The elution profile for the peptidylglutamyl-peptide bond hydrolyzing activity of turtle liver MPC on Sepharose CL-6B is shown in Figure 33. Peptidylglutamyl-peptide bond hydrolyzing, trypsin-like and chymotrypsin-like activities co-eluted during gel filtration at a molecular weight of $940 \text{ kD} \pm 46 \text{ kD}$ ($n = 9$), somewhat higher than the approximately 700 kD that is generally typical of the vertebrate enzyme although masses ranging from 550 to 900 kD have been reported (Orlowski 1990). The elution profile was the same for liver extracts from control, anoxia-exposed, and aerobic recovery turtles.

The partial purity of the MPC was verified by native PAGE (Figure 34A). Silver stain revealed that the MPC migrated as a single band under nondissociating conditions. When the gel was sliced into 6 mm segments and proteolytic activity was determined (Figure 34B) in each segment, proteinase activity corresponded to a single band in the 4.5 % gel. Specific activity increased from 10.6 mU/mg protein in the crude homogenate to 356.4 mU/mg protein in the peak fraction off of gel filtration resulting in a 34 fold purification.

Catalytic activity of partially purified MPC during anoxia exposure and recovery from anoxia

Pooled peak fractions of turtle liver MPC activity from the Sepharose CL-6B column were tested for three individual catalytic activities (Figure 35). Peptidylglutamyl-peptide bond hydrolyzing, trypsin-like and chymotrypsin-like activities, measured by monitoring the hydrolysis of Z-LLE-MNA, Z-ARR-MNA or Suc-LLVY-AMC respectively, showed an activity ratio of 6.2:2.7:1. This differs considerably from the ratios reported for bovine pituitaries (1:1.2:1.9) (Wilk and Orlowski 1989) or avian

erythrocytes (1:7.7:4.1) (Strack et al. 1992). The prominence of the peptidylglutamyl-peptide bond hydrolyzing activity in turtle liver may reflect an organ-specific or species-specific function of the enzyme. Proteins hydrolyzed by this activity include β -casein and lysozyme and previous studies, in examining the effects of SDS on proteolysis, have indicated that this activity is the major one responsible for the degradation of proteins by the MPC (Orlowski and Michaud 1989).

Anoxia exposure had no effect on any of the three proteolytic activities of the semi-purified MPC (Figure 35). However, during aerobic recovery after anoxia the peptidylglutamyl-peptide bond hydrolyzing activity was significantly increased, rising by 29 % compared with control values. This clearly shows that MPC activity can be modified in vivo in response to a change in the physiological state of liver and indicates that the MPC may be involved in the metabolic recovery from anoxia stress. The mechanism of this change in MPC activity is not yet known. The chymotrypsin-like activity of the MPC has been shown to be regulated by phosphorylation via a cAMP-dependent protein kinase that copurifies with the complex (Pereira and Wilk 1990). Two subunits, phosphorylated by the copurifying protein kinase and/or tyrosine kinase, suggest that protein phosphorylation may represent a mechanism for regulating the activity of the MPC. However, whether similar posttranslational regulation of subunits catalyzing peptidylglutamyl-peptide bond hydrolyzing activity can occur has not been reported to date.

Possible role of the MPC in recovery from anoxia

It has been suggested that the MPC may be at least partially responsible for the rapid degradation of proteins subjected to oxygen free radical damage (Rivett 1985, Pacifici and Davies 1988). For example, complete removal of the MPC from human erythrocyte extracts by immunoprecipitation or by inhibitors of its proteolytic activity decreased degradation of hydroxyl- and superoxide-damaged proteins by 30-40 %

(Matthews et al. 1989). One of the realities of post-anoxic recovery is the rapid reoxygenation of tissues which creates a relative hyperoxia and a burst of oxygen free radicals generation. Indeed, oxygen free radical damage appears to be a primary cause of post-ischemic reperfusion injury reported in many anoxia-intolerant species, including mammals (Eckenhoff et al. 1992). It is reasonable to assume that anoxia-tolerant species must have developed mechanisms to limit oxygen free radical damage during recovery. One of these mechanisms may be an increase in the MPC peptidylglutamyl-peptide bond hydrolyzing activity during recovery to quickly and selectively degrade proteins damaged by free radicals.

Fig. 33. Elution profile on Sepharose CL-6B of the Z-LLE-MNA hydrolyzing activity of multicatalytic proteinase complex (MPC) from Trachemys scripta elegans liver. Enzyme activity (minus blank) is shown as $\text{nmol product} \cdot \text{h}^{-1} \cdot \text{ml}^{-1}$ for liver samples from control (open circles), 20 h anoxic (closed circles), and 24 h aerobic recovery (open triangles) turtles. Closed squares show the molecular weight standards: blue dextran, thyroglobulin, apoferritin, pyruvate kinase and lactate dehydrogenase.

Fig. 34. Nondissociating PAGE of the partially purified MPC from Trachemys scripta elegans liver. A. Nondissociating PAGE of the partially purified MPC (2.3 to 5.3 μg) stained for protein with Silver stain. Numbers below individual lanes represent fraction number off of the Sepharose CL-6B gel filtration column. B. Proteolytic activity (in Fluorescence units) of slices (6 mm) of the 4.5 % gel. Gel slices were incubated in 500 μl of 67 $\mu\text{mol l}^{-1}$ Z-LLE-MNA for 36 h at room temperature and then assayed for proteolytic activity. Background fluorescence (ΔF of 136 fluorescence units) was subtracted from reported activities.

A

36 37 38 39 40 41 42

B

Fig. 35. Peptidylglutamyl peptide bond hydrolyzing (Z-LLE-MNA), trypsin-like (Z-ARR-MNA) and chymotrypsin-like (Suc-LLVY-AMC) activities of partially purified liver MPC in pooled peak fractions taken from Sepharose CL-6B gel filtration from control (open bars), 20 h anoxic (striped bars) and 24 h recovered (filled bars) *Trachemys scripta elegans*. Data are means $\pm$ S.E.M., n = 3-9, expressed per μ g protein. * - Significantly different from the corresponding control with the same substrate, $P < 0.01$.

CHAPTER EIGHT

GENERAL DISCUSSION

The transition into a hypometabolic state in anoxia tolerant organisms not only involves a change in oxygen content and pH, but also a change in redox status in tissues. As oxygen availability drops, NADH levels begin to rise and the intracellular redox system becomes more reduced (Clark and Miller 1973). Thus, diving turtles must endure the two extremes of redox state; the highly reducing state of the later stages of anoxia and the highly oxidative state of recovery from anoxia. The maintenance of redox balance within the cell is crucial to enzymatic function during turtle anoxia. To conclude this thesis, a number of adaptations that turtles have shown (based on my data and/or on the existing literature) to maintain redox balance will be discussed.

Metabolic Flux Potential and Redox Balance

The changes that occur in support of metabolic depression during turtle anoxia are best illustrated by the responses of enzyme maximal activities. The majority of enzyme activities remained unchanged or decreased in response to anoxia/recovery, indicating that the turtle is well suited with constitutive enzyme activities to meet the demands of anoxic excursions. Of the 17 enzymes which showed changes in activities between control and anoxic tissues of T. s. elegans, 14 showed reduced activities whereas only 3 showed increases. The majority of the enzymes showing decreased activities were those associated with either carbohydrate or amino acid metabolism. Half of the instances of reduced enzyme activities during anoxia were recorded in brain. Brain, in general, is a highly metabolically active tissue, and would therefore require stringent control over its enzyme activities during energy deficiency. In many cases, enzyme activities remained depressed during recovery from anoxia and did not return to normoxic levels.

De novo protein synthesis under anoxic conditions in turtles would be energetically costly and therefore only enzymes essential to survival would be synthesized. The three enzymes which revealed increased activity during anoxia include G6PDH in heart, MDH in white muscle and CPT in kidney. The increase in G6PDH activity in heart during anoxia is possibly due to an increased need for reducing equivalents in the form of NADPH in this tissue. NADPH is utilized by GR to reduce GSSG as well as in cholesterol biosynthesis, deoxyribonucleotide formation and fatty acid synthesis. The main purpose for potentially increased shuttling of carbon through the Pentose Phosphate Pathway in turtle heart during anoxia remains yet to be determined.

A possible reason for the increase in NADPMDH activity in white muscle also remains unknown. In the muscle of diving vertebrates, the NAD-linked enzyme provides NAD^+ for GAPDH in order to maintain anaerobic glycolysis during extended dives (Hochachka et al. 1974, Owen and Hochachka 1974, Storey 1974). Lactate accumulates to such an extent that pyruvate is transaminated to alanine (Hochachka et al. 1974). Oxaloacetate is provided to NADMDH by a 1:1 coupling of GPT and GOT (Owen and Hochachka 1974). NADPMDH however, being a cytosolic enzyme, serves a different function. The increase in the NADP-linked activity in anoxia made this activity greater than the NAD-linked activity in white muscle. If NADPMDH activity increases without a corresponding increase in NADMDH, then it is possible that the NADP^+ activity belongs to a different enzyme protein that is specifically enhanced in anoxia.

An increase in CPT activity in turtle kidney during anoxia is a unique finding. The increase in CPT activity in the kidney during anoxia may indicate that fatty acids are

being utilized as a metabolic fuel during the extreme stages of anoxic treatment when tissues are severely hypoxic. Renal tubule cells have large numbers of mitochondria on their serosal side and may have a well developed carnitine shuttle. In mammals, severe hypoxia is marked by increased carbohydrate and decreased fatty acid consumption. In turtles, it is the opposite with decreased carbohydrate consumption. β -oxidation of fatty acids may increase during the severe hypoxia in key organs. The implications of increased activity of CPT in anoxic turtle kidney remains to be determined. It is, however, possible that kidney is one of the organs that has preferential access to remaining oxygen supplies well into submergence so maybe it can keep up an aerobic, lipid-based metabolism for longer than other tissues.

Antioxidant and Prooxidant Strategies

The strategy which *T. s. elegans* uses to combat the potential for oxidative stress upon recovery from anoxia is the maintenance of consistently high antioxidant defenses in order to immediately quench or limit the formation of ROS. With these defenses, the formation of ROS during reperfusion may be effectively quenched so that no net oxidative damage (and therefore no oxidative stress) occurs. The maintenance of constitutively high antioxidant enzyme activities can be seen when comparing the maximal activities of turtle antioxidant enzymes to those in the same tissues of other lower vertebrates. Activities in turtle organs were similar to mammalian levels but well above those of other ectothermic vertebrates. In particular, turtle liver and skeletal muscle SOD, CAT and GPOX activities are substantially higher than those found in the same tissues from other ectothermic vertebrate sources (Storey 1996b, Willmore and

Storey 1997a). Similarly, the levels of GSH in liver and skeletal muscle are much higher than those found in the same tissues of other organisms (Storey 1996b, Willmore and Storey 1997b). High levels of GSH also provide cells with high reducing power in order to maintain the redox state (as well as a high GSH/GSSG ratio) in cells. Other low molecular weight antioxidants are also present in high concentrations in turtle tissues; for example, ascorbate in turtle brain (Rice and Cammack 1991). The same group, however, did not find high levels of glutathione in turtle brain (Rice et al. 1995).

Turtle XO was found to comprise a large portion of the total XO+XDH present in tissues. Although the ratio XO / XO+XDH did not change in anoxia, the potential for radical production from this source is high. Since the levels of ATP fall only marginally in turtle tissue during anoxia (as ATP turnover decreases), the production of xanthine is also not expected to change. However, a slight dip in the levels of ATP occurs in the early stages of anoxic submergence which causes a transitory increase in the levels of adenosine in turtle brain (Nilsson and Lutz 1992). It has been suggested that this transitory increase in adenosine provides the signal for metabolic depression in this tissue. The adenosine could also be further broken down to xanthine. XO in turtle tissues would utilize the xanthine and produce H_2O_2 in the process. Recent evidence suggests that moderately high concentrations of certain forms of ROS (such as H_2O_2) may act as signal transduction messengers (Sen and Packer 1996; Lander 1997). At least two well defined transcription factors, nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) (Flohé et al. 1997) and activator protein-1 (AP-1) (Abate et al. 1990), are regulated by the intracellular redox state. In some cases, ROS are required for ligand-stimulated gene expression, whereas in others, ROS themselves initiate a cellular response. It has been well established that

reactive nitrogen species (nitric oxide; NO) regulate blood pressure through the generation of cGMP. In the same respect, ROS may interact with iron or thiol based targets in order to modify G-proteins, various transcription factors, ion channels, cyclooxygenase and ribonucleotide reductase. An increase in ROS production from an XO source during the hypoxia transition period could provide the signal for transduction cascades which would initiate mechanisms of metabolic depression that are brought into play as oxygen levels fall towards zero. Further tests are required to determine if this is the case.

Changes in the maximal activities of the glutathione related enzymes in liver during anoxia led to the purification and characterization of two enzymes. By purifying turtle liver GSTs and GR, it could be determined whether changes in activities during anoxia were due to the formation of new isozymes with either increased or decreased activity.

The glutathione S-transferases belong to a multigene enzyme superfamily which catalyze the addition of GSH to various electrophiles. The products of cellular damage due to peroxidation by prooxidants are included amongst these such as compounds resulting from peroxidation of lipids in biological membranes (reactive alkenes, epoxides, hydroperoxides and aldehydes). Purification of turtle liver GSTs resulted in the separation of at least two isozymes; one isozyme was a class Alpha GST whereas the other was a novel Alpha-like GST with no detectable peroxidase activity. No new isozymes were formed during anoxia. Hence, the lower maximal activities of GST seen in turtle liver during anoxia are due solely to decreased enzyme synthesis or increased enzyme degradation.

Glutathione reductase is a homodimeric, FAD-dependent enzyme which utilizes NADPH in order to reduce oxidized glutathione (GSSG). The enzyme is important to the redox status of the cell in that it produces GSH which can maintain the reduced state of proteins, can act directly against prooxidants, and can be utilized by other antioxidant enzymes. Purification of turtle liver GR resulted in a single isozyme being isolated. No new isozymes were formed during anoxia. Increased activities of the enzyme during anoxia in liver is, therefore, due solely to the formation of new enzyme protein. The high activities of this enzyme in turtle tissues preserves the GSH/GSSG ratio in cells at a very high level.

Protease Function During Anoxia and Recovery From Anoxia

Control over protein turnover during anoxia is an important aspect of hypometabolism in turtles. Coordinated suppression of the rates of both protein synthesis and protein degradation is key for long term homeostasis in the anoxic state. Protein degradation must be tightly regulated in order to match decreased protein synthesis. In anoxia tolerant turtle hepatocytes, proteolysis has been shown to be largely energy dependent under normoxia, whereas under anoxia there is a shift to a slower overall proteolytic rate that is largely energy independent and represents loss mostly from the labile (as opposed to stable) protein pool (Land and Hochachka 1994).

One of the major cytosolic proteases is the multicatalytic proteinase complex. In partially purified turtle liver MPC, the only activity which responded to anoxia/recovery was the peptidylglutamyl-peptide hydrolyzing activity which increased during recovery from anoxia. The peptidylglutamyl-peptide hydrolyzing activity, one of the main

activities of the MPC, is only partially expressed and can be greatly activated by low concentrations of SDS and fatty acids (Orlowski et al. 1991). The activity is under positive cooperative control and is inhibited by the presence of such proteins as albumin, lysozyme or casein (Orlowski et al. 1991). The increase in this activity may be as a result of activation by proteins oxidatively damaged during anoxia. Whereas no products from damaged lipids were accumulated by anoxic or recovered liver (Chapter 3), the GSH/GSSG ratio actually decreased (due to an increase in GSSG) (Chapter 4) which indicates that oxidative stress may have occurred in this tissue during extreme anoxia. The effects of such oxidative stress may have manifested themselves in injury to proteins rather than lipids. Activation of the peptidylglutamyl-peptide hydrolyzing activity of MPC may also be due to an excess of free fatty acids during recovery from anoxia in liver (Orlowski et al. 1991). In either case, the role of the MPC in recovery from anoxia remains to be determined.

In summary, anoxia tolerant turtles have evolved some impressive metabolic adaptations that allow them to deal effectively with wide variations in oxygen tension in their bodies, allowing effective reorganization of metabolism to ensure that cell viability is maintained when oxygen is lacking and that cellular metabolism and macromolecules are protected from wide variation in the production of reactive oxygen species.

PUBLICATION LIST

Published Research Articles

- Hermes-Lima, M., W.G. Willmore and K.B. Storey. 1995. Quantification of lipid hydroperoxides in tissue extracts based on Fe (III) xylenol orange complex formation. Free Rad. Biol. Med. **19** (3): 271-280.
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- Willmore, W.G. and K.B. Storey. 1997. Antioxidant systems and anoxia tolerance in a freshwater turtle Trachemys scripta elegans. Mol. Cell. Biochem. **170**: 177-185.
- Willmore, W.G. and K.B. Storey. 1997. Glutathione function, glutathione-related enzymes and anoxia tolerance in a freshwater turtle Trachemys scripta elegans. Am. J. Physiol. (Reg. Integr. Comp. Physiol.) (in press).

Research Articles In Preparation

- Willmore, W.G. and K.B. Storey. Purification and characterization of glutathione S-transferase from the slider turtle Trachemys scripta elegans.
- Willmore, W.G. and K.B. Storey. Purification and characterization of glutathione reductase from the slider turtle Trachemys scripta elegans.
- Willmore, W.G., K.J. Cowan and K.B. Storey. Changes in metabolic flux potential during anoxia and recovery in the anoxia tolerant turtle Trachemys scripta elegans.
- Willmore, W.G. and K.B. Storey. Mitochondrial gene expression during anoxia in brain of the slider turtle Trachemys scripta elegans.

Willmore, W.G. and K.B. Storey. Ornithine decarboxylase antizyme expression in anoxic turtle brain.

Communications Presented at Scientific Meetings/Abstracts

Willmore, W.G. and K.B. Storey. 1994. Antioxidant enzyme function during anoxia and recovery in an anoxia tolerant freshwater turtle. (abstract). Canadian Federation of Biological Societies 37th Annual Meeting, Montreal, Quebec, June 16-18, 1994.

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APPENDIX

Fig. 35. Densitometry of bands from SDS-PAGE (15% w/v acrylamide gel) of purified GSTs from control Trachemys scripta elegans liver stained with Coomassie blue (see Fig. 19.). Closed circles represent densitometry of turtle liver GST from Peak 1 (Lane 3, Fig. 19) while open circles represent densitometry of turtle liver GST from Peak 2 (Lane 4, Fig. 19).

Table 13. Kinetic parameters of crude supernatant GR and GST isolated from liver of Trachemys scripta elegans. Results are means of 4 determinations $\pm$ S.E.M.

Turtle Liver GR Crude Kinetics				
		Control	Anoxic	Two-tailed Student's T
GSSG	Km	0.0502 +/- 0.00474	0.0408 +/- 0.00105	Not significantly different
NADPH	Km	0.0131 +/- 0.00185	0.0177 +/- 0.00500	Not significantly different
GSH	I50	8.739 +/- 0.226	7.96 +/- 0.452	Not significantly different
Turtle Liver GST Crude Kinetics				
		Control	Anoxic	
GSH	Km	0.333 +/- 0.0210	0.280 +/- 0.00843	Not significantly different
CDNB	Km	0.563 +/- 0.120	0.363 +/- 0.0610	Not significantly different
GSSG	I50	0.638 +/- 0.0174	0.822 +/- 0.0331	p<0.005

IMAGE EVALUATION TEST TARGET (QA-3)

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