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**Carbon-13 Nuclear Magnetic Resonance
Studies of D-Glucose Metabolism
and
Purification of Gluconokinase from
*Schizosaccharomyces pombe***

by

Honggang Ye, B.Sc.

(Peking University)

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

Master of Science

**O.C.C.I.
Department of Chemistry
Carleton University
Ottawa, Ontario
August, 1993**

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submitted by Honggang Ye in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Metabolic pathways of D-glucose by *Schizosaccharomyces pombe* were studied by D-[^{13}C]-glucose nuclear magnetic resonance (NMR) spectroscopy. The activities of several enzymes in the glucose metabolic pathways were examined. The glycolysis, the pentose phosphate pathway and the direct oxidation route of glucose to gluconate (the gluconate route) were operative. Gluconokinase which phosphorylates D-gluconate to D-6-phosphogluconate was purified and partially characterized. The purified enzyme was electrophoretically homogeneous with a subunit molecular weight of 24,000 dalton. It has relatively high affinity for ATP ($K_{\text{ATP}} = 0.056 \text{ mM}$) and low for gluconate ($K_{\text{gluconate}} = 1.6 \text{ mM}$). The gluconokinase catalyzed reaction appears to follow a random bi bi mechanism with a formation of the dead-end E-gluconate-ADP complex. Gluconate formed may be metabolized further through the pentose phosphate pathway. NADPH formed by direct glucose oxidation and the oxidative branch of the pentose phosphate pathway may serve as the reductive equivalent for biosynthetic processes.

ACKNOWLEDGEMENT

It is a pleasure to acknowledge the guidance, instruction, encourage, criticism, and friendship I have received from Dr. C. S. Tsai throughout the course of this work. I also wish to thank Dr. H. Yamazaki, Dr. K. B. Storey, Dr. R. G. Barradas, and Dr. Buchanan, and K. Diedrich, R. Kehle, and L. Klimpel.

I am grateful to Keith Bourque for running NMR spectra. Thanks to F. Cassalman for the use of the equipment.

Special thanks to my good friend Shi for his help and encouragement. I also appreciate the help from every member in Steacie Building.

Financial support was provided by the Faculty of Graduate Studies, the Department of Chemistry.

I am especially thankful for the love and support I have received from my parents.

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LIST OF ABBREVIATIONS

2',5'-ADP	Adenosine 2',5'-diphosphate
6PGA	6-phosphogluconate
6PGDH	6-Phosphogluconate dehydrogenase
6PGL	6-phosphogluconolactonase
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
C	Cross-linker concentration
CoA	Coenzyme A
DTT	Dithiothreitol
[E] _t	Total enzyme concentration
EDA	2-keto-3-deoxy-6-phosphogluconate aldolase
EDD	6-phosphogluconate dehydratase
EDTA	Ethylenediamine-tetraacetate
ENO	Enolase
Er-4-P	Erythrose-4-phosphate
F-6-P	Fructose-6-phosphate
FDA	Fructose-1,6-bisphosphate aldolase
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GAP	Glycolaldehyde-3-phosphate
GD	Glucose dehydrogenase
GL	Gluconolactonase
GNK	Gluconokinase
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HK	Hexokinase
Kdal	Kilodalton
NAD(P) ⁺	β-Nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced form of NAD(P) ⁺
nm	nanometer
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
PGI	Phosphoglucose isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
P _i	Inorganic phosphate
PK	Pyruvate kinase
R-5-P	Ribose-5-phosphate
RPI	Ribose-5-phosphate isomerase
rpm	Round per minute

Ru-5-P	Ribulose-5-phosphate
S-7-P	Sedoheptulose-7-phosphate
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	Sodium dodecyl sulfate
T	Total monomer concentration
TA	Transaldolase
TCA	Tricarboxylic acid
TDH	Glyceraldehyde-3-phosphate dehydrogenase
TK	Transketolase
TPI	Triose-phosphate isomerase
V_{max}	The limiting maximal velocity
$V_{max}/[E]_t$	Turnover number
v	Initial reaction rate
Xu-5-P	Xylulose-5-phosphate
σ	Standard deviation
δ	Chemical shift

CHAPTER 1

INTRODUCTION

Fission yeast, *Schizosaccharomyces (S.) pombe* utilizes sugar for growth and energy metabolism. D-Glucose, which is the most common source of energy, is catabolized mainly *via* glycolysis which is undoubtedly the most ubiquitous pathway of carbon metabolism. An alternative pathway to glycolysis is the pentose phosphate pathway (Figure 1). The presence of the pentose phosphate pathway is implicated in *S. pombe* by its ability to ferment D-xylulose to ethanol (Wang *et al*, 1980; Ueng *et al*, 1981).

Previous investigation concerning glucose catabolism has shown that *S. pombe* possesses capabilities for operation of the glycolytic pathway, the pentose phosphate pathway and the tricarboxylate cycle (Tsai *et al*, 1992). Glucose catabolism is initiated by phosphorylation followed by oxidation. Alternatively, it can be oxidized to gluconate, followed by phosphorylation. This latter route is designated as the gluconate route in this study. The conversion of D-glucose to D-gluconate is mediated by either NADP-linked glucose dehydrogenase in *S. pombe* (Shi, 1993) or membrane-associated glucose dehydrogenase in bacteria (Allenza and Lessie, 1982). D-Gluconate is phosphorylated to D-6-phosphogluconate by gluconokinase. The product, D-6-phosphogluconate, which is produced either from phosphorylation or oxidation of D-glucose, is further metabolized through the Entner-Doudoroff pathway and/or the pentose phosphate pathway (see Figure 1).

Figure 1. Glycolysis, the pentose phosphate pathway, and the Entner-Doudoroff pathway. The reactions are schematized, but cofactors and cosubstrates (ADP, Pi, NAD, etc.) are not shown. The glycolytic enzymes, according to their sequential order in the pathway are: HK, hexokinase (EC 2.7.1.2); PGI, phosphoglucose isomerase (EC 5.3.1.9); PFK, phosphofructokinase (EC 2.7.1.11); FDA, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); TPI, triose-phosphate isomerase (EC 5.3.1.1); TDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, phosphoglycerate kinase (EC 2.7.2.3); PGM, phosphoglycerate mutase (EC 2.7.5.3); ENO, enolase (EC 4.2.1.11); and PK, pyruvate kinase (EC 2.7.1.40). The pentose phosphate pathway enzymes are: G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 6PGL, 6-phosphogluconolactonase (EC 3.1.1.31); 6PGDH, 6-phosphogluconate dehydrogenase (EC 1.1.1.44); RPI, ribose-5-phosphate isomerase (EC 5.3.1.6); and TK, transketolase (EC 2.2.1.1). EDD, 6-phosphogluconate dehydratase (EC 4.2.1.12) and EDA, 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) are the two enzymes in the Entner-Doudoroff pathway. GD, glucose dehydrogenase (EC 1.1.1.42); and GNK, gluconokinase (EC 2.7.1.12) are also shown.

1.1.1 Glycolysis (Embden-Meyerhof-Parnas Scheme) and hexokinases

Glycolysis, which had evaded elucidation by the scientific community for several decades, is presently the best understood biological carbohydrate catabolic pathway. For each mole of glucose consumed, 150.7 KJ (36 kcal) energy is released. To a first approximation, the net equation for one turn in glycolysis is:

According to this equation, two molecules of ATP are generated and two NAD^+ molecules are reduced to NADH per molecule of D-glucose oxidized to pyruvate. While this equation partially reflects the needs of the cell under certain conditions, it is an oversimplification.

Louis Pasteur first showed that a balance existed between the amount of glucose fermented by yeast and the alcohol and carbon dioxide formed. The reaction sequence in yeast, outlined in Figure 1, was the first major pathway to be understood by biochemists and occurs in many other prokaryotic and eukaryotic organisms. Sugars are converted to the key intermediary product, *pyruvic acid*. The essential features of the pathway are:

- i. Glucose is activated by phosphorylation (either during or after entry to the cell), in a step requiring the expenditure of ATP. A second phosphorylation forming fructose-1,6-bisphosphate uses a second molecule of ATP. There is, however, net synthesis of two ATP molecules per molecule of glucose oxidized, since two 3-carbon sugar fragments are formed in the step catalysed by aldolase, and the conversion of each of

these to pyruvate leads to the phosphorylation of two ADP molecules.

ii. The phosphorylation steps involve substrate-level coupling of the energy of oxidation to ATP synthesis, at a thermodynamic efficiency of about 30%.

iii. During conversion of glucose to pyruvate, NAD^+ is reduced to NADH. There are many reactions found in different microorganisms for converting pyruvate to final fermentation products with the balancing re-oxidation of NADH. The same organism may produce different compounds when grown in excess glucose. Yeast cytoplasmic enzymes ferment glucose to ethanol, but in the presence of bisulphite, forming an addition product with acetaldehyde, they convert glucose to glycerol by the NADH-linked reduction of glyceraldehyde to glycerol-1-phosphate.

Phosphorylation of glucose is the first step in glycolysis. A family of hexose phosphorylating enzymes, hexokinases (ADP:D-hexose-6-phosphotransferase, EC 2.7.1.1) carry out this important process, converting glucose to glucose-6-phosphate with ATP as the phosphate donor. It is generally accepted that this reaction is one of the principle control points of glycolysis, together with the reaction catalyzed by phosphofructokinase (EC 2.7.1.11). Phosphofructokinase is the rate-limiting enzyme in the metabolism of most hexoses by the Embden-Meyerhof-Parnas pathway, and its activity is influenced by many effectors including adenine nucleotides (Uyeda, 1979) and the recently discovered activator, fructose-2,6-bisphosphate (Van Schaftingen *et al.*, 1980; Uyeda *et al.*, 1981; Pilkis *et al.*, 1981). Both reactions are accompanied with large changes in free energy.

i.e., they are far displaced from equilibrium, and both enzymes are regulatory enzymes (Newsholme and Start, 1973). Glycolysis can also be influenced by the activity of pyruvate kinase (EC 2.7.1.40) which is also subject to extensive regulation (Engström *et al.*, 1981).

In yeast, three forms of hexokinase exist, hexokinases P-I and P-II, and glucokinase. Like other enzymes involved in glycolysis, hexokinases are soluble proteins found mainly in the microbial cytoplasm or associated with the cytoplasmic membrane. These enzymes differ from one another in their activities with various hexoses. Both hexokinases P-I and P-II can use a range of hexose substrates and can be distinguished from one another by their activity differences with certain substrates, notably fructose (Lazarus *et al.*, 1966). Mutants lacking either hexokinase P-I or P-II grow on fructose while the double mutant does not. Similarly, double mutants lacking any two of the three enzymes grow on glucose while the triple mutant does not. Thus the individual enzymes are adequate for growth (Lobo and Maitra, 1977). Yeast glucokinase, on the other hand, shows a rather narrow substrate preference, activities being highest with D-glucose and D-mannose (Maitra, 1985).

Both yeast hexokinases P-I and P-II exist as dimers, although both are readily dissociated to monomers of 50 Kdal by the addition of glucose, or by raising the pH or the ionic strength. Yeast glucokinase appears to exist only as a monomer and has a slightly higher molecular weight (51 Kdal). Hexokinase P-II is the predominant isoenzyme in the cell. In addition to its hexose phosphorylating function, P-II activity is necessary for carbon catabolic repression (Rose *et al.*, 1991) and together with P-I, may

be subject to allosteric controls (Kopetzki and Entian, 1985). Whether the glucokinase isoenzyme plays a different physiological role in the yeast cell from the other two isoenzymes is not clear.

1.1.2 Entner-Doudoroff pathway

Isotopic studies on *Pseudomonas (Zymomonas) lindneri* showed that pyruvate could not have been formed by the reactions of the EMP pathway, although the initial reaction, the formation of glucose-6-phosphate by hexokinase reaction, is the same. Instead glucose-6-phosphate is catabolized via Entner-Doudoroff pathway. The existence of the Entner-Doudoroff pathway is confined to prokaryote and is a feature of the metabolism of *Pseudomonas*, *Rhizobium* and *Xanthomonas* species (Dawes and Sutherland, 1992).

The characteristics of this pathway are:

- i. The formation of 2-keto-3-deoxy-6-phosphogluconate.
- ii. The labelling pattern in which the carboxyl group of one mole of pyruvate is derived from C1 of the hexose and that of the second pyruvate from C4.
- iii. The lower energy yield of one mole of ATP per mole glucose, due to the oxidation of a single mole of triose phosphate as opposed to the two moles available in the Embden-Meyerhof-Parnas pathway.

The Entner-Doudoroff pathway is initiated by the conversion of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate by 6-phosphogluconate

dehydratase. Cleavage of this compound by a specific aldolase, 2-keto-3-deoxy-phosphogluconate aldolase, results in the formation of glyceraldehyde-3-phosphate and pyruvate. The pathway is inducible and employed for metabolism of gluconate, which is converted to 6-phosphogluconate by gluconokinase (Eisenberg and Doborogosz, 1967; Fraenkel and Horecker, 1964; Fraenkel and Levisohn, 1967).

The process is much less widely used by microorganisms than the Embden-Meyerhof-Parnas pathway, but occurs in a number of *Pseudomonas* species and a few other Gram-negative bacteria. In *Zymomonas lindneri*, the major products of glucose catabolism are ethanol and CO₂, which are produced from pyruvate by the same mechanism as in *Saccharomyces cerevisiae* (Dawes and Sutherland, 1992).

1.1.3 Pentose phosphate pathway

This pathway is found in many bacteria and in most eukaryotic organisms. It can serve several functions in cells (Table 1.). The initial steps in this pathway involve the oxidation of glucose-6-phosphate. Figure 2 shows how six pentoses can be converted to five hexoses by the combined activities of the enzymes transketolase, transaldolase and aldolase. The overall reaction for six glucose-6-phosphate molecule is:

The NADPH can be re-oxidized in the respiratory chain providing 35 ATP molecules. It is important to consider, however, that the primary role of the pentose phosphate pathway may well be in biosynthesis rather than oxidative energy metabolism.

Figure 2. The classical formulation of the pentose phosphate pathway, showing all enzymes, substrates, intermediates, and products (Horecker and Mehler, 1955).

The pentose phosphate pathway can be divided into two branches: the oxidative branch and the nonoxidative branch.

Studies, primarily by Warburg (1935), Lipmann (1936), Dickens (1938), and Horecker (1954), defined the oxidative portion of this pathway. Carbon-1 of glucose-6-phosphate is oxidized to CO_2 , and pentose 5-phosphate is formed, *i.e.*,

In most circumstances in which the oxidative portion operates, the need for NADPH should exceed the need for pentose-5-phosphate. To meet the demand of the NADPH, the pentose-5-phosphate has to be further metabolized in order to avoid its accumulation. An early indication of the cyclic nature of the pathway was the demonstration that degradation of pentose nucleosides by red cells was accompanied by hexose phosphate and triose phosphate formation (Dische, 1938). Subsequently, glucose-6-phosphate was found to be a product of ribose-5-phosphate metabolism by liver, bone marrow, and spinach leaf preparations.

Subsequent studies which defined the activities of transaldolase as well as transketolase and demonstrated that sedoheptulose-7-phosphate appeared to be an intermediate in the conversion of pentose-5-phosphate to hexose-6-phosphate by liver enzyme preparation. This led to the postulate (Horecker *et al.*, 1954; Horecker and Mehler, 1955) of the sequence of reactions that now comprise the nonoxidative branch of the classical pathway (see Figure 2):

sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate →

erythrose-4-phosphate + fructose-6-phosphate

xylulose-5-phosphate + erythrose-4-phosphate →

glyceraldehyde-3-phosphate + fructose-6-phosphate

The overall reaction for the nonoxidative branch can be represented by:

3 pentose-5-phosphate → 2 fructose-6-phosphate + glyceraldehyde-3-phosphate.

Since phosphohexose isomerase is a relatively active enzyme in tissues where it is present, the pathway is a cycle (Wood *et al.*, 1963) in which

3 glucose-6-phosphate →

3 CO₂ + glyceraldehyde-3-phosphate + 2 glucose-6-phosphate.

If six molecules of glucose-6-phosphate are metabolized in the pathway and two molecules of the glyceraldehyde-3-phosphate formed are subsequently converted to glucose-6-phosphate, the overall reaction can be written as:

6 glucose-6-phosphate → 6 CO₂ + 5 glucose-6-phosphate.

Designating the carbonyl carbon of glucose by 1 and succeeding carbons in sequence by 2-6, the movement of carbons in the nonoxidative branch of the pathway is shown in Figure 3.

Beside viewing the pathway in the pentose synthesis (Capriol and Rittenberg, 1969, Johnson *et al.*, 1973 and Josephson and Fraenkel, 1974), a second possible function of the oxidative branch is to provide NADPH for biosynthesis, since both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase specifically utilize NADP⁺ as the coenzyme (Csonka and Fraenkel, 1977). In *Saccharomyces cerevisiae*, the

Figure 3. Rearrangement of carbons in the nonoxidative branch of pentose phosphate pathway.

pentose phosphate pathway accounts for the production of almost all the requirements of the NADPH (Lagunas and Gancedo, 1973). It was calculated that the pathway could provide 80-100% of the NADPH required for fatty acid synthesis in rat mammary gland (Katz and Wals, 1972) and 60% of the NADPH requirement in rat adipose tissue (Kather *et al.*, 1972b).

The oxidative branch generates pentose phosphate and NADPH *via* the two dehydrogenases at the start of the pathway. The nonoxidative branch equilibrates the pentose phosphates with the hexose phosphate of glycolysis *via* the remaining pathway enzymes, in particular transketolase and transaldolase. Pentose phosphate which is formed by the oxidative branch and not further utilized, is converted back to hexose phosphate by the nonoxidative branch and re-enters the glycolysis pathway.

The nonoxidative branch of the pentose phosphate pathway clearly does have a major catabolic role in growth of microorganisms on pentoses, as well as being used for the portion of gluconate metabolism not employing the Entner-Doudoroff pathway in *E. coli* (Orozco de Silva and Fraenkel, 1979) and *Pseudomonas cepacia* (Allenza and Lessie, 1982).

Since the need for NADPH (and ribose phosphate) is variable, mechanisms must exist which adjust the rate of the pentose phosphate pathway to requirements. Quantitatively, the main NADPH consuming process is the synthesis of fatty acids from carbohydrate where two molecules of NADPH are used for each acetyl CoA added to a fatty acid chain. Whether fatty acids are synthesized and thus whether NADPH is needed depends on the presence in the diet of excess carbohydrates, *i.e.*, more carbohydrate than

can be broken down, or deposited as glycogen.

A major factor in the regulation of the pentose phosphate pathway is known to be the adaptive change in the capacity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Glucose-6-phosphate dehydrogenase is considered to be the regulatory enzymes of the oxidative branch of the pathway, the flux being controlled by the amounts of enzymes and by the NADPH/NADP⁺ ratio (Gumaa and Mclean, 1971; Kather *et al.*, 1972). Glucose-6-phosphate dehydrogenase has a distinguished history in that it was not only the first dehydrogenase to be highly purified (by Negelein and Hass in the laboratory of Otto Warburg in 1935) but also the enzyme the study of which led Warburg's discovery of NADP⁺ and to the identification of nicotinamide as the reactive component of the enzyme.

Assays of the enzymes in a variety of tissues and species indicate that transaldolase is the rate-limiting enzyme for the non-oxidative branch of the pathway. However, the control of the nonoxidative branch at the transketolase step seems less well-established, particularly because of a possible coupling with transaldolase and the manner in which transaldolase activity has been determined (Wood, 1985).

Unlike the oxidative reactions catalysed by glucose-6-phosphate dehydrogenase (Jeffery *et al.*, 1993) and 6-phosphogluconate dehydrogenase (Sheldon *et al.*, 1990), which has been described in detail, the gluconate route from glucose to 6-phosphogluconate still requires further investigation.

1.2 Functions of glycolysis and pentose phosphate pathway

TABLE 1. Function of Different Pathway of Glucose Degradation

Embden-Meyerhof-Parnas pathway plus associated reaction TCA cycle	Pentose phosphate pathway
<p style="text-align: center;">supplies</p> <p>ATP Carbon skeletons for biosynthesis of non-essential amino acids acetyl CoA (fat, steroids, acetylations) glycerophosphate (fat)</p>	<p style="text-align: center;">supplies</p> <p>Ribose-5-phosphate NADPH for reductive synthesis hydroxylation of steroids reduction of Met-Hb reduction of GSSG</p>

Taken from Krebs, *et al.* (1974).

Functions of the hexose diphosphate pathway (Embden-Meyerhof-Parnas Scheme for glucose fermentation) and the pentose phosphate pathway are different (Table 1). The main function of the Embden-Meyerhof-Parnas pathway, in conjunction with the tricarboxylic acid (TCA) cycle, is the supply of energy in the form of ATP. An additional function, usually minor in quantitatively terms, is the supply of cell constituents, such as glutamate, alanine, aspartate, galactose, glucosamine and acetyl CoA. The main function of the pentose phosphate pathway and its associated reaction is not the supply of energy but the supply of NADPH to be used as reducing agent in many biosyntheses, and a supply of ribose phosphate for the synthesis of nucleic acids (Krebs *et al.*, 1974). The importance of the latter pathway is evident since pentose phosphates, beside being essential components of nucleic acid and nucleotides, are crucial in the initial step in the synthesis of purine bases (Severin and Stepanova, 1980).

1.3 Metabolic studies using ^{13}C nuclear magnetic resonance (NMR) spectroscopy

Increased availability of NMR instrumentation in biochemical laboratories and clinical environments has led to an increase in the use of this technique for metabolic analysis. Although many of these studies can be carried out without the introduction of isotopic labels, labelling often provides essential gains in sensitivity as well as unique insight to metabolic transformations, and can allow the resolution of ambiguous data. The use of ^{13}C -NMR as a tool for monitoring metabolic sequences, by following the fate of a particular carbon atom of an enriched substrate molecule, has become feasible.

Typically, ^{13}C NMR studies involving enriched compounds monitor one or more

of the following properties: (a) the rate of incorporation or degradation of a label, (b) the level of steady-state labelling, and (c) the position of the label in the carbon skeleton. The entry of a ^{13}C -labelled precursor into different metabolic processes can be followed simultaneously by ^{13}C NMR, without the need for chemical isolation or purification; the individual carbons which are specifically labelled in each individual metabolite can be resolved, providing a sufficient signal/noise ratio is obtained. Classical methods using ^{14}C -labelled precursors remain much more sensitive than ^{13}C -NMR, due to the relative insensitivity of the latter technique. However, it is the exceptional chemical resolution that renders ^{13}C NMR uniquely suitable for metabolic studies. In the area of pathway elucidation, the ^{13}C labelling/NMR detection approach offers the overwhelming advantage over more traditional ^{14}C studies of allowing quantitation and qualification of the ^{13}C labelling at any site in the product without the need of chemical degradation. Information relating to the metabolic flux through various pathways can be obtained from studies of the distribution of the label in the various carbon positions of the biosynthesized metabolites, and from the direct observation of metabolic intermediates. Intermediates present in a larger 'pool' with a metabolic half-life greater than 0.5 min are, however, accessible by ^{13}C -NMR (Scott and Baxter, 1981).

In general, much of the information of a ^{13}C labelling study can be extracted from an analysis of the isotopic composition of the products, either *in vivo* (if possible) or *in vitro*. It is worth emphasizing that useful data related to regulation problems can also be obtained by analyzing excreted or extracted metabolites.

Sonawat *et al.* (1990) have used intracellular and crude cell extracts ^{13}C NMR to

demonstrate the presence of glucose dehydrogenase activity in *H. halobium* grown in the presence of glucose. Their study has provided evidence for the existence of Entner-Doudoroff and glycolytic enzymes in *H. halobium* cells grown in the presence of glucose.

Carbon-13 NMR spectroscopy was used to study the metabolism of [2-¹³C]acetate in a diploid strain of *Saccharomyces cerevisiae* (Dickinson and Hewlins, 1991). By analyzing the pattern of ¹³C-labelling in glutamate it was deduced that the glyoxylate cycle is responsible for most of the acetate utilization and that there is very little TCA cycle activity. The labelling α',α' -trehalose indicated that glycogenesis and the pentose phosphate pathway operate in a similar way in the wild-type yeast.

Carbon-13 NMR of isotopically enriched metabolites had been used to study the metabolism of *Microbacterium ammoniophilum*, a bacterium which excretes large quantities of L-glutamic acid into its medium (Walker *et al.*, 1982). Biosynthesis from 90% [1-¹³C]glucose results in relatively high specificity of the label, with [2,4-¹³C]glutamate as the major product. The predominant biosynthetic pathway for glutamate from glucose was determined to be the Embden-Meyerhof-Parnas pathway followed by phosphoenolpyruvate carboxylase and the first third of the TCA cycle. Different metabolic pathways are associated with different correlations in the enrichment of the carbons, reflected in the spectrum as different ¹³C-¹³C scalar multiplet intensities. It was demonstrated that 13% of the glucose metabolized by *M. ammoniophilum* occurs via the pentose phosphate pathway. This agrees well with the 10% value determined by analysis of the pyruvate carboxyl group in metabolically blocked cells (Shiio, *et al.*, 1960a and 1960b).

1.4 Gluconokinase (ATP:D-gluconate-6-phosphotransferase, EC 2.7.1.12)

The formation of D-gluconate by direct oxidation of D-glucose is catalyzed by glucose dehydrogenase which is present in many animal tissues and microorganisms (Dickens, 1938; Lipmann, 1936; Warburg, 1937). More recently Shi (1993) has demonstrated that gluconate may be formed by an NADP⁺-linked dehydrogenase in *S. pombe*. Gluconate thus formed may be metabolized further. The first step in its utilization is a phosphorylation reaction:

In *Escherichia coli*, the initial steps of gluconate metabolism are its entry into the cell and its subsequent phosphorylation to 6-phosphogluconate (Cohen, 1951). 6-phosphogluconate is catalyzed primarily by the Entner-Doudoroff pathway, but also by the pentose phosphate pathway (Eisenberg and Dobrogosz, 1967). The gluconokinase activity is induced when the cells grow on gluconate (Cohen, 1951; Fraenkel and Levisohn, 1967; Pouysségur *et al.*, 1974). Gluconokinase was also found in other bacteria and yeasts (Coffee and Hu, 1970; Zacharion and Scopes, 1985; Sable and Guarino, 1952).

Gluconokinase has been partially purified and completely purified from different bacteria and yeasts (Cohen, 1951; Sable and Guarino, 1952; Coffee and Lu, 1970; Zacharion and Scopes, 1985). As is the case for all known kinase reactions, gluconokinase exhibits an absolute requirement for a divalent cation. The enzyme from *E. coli* was optimally active at 1 mM Mg²⁺, that from yeast at 5 to 7 mM Mg²⁺. Mn²⁺ also activated this enzyme although to a lesser extent (Cohen, 1955).

The Michaelis constants for the yeast enzyme are $K_m = 1.68$ mM for magnesium,

1.74 mM for gluconate, and 0.20 mM for ATP (Sable and Guarino, 1952). The Michaelis constants for *Pseudomonad* enzyme are 1.04 mM and 0.12 mM for ATP and gluconate respectively. It was found that either ATP or gluconate alone can stabilize the enzyme to heat inactivation (Coffee and Hu, 1970). The kinetic data from *Pseudomonad* suggested that the catalytic reaction appears to follow a sequential (probably random) order of addition of substrates to the enzyme (Coffee and Hu, 1972). The molecular weight of gluconokinase varies from 18,000 to 112,000. Fujita *et al.* (1986) showed that the second open reading frame (OPF *gntK*) in the *B. subtilis* *gnt* operon encodes gluconokinase, which has 513 amino acids and a molecular weight of 57,169. Sequence comparisons of gluconokinase of *Bacillus subtilis* with carbohydrate kinases of *E. coli* revealed that *B. subtilis* gluconokinase is homologous to xylulose kinase (484 amino acids, M.W. 52,618), glycerol kinase (501 amino acids, M.W. 56,099), and fucose kinase (482 amino acids, M.W. 53,235) of *E. coli* (Reizer *et al.*, 1991). The results strongly argue that these four kinases share an evolutionary ancestry (Reizer *et al.*, 1991). The characterizations of gluconokinase from different sources are summarized in Table 2.

The bacterial gluconokinase is involved in the Entner-Doudoroff pathway of glucose and/or gluconate metabolism. The product of gluconokinase catalyzed reaction, 6-phosphogluconate, is converted to pyruvate and glyceraldehyde 3-phosphate primarily by the Entner-Doudoroff pathway. The pentose phosphate pathway does not seem to be the only pathway for the 6-phosphogluconate metabolism in *E. coli* and *Pseudomonad*.

The use of the Entner-Doudoroff pathway for glucose degradation appears not to

Table 2. Characterizations of Gluconokinase

Molecular Weight	Michaelis Constants Km (mM)		Reference
	ATP	gluconate	
N/A	0.2	1.7	Cohen, 1955
112,000	1.0	0.12	Coffee and Hu, 1972
18,000	1.5	0.33	Zachariou and Scopes, 1985*

* The Km for ATP and gluconate were obtained at 5 mM gluconate and 2 mM ATP respectively.

be confined to *S. pombe*. The overall activity of the Entner-Doudoroff pathway enzymes such as 6-phosphogluconate dehydratase and 2-keto-3-deoxy-phosphogluconate aldolase have not been detected in the cell-free extract of *S. pombe*. Cell-free extract of *S. pombe* contains a high level of 6-phosphogluconate dehydrogenase, a key enzyme in the pentose phosphate pathway (Tsai, 1992), implying that the 6-phosphogluconate dissimilation occurs exclusively *via* the pentose phosphate pathway. Data will be presented on the specificity, kinetic study, and the purification of gluconokinase, as well as the ^{13}C -NMR study of the glucose metabolism in the cell-free extract of *S. pombe*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Malt extracts was obtained from Oxoid. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, hexokinase (EC 2.7.1.1) from baker's yeast, adenosine 2',5'-diphosphate (2',5'-ADP) agarose, adenosine 5'-triphosphate (ATP-) agarose, adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), β -nicotinamide adenine dinucleotide, reduced form (NADH), β -nicotinamide adenine dinucleotide phosphate (NADP⁺), potassium gluconate, sodium 6-phosphogluconate, SDS molecular weight marker, D-[1-¹³C]Glucose (99 atom % ¹³C), D-[2-¹³C]glucose (99 atom % ¹³C) and glass beads (0.45 - 0.50 mm) were from Sigma Chemical Co. Glucose-6-phosphate dehydrogenase (G6PDH), pyruvate kinase, lactate dehydrogenase were from Boehringer Mannheim GmbH, Germany. D-[U-¹³C]Glucose (68.1 atom % U-¹³C) was from MSD isotopes, Division of Merck Frost Canada Inc, Montreal. D-Glucose and dithiothreitol (DTT) were provided by Calbiochem Behring. Agarose Blue-A was purchased from Amicon Corp. Bio-Gel P-6 and P-100 were from Bio-Rad Laboratories. Reagent grade chemicals used in the preparation of culture media and gluconokinase purification were purchased from various commercial sources.

2.2 Yeast culture

The fission yeast *S. pombe* NCYC 132S₂-2 was maintained by periodic transfer

to 2% malt extract broth at 30°C (Tsai *et al.*, 1987). The minimal salts medium (EMM2) of Mitchison (1970) was modified to contain 2.0 mg/ml D-glucose which was the only carbon source. Batch cultures (500 ml each flask) of the fission yeast were grown in 2 litres Erlenmyer flasks with constant shaking at 150 rpm, 30°C as described (Tsai *et al.*, 1989). Cells were harvested by centrifugation carried out by Sorvall RC2-B automatic refrigerated centrifuge at 4°C (4,000 rpm, 20 min) and washed three times with extraction buffer, 25 mM Hepes (pH 7.2), containing 2 mM DTT, 1 mM 6-aminocaproic acid, 10 mM MgCl₂, and 1 mM EDTA.

2.3 Preparation of cell-free extract

One gram of yeast cells in 4.0 ml of extraction buffer, pH 7.2, was mixed with approximately 5 g of acid-washed glass beads (0.45-0.50 mm) and shaken in a prechilled cell homogenizer MSK (Braun) at 4,000 rpm for 80 s to disrupt cells. The homogenate was extracted with 5 ml of the same buffer at least three times. The combined extracts were centrifuged at 10,000 rpm for 20 min twice to remove unbroken cells and cell debris. The supernatant was used immediately as the cell-free extract for enzymic assays, gluconokinase purification, and in ¹³C NMR experiments.

2.4.1 Assay of enzymic activities

All enzymic assays were carried out in a Varian Spectrophotometer (Model Cary 3) at room temperature (20°C). Reactions were initiated by the addition of cell-free extracts of *S. pombe* or purified gluconokinase. Rates of NADP⁺ reduction or NADH

oxidation were followed spectrophotometrically at 340 nm ($\epsilon_{340} = 6.22 \text{ cm}^{-1} \text{ mM}^{-1}$). Triplicate sample of assays gave a reproducibility of $\pm 10 \%$. Total reaction volume used in each assay was 1.0 ml.

Glucokinase activity of hexokinase (EC 2.7.1.1) was assayed by G6PDH formation of NADPH in a reaction mixture containing 5 mM glucose, 1.0 mM ATP, 0.5 mM NADP⁺, and 2 units of yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PDH) in 25 mM HEPES buffer (buffer A), which contained 25 mM HEPES, 10 mM MgCl₂, 1 mM DTT and 1 mM EDTA, pH 7.2 (Barnard, 1975). An assay mixture of glucose dehydrogenase (EC 1.1.1.47) contained 5.0 mM glucose, 0.5 mM NADP⁺ in buffer A (Shi, 1993). G6PDH activity was measured in a reaction mixture containing 5 mM glucose-6-phosphate, 0.5 mM NADP⁺ in buffer A (Kuby and Noltmann, 1966). For the assay of 6-phosphogluconate dehydrogenase (EC 1.1.1.44), the reaction mixture was composed of 2.5 mM 6-phosphogluconate, 0.5 mM NADP⁺ in buffer A (Glock, 1964).

The overall activities of the Entner-Doudoroff pathway enzymes, 6-phosphogluconate dehydratase (6PGA) and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase were determined by measuring 6PGA-dependent formation of pyruvate, which was determined spectrophotometrically as its dinitrophenylhydrazone. The assay mixture (0.5 ml) contained 5 mM 6PGA in buffer A. After incubation for 30 min at 30°C, the reactions were terminated by the addition of 0.5 ml of HCl containing 0.02% (w/v) dinitrophenylhydrazine. After an additional 10 min of incubation at room temperature, 1 ml of 2 N NaOH was added, and the absorbance at 450 nm was determined (Lessie and Whiteley, 1969).

Gluconokinase (EC 2.7.1.12) activity was measured in a reaction mixture containing 5 mM gluconate, 1.0 mM ATP and 0.5 mM NADP⁺, and 0.5 unit partially purified yeast 6PGDH (see 2.9) in buffer A (Bermeyer *et al.*, 1974). One units of gluconokinase activity will convert 1.0 μ mole of D-gluconate to 6-phosphogluconate per min at pH 7.2 and 20°C.

When studying the product inhibition due to 6-phosphogluconate, another coupled assay was used in which the formation of ADP was coupled to the oxidation of NADH (0.25 mM) by addition of pyruvate kinase (1.0 unit) and lactate dehydrogenase (4.0 units), and excess phosphoenolpyruvate (PEP, 1 mM). The reaction was carried out in buffer A, pH 7.2, 1 mM PEP, 0.25 mM NADH, and other additions as indicated. The mixture was pre-incubated for 5 min to remove traces ADP present in the ATP, and the absorbance change was measured as previously (Denise *et al.*, 1985).

2.4.2. Protein analyses

Protein concentration was determined by the Bradford method (dye-binding methods) using Coomassie Brilliant Blue-G (Bradford, 1976). Crystalline bovine albumin was used as the standard. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 nm (red form) to 595 nm (blue form). The standard curve was constructed by using bovine albumin as standard protein, ranging from 10 μ g to 100 μ g. The absorbance of protein-dye mixture at 595 nm was measured. Alternatively, the protein concentration was monitored by the absorbance at 280 nm.

2.5 Carbon-13 NMR studies of glucose metabolism in the cell-free extract of *S. pombe*

Cell-free extracts were prepared as described above (see section 2.3). ^{13}C -labelled D-glucose was added to 700 μl of cell-free extract to give a concentration of 5.0 mM. After a fixed time, one and two hours, the incubation was stopped by adding 50 μl HClO_4 solution (10 % v/v) at 4°C for 1 hour. The precipitate was removed by centrifugation at 340,000 rpm for 30 min, in a Beckman L8-70 Ultracentrifuge. To the supernatant were added 200 μl D_2O to a final concentration of 20% (v/v), and 50 μl dioxane (as external standard). The mixture was neutralised with 1 N KOH (pH 7) and centrifuged to remove any precipitate at 7000 rpm for 10 min. The supernatant was then used as the NMR sample.

Carbon-13 NMR spectra were recorded at 100.6 MHz on a Bruker AMX-400 spectrometer, operating in the Fourier transform mode. Dioxane was used as an external standard (67.4 p.p.m., corresponding to tetramethylsilane 0 p.p.m.). All test samples contained 20% (v/v) D_2O to provide the field frequency lock signal. Scans (1000 -2000 field-induction) were acquired with the use of 45° pulses repeated at 0.5 s intervals over 26000 Hz spectral width.

To investigate effects of cofactors, NADP^+ and/or ATP were added to the ^{13}C -labelled D-glucose and cell-free extract mixture.

2.6 Gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out according to the procedure of Laemmli (1970) by using 4%

to 12% T (total monomer concentration), 2.67% C (cross-linker concentration) slab gel of 1.5 mm thickness. SDS-PAGE was conducted in a vertical slab gel electrophoresis chamber (Model Protein II xi, Bio-Rad Laboratories) at constant current of 25 mA for 5 hours at 20°C. Proteins were visualized using the silver staining procedure of Nielson and Brown (1984). Determination of the subunit molecular weight was made relative to the standard protein (bovine albumin, 66K; egg albumin, 45K; trypsinogen, 24K; and lysozyme, 14.3K were indicated) by plotting the log molecular weight against mobility (Friefelder, 1976).

2.7 Purification of gluconokinase

All procedures were performed at 0-4°C with 25 mM HEPES buffer (buffer A), which contained 25 mM HEPES, 10 mM MgCl₂, 1 mM DTT and 1 mM EDTA, pH 7.2 unless stated otherwise. To the cell-free extract were added ammonium sulphate to give a saturation of 45%. The solution was left stirring for 2 hours. The mixture was centrifuged at 10,000 rpm for 30 min. After centrifugation, further ammonium sulphate was added to the supernatant to give a saturation of 70%. The mixture was left stirring for another 2 hours. The mixture was centrifuged at 10,000 rpm for 30 min. The precipitate was dissolved in buffer A, twice the volume of the precipitate. The mixture was applied to a Bio-gel P-6 column (20 cm x 2.6 cm) equilibrated with buffer A. The peak fractions of gluconokinase were pooled and applied to a Blue-A agarose column (5.0 cm x 1.0 cm) equilibrated with buffer A. After extensive buffer washing, the active enzyme was eluted on addition of 0 - 1.0 M KCl gradient in buffer A. The active

fractions were pooled and concentrated in a S&S Collodion dialysis bag (molecular weight cutoff: 10,000 dalton) against buffer A by applying a vacuum to the outside of the dialysis bag. The enzyme solution was then delivered to a 2',5'-ADP agarose column (4.0 cm x 1.0 cm) equilibrated with buffer A. The active fraction was pooled. The enzyme was applied to a Bio-gel P-100 column (30 cm x 2.6 cm) after having been concentrated in the dialysis bag as described above. The eluted enzyme was located by measurement of gluconokinase activity and peak fractions were pooled. The peak fractions were applied to an ATP-agarose column (6.0 cm x 1.0 cm) equilibrated with 25 mM HEPES buffer, pH 7.2, containing 1 mM DTT and 1 mM EDTA. The column was washed by the same buffer until the absorbance at 280 nm was less than 0.01. The enzyme was eluted immediately on addition of the same buffer containing 0.5 mM ATP. Alternatively, the enzyme can be eluted by the addition of 10 mM magnesium. The active fractions were pooled. The fractions were concentrated and the cofactor was removed by dialysis against three changes of 300 ml buffer A for 24 hours as described above.

2.8 Partial purification of hexokinase

The active fractions from Blue-A agarose column were pooled and concentrated as described above. Two peaks which exhibited hexokinase activities (hexokinase I and II) were eluted from Blue-A agarose column. Hexokinase I was eluted prior to the addition of 0 - 1.0 M KCl gradient. Hexokinase II was eluted immediately after the application of KCl gradient. The active fractions were pooled and applied to Bio-gel P-

100 agarose column. Hexokinase II was eluted before gluconokinase. The active fractions were pooled. Both hexokinases were free of gluconokinase and 6PGDH activities.

2.9 Partial purification of 6-phosphogluconate dehydrogenase

After extensive buffer washing of the ADP-Agarose column, 0 - 1.0 M KCl gradient in buffer A was used to elute the 6-phosphogluconate dehydrogenase. The peak fraction of 6-phosphogluconate dehydrogenase, free of gluconokinase, was pooled and concentrated as described above. This partially purified 6-phosphogluconate dehydrogenase was used as the coupled enzyme in the determination of gluconokinase activity.

CHAPTER 3

RESULTS

3.1 Enzymic activity in cell-free extracts of *S. pombe*

Activities of several enzymes that are members of the glycolytic pathway, the pentose phosphate pathway, and the gluconate route were measured in cell-free extracts of *S. pombe*. Yeast cells were grown in 0.2% (w/v) D-glucose as the sole carbon source. Figure 4 shows the time course of enzymic activity from 24 to 144 hours. It revealed the presence of the Embden-Meyerhof-Parnas pathway, and the pentose phosphate pathway but the absence of the Entner-Doudoroff pathway. The overall 6-phosphogluconate dehydratase and 2-keto-3-deoxy-phosphogluconate aldolase activities of Entner-Doudoroff pathway was not detected in the cell-free extract of *S. pombe*.

Among the enzymes assayed, the key enzyme of the pentose phosphate pathway, 6-phosphogluconate dehydrogenase exhibited the highest specific activity after 36 hours' growth on D-glucose. While hexokinase activities dropped its specific activity to a steady-state after 36 hours' growth on glucose. Gluconokinase increased its activity after the cell growth reached the stationary phase. Glucose dehydrogenase and glucose-6-phosphate dehydrogenase maintained relative constant specific activities during the course of growth.

3.2 Carbon-13 NMR spectroscopy of cell-free extract of *S. pombe*

Carbon-13 labeled D-glucose was added to the cell-free extract of *S. pombe* grown for 2 to 3 days in D-glucose. A series of spectra were obtained after periods of 0, 1 and

Figure 4. Time course of the enzymic activities in the cell-free extract of glucose-grown *S. pombe* batch culture. The activities of gluconokinase (GNK —●—), hexokinase (HK —◆—), 6-phosphogluconate dehydrogenase (6PGDH —◆—), glucose dehydrogenase (GD —○—), and glucose-6-phosphate dehydrogenase (G6PDH —□—) were measured during the cell growth. The error of measurements is $\pm 15\%$.

2 hours of incubation. The ^{13}C -NMR experiments were performed under identical condition except for the incubation time or the additions of co-factors, *i.e.*, ATP and/or NADP^+ .

Typical 100.6 MHz ^{13}C -NMR spectra of yeast cell-free extract, carried out in the presence of D-[1- ^{13}C]glucose, are shown in Figure 5. Each spectrum represents an average of 2000 free induction decays. In view of the ^{13}C -NMR spectrum obtained from incubation of glucose oxidase and D-[U- ^{13}C]glucose (see Figure 6), the resonance peak at $\delta 180$ ppm resonance can be assigned to C-1 of D-gluconate.

Figure 7 shows ^{13}C -NMR spectral analyses of D-[U- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*, incubated for 0, 1, and 2 hours. The broad triplet at $\delta 65.5$ ppm indicates the presence of C-6 of gluconate and/or 6-phosphogluconate (the resonance assignments for gluconate and 6-phosphogluconate were made ambiguously because the shifts are very similar in C-6 of those two compounds).

Effects of co-factors, ATP and NADP^+ on glucose metabolism by cell-free extract of *S. pombe* grown on glucose incubated with ^{13}C -labeled glucose are presented in Figures 8-10. Figure 8 shows ^{13}C -NMR spectral analyses of D-[U- ^{13}C]-glucose metabolism with addition of cofactor, ATP at the concentration of 0 mM, 1 mM, and 5 mM, by incubation with the cell-free extract of *S. pombe* for 2 hours. The chemical shift at $\delta 19.8$ ppm is typical of a methyl group in a small metabolite such as ethanol (Narbad *et al.*, 1989). The broad peaks at $\delta 66.7$ ppm has previously been assigned to C-1 of fructose-1,6-bisphosphate (FDP) in *E. coli* cells (Ugurbil *et al.*, 1978) and to C-3 of 2,3-diphosphoglycerate (DPG) in erythrocytes (Styles *et al.*, 1979). However, a triplet peaks

Figure 5. ^{13}C -NMR spectral analyses of D-[1- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*. Mixture was incubated for a.) 0 hour as controlled, b) 1 hour, and c.) 2 hours. Resonance assignments are (1) [1- ^{13}C] gluconate; (2) and (3) [1- ^{13}C] glucose (α and β); and (4) dioxane.

Figure 6. ^{13}C NMR spectrum of the product D-gluconate from the incubation of D-[U- ^{13}C]glucose (5.0 mM) with glucose oxidase (2 units) for 2 hours at room temperature. Resonance assignments are (1) [1- ^{13}C]gluconate, (2) [1- ^{13}C]gluconolactone, (3) [2 $\rightarrow$ 5- ^{13}C]gluconolactone, (4) dioxane, (5) [6- ^{13}C]gluconate, and (6) [6- ^{13}C]gluconolactone.

Figure 7. ^{13}C NMR spectral analyses of D-[U- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*. Mixture was incubated for a.) 0 hour as controlled, b.) 1 hour, and c.) 2 hours. Resonance assignments are (1) [1- ^{13}C] of α -keto acid(s); (2) [1- ^{13}C] gluconate; (3) and (4) [1- ^{13}C]glucose (α and β); (5)-(8) α and β of [2 $\rightarrow$ 5- ^{13}C]glucose; (9) α and β [6- ^{13}C]glucose; (10) dioxane; (11) [6- ^{13}C]gluconate or [6- ^{13}C]6-phosphogluconate. Inset is the $\delta 60 - 70$ ppm expansion.

Figure 8. ^{13}C -NMR spectral analyses of the effect of ATP on D-[U- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*. Mixture was incubated for 2 hours. ATP concentrations were a.) 0 mM as controlled, b.) 1.0 mM, and c.) 5 mM. Resonance assignments are (1) [1- ^{13}C]gluconate or 6-phosphogluconate; (2) and (3) [1- ^{13}C] glucose (α and β); (4)-(7) α and β of [2 $\rightarrow$ 5- ^{13}C]glucose; (8) α and β [6- ^{13}C]glucose; (9) dioxane; (10) [6- ^{13}C]gluconate or [6- ^{13}C]6-phosphogluconate; (11) [6- ^{13}C]fructose-1,6-diphosphate, [6- ^{13}C]glucose-6-phosphate and [3- ^{13}C]2,3-diphosphoglycerate; (12) methyl group in a small metabolite, such as ethanol. Inset is the $\delta 60 - 70$ ppm expansion.

is evident at $\delta 66.7$ ppm. from the spectrum of yeast hexokinase and D-[U- ^{13}C]glucose reaction mixture. An increase in the signal at $\delta 66.7$ ppm corresponds to the accumulation of D-[6- ^{13}C]glucose-6-phosphate as the product of hexokinase and other intermediates of glycolysis.

Figure 9 shows ^{13}C -NMR spectral analyses of D-[U- ^{13}C]-glucose metabolism in the presence of cofactors 5 mM of ATP, with or without 5 mM of NADP $^+$, by the cell-free extract of *S. pombe* with 2 hours incubation. A broad resonance at $\delta 66.5$ ppm along with peaks at $\delta 67$ ppm (C-1 of ketose phosphates) were observed in the ATP incubation mixture in comparison to a triplet in the experiment of ATP + NADP $^+$ incubation. The triplet at $\delta 66.5$ ppm could not be assigned.

Figure 10 shows ^{13}C -NMR spectral analyses of D-[2- ^{13}C]-glucose metabolism with the addition of 5 mM of ATP with or without 5 mM of NADP $^+$, by incubating with the cell-free extract of *S. pombe* for 2 hours. The peak at $\delta 76.8$ ppm is assigned to D-6-phosphogluconate according to the data obtained from glucose-6-phosphate dehydrogenase reaction (Jarori and Maitra, 1991). The C-1 of pentose (or pentose phosphate) at $\delta 100.8$ ppm can be detected in the cell-free extract incubated with either ATP or ATP + NADP $^+$. The fate for D-[2- ^{13}C]glucose to enter the pentose phosphate pathway is first decarboxylated at the C-1 position to ribulose. This decarboxylation results in the loss of the C-1. As the result, the C-1 of pentose came from C-2 of glucose. A small, broad, resonance was observed at $\delta 104$ ppm, probably due to the C-1 peak of glycogen. The result of ^{13}C label at glycogen from C-2 of glucose is the recycling of glucose in the pentose phosphate pathway (Kunnecke and Seelig, 1991).

Figure 9. $\delta 60 - 70$ ppm expansion of the ^{13}C -NMR spectral analyses of the effects of ATP and NADP^+ on D-[U- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*. Mixture was incubated for 2 hours. Concentrations of Cofactors added to the incubation mixture were a.) 0 mM as controlled, b.) 5 mM ATP, and c.) 5 mM ATP and 5 mM NADP^+ . Resonance assignments are (1) dioxane; (2) [6- ^{13}C]gluconate or [6- ^{13}C]6-phosphogluconate; (3) [6- ^{13}C]fructose-1,6-diphosphate, [6- ^{13}C]glucose-6-phosphate and [3- ^{13}C]2,3-diphosphoglycerate; (4) α and β of [6- ^{13}C]glucose.

c.)

b.)

a.)

70

65

60

Figure 10. $\delta 73-78$ ppm (right) and $\delta 100 - 108$ ppm (left) expansions of the ^{13}C -NMR spectral analyses of the effects of ATP and NADP^+ on D-[2- ^{13}C]-glucose metabolism by the cell-free extract of *S. pombe*. Mixture was incubated for 2 hours. Concentrations of Cofactors added to the incubation mixture were a.) 5 mM ATP, and b.) 5 mM ATP and 5 mM NADP^+ . Resonance assignments are (1) and (2) α and β -[2- ^{13}C]-glucose; (3) [2- ^{13}C]-6-phosphogluconate; (4) [1- ^{13}C] of pentose (or phosphate); (5) [1- ^{13}C] of glycogen; (6)-(8) unknown.

3.3 Purification of gluconokinase

The fractionation of gluconokinase with ammonium sulfate between 45-70% saturation, is illustrated in Figure 11. In Figures 11 - 32, the enzymic activity was expressed in the absorbance changing at 340 nm per min at room temperature. Figures 12 to 15 show the chromatographic profiles of protein and gluconokinase by Bio-gel P-6, Blue-A agarose, 2',5'-ADP agarose, and Bio-gel P-100 chromatographic separations. Enzyme profile of affinity chromatography using ATP agarose is shown in Figure 16. ATP (0.5 mM in 25 mM Hepes buffer, without Mg^{2+}) was used to elute gluconokinase. A comparison of elution profiles employing 0.5 mM ATP or 10 mM Mg^{2+} as an eluent is shown in Figure 17.

The elution profile from Bio-gel P-100 column revealed that gluconokinase was eluted after hexokinase, which was excluded at the void volume of the column. In the procedure without using 2',5'-ADP column, 6-phosphogluconate dehydrogenase was also eluted before gluconokinase. Bovine albumin (M.W. 66,000 dal) used as the standard was also eluted earlier than gluconokinase. The estimated native molecular weight of gluconokinase is 50,000 dal. The enzyme activity was lost during the concentration using Amicon centricon®30 microconcentrator with molecular weight cutoff at 30,000 dal). This suggests that the native enzyme could be a dimer which dissociated during the concentration. The purified gluconokinase is free of hexokinase and 6-phosphogluconate dehydrogenase activities.

Table 3 summarizes the purification procedure for gluconokinase. After the six-step purification procedure, gluconokinase was recovered (18 ± 7)% with a specific

Figure 11. Fractionation of gluconokinase by ammonium sulfate. The protein content (+) was measured by dye-binding method at 595 nm. GNK activity (Δ) was assayed with the coupled enzyme 6PGDH as described.

Figure 12. Bio-gel P-6 filtration chromatography of gluconokinase. Fractions (5 ml each) were collected and assayed for GNK (Δ), 6PGDH ($\square$) activities as well as protein (+) by dye-binding method at 595 nm.

Figure 13. Blue-A agarose ligand chromatography of gluconokinase. Fractions (5 ml) were collected and assayed for GNK (Δ) and 6PGDH ($\square$) activities as well as the protein (+) by the dye-binding method at 595 nm.

Figure 14. 2',5'-ADP agarose affinity chromatography of gluconokinase. Fractions (5 ml) were collected and assayed for GNK (Δ) and 6PGDH ($\square$) activities as well as the protein (+) by the dye-binding method at 595 nm.

Figure 15. Bio-gel P-100 filtration chromatography of gluconokinase. Fractions (2.5 ml) were collected and assayed for GNK activity (Δ) and protein at 280 nm (...).

Figure 16. ATP-agarose affinity chromatography of gluconokinase. 0.5 mM ATP used as an eluent at the arrow indicated. Fractions (2.5 ml) were collected and assayed for GNK activity (Δ).

Figure 17. Comparison of 0.5 mM ATP (Δ) and 10 mM Mg^{2+} (+) elution profiles on ATP agarose column. The elution with cofactors started at the arrow indicated. Fractions (2.5 ml) were collected and assayed for GNK activity.

activity of 140 ± 20 units/mg protein.

3.4 Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

The purified gluconokinase was homogenous as shown by gel electrophoresis (Figure 18). A single band was detected at the gel. Figure 19 shows the subunit weight measurement of gluconokinase. The subunit molecular weight of gluconokinase was estimated at 24,000 dal.

Table 3. Purification of gluconokinase from *Schizosaccharomyces pombe*

The representative purification scheme (three preparations) for gluconokinase yeast cells grown on 4 litres of 0.2% D-glucose culture for 3 days. The activity of the gluconokinase was measured with the standard assay coupled with 6-phosphogluconate dehydrogenase. The protein concentration was determined with dye-binding method.

Step	Specific activity $\pm \sigma$	% $\pm \sigma$ (single step recovery)	total activity $\pm \sigma$	purification fold
Crude cell-free extract	0.34 ± 0.04	100	71 ± 7	1
ammonium sulfate 45-70% salt-out	0.46 ± 0.05	-	-	1.4
Bio-Rad P-6 desalt column	1.1 ± 0.2	$66 \pm 2 (66 \pm 2)$	47 ± 6	3.2
Blue-A agarose	4.3 ± 0.4	$51 \pm 4 (77 \pm 3)$	36 ± 6	13
2',5'-ADF agarose	9.6 ± 2.4	$44 \pm 4 (87 \pm 5)$	31 ± 6	28
Bio-Rad P-100 gel filtration	32 ± 5	$37 \pm 7 (84 \pm 8)$	26 ± 7	94
ATP agarose affinity chromatography	140 ± 20	$26 \pm 7 (70 \pm 7)$	18 ± 6	410

σ : standard derivation, $n = 3$.

Figure 18. SDS-PAGE of the purified gluconokinase from *S. pombe*

Electrophoresis was carried out on a 4 - 12% acrylamide gel, as described. Lane 1 was the purified GNK from ATP agarose affinity chromatography (1 μ g protein). Lane 2 was the GNK active fraction from Bio-gel P-100 column (1 μ g protein). Lane 3 contained the molecular weight markers bovine albumin (66 Kdal), egg albumin (45 Kdal), trypsinogen (24 Kdal) and lysozyme (14.3 Kdal).

Figure 19. The standard curve for SDS-PAGE.

Data taken from Figure 18 were plotted as the logarithm of the molecular weight of standard proteins *versus* the relative mobility. The molecular weight markers (O) were, from left to right, bovine albumin (66 Kdal), egg albumin (45 Kdal), trypsinogen (24 Kdal) and lysozyme (14.3 Kdal). Gluconokinase is shown as (Δ).

3.5.1 Initial rate studies of gluconokinase

The dependence of the gluconokinase activity on gluconate and ATP was studied with a set of combination by varying both substrates in the concentration ranges of 0.3 mM to 5.0 mM for gluconate and 0.03 mM to 1.0 mM for ATP. The concentration of total magnesium was held constant at 10.0 mM. The data, for primary plots are averaged values from three determinations of three enzyme preparations. Double-reciprocal plots of initial rates *versus* concentrations of ATP and gluconate (Lineweaver and Burk, 1934) are presented in Figures 20 and 21. They fit converging straight lines indicating a hyperbolic responses of the enzyme activity to gluconate as well as to ATP. Therefore a ping-pong mechanism can be ruled out (Cleland, 1966).

A bisubstrate reaction with a random binding of A and B, providing that the binding of one substrate changes the dissociation constant for the other substrate by a factor α , the system can be described by the equilibria (Segel, 1975) shown below

Assuming that the rate-limiting step is $EAB \rightarrow EPQ$, k_p as the rate constant for the breakdown of EPQ to $E + P + Q$, the equation for the random system can be represented by the following equation:

$$v = k_p [EAB] \quad (1)$$

and

$$\frac{v}{[E]_t} = \frac{k_p}{[E] + [EA] + [EB] + [EAB]} \quad (2)$$

where $[E]_t$ is the total enzyme concentration, $[E]$ is the free enzyme concentration, $[EA]$, $[EB]$, $[EAB]$ are the concentrations of E-ATP, E-gluconate, and E-ATP-gluconate complexes. Substituting and designating $k_p[E]_t$ as $V_{\max}$, the limiting maximal velocity that would be observed when all the enzyme is present as EAB; the equations (2) becomes:

$$v = \frac{V_{\max} [A] [B]}{\alpha K_a K_b + \alpha K_b [A] + \alpha K_a [B] + [A] [B]} \quad (3)$$

where K_a and K_b are the dissociate constants of EA and EB complex respectively.

The equation (3) can be rearranged to reflect the dependence of the initial velocity on the concentration of the varied ligand:

$$\frac{v}{V_{\max}} = \frac{[A]}{\alpha K_a (1 + \frac{K_b}{[B]}) + [A] (1 + \frac{\alpha K_b}{[B]})} \quad (4)$$

$$\frac{v}{V_{\max}} = \frac{[B]}{\alpha K_b (1 + \frac{K_a}{[A]}) + [B] (1 + \frac{\alpha K_a}{[A]})} \quad (5)$$

For initial steady state rate studies, the concentration of products P and Q is equal to 0 and the above is valid.

Initial rates (v) are measured and plotted against variation of one substrate (say A) in a double reciprocal plot. The second substrate (B) is held constant. The inverse

of the equation 5 becomes:

$$\frac{1}{v} = \frac{\alpha K_a}{V_{\max}} \left(1 + \frac{K_b}{[B]}\right) \frac{1}{[A]} + \frac{1}{V_{\max}} \left(1 + \frac{\alpha K_b}{[B]}\right) \quad (6)$$

For an enzyme following bisubstrate kinetics in which the above relations are true, the double reciprocal plot will yield a family of converging lines, each being obtained for a different fixed concentration of B. The slopes of each line are given by:

$$\text{slope} = \frac{\alpha K_a}{V_{\max}} \left(1 + \frac{K_b}{[B]}\right) \quad (7)$$

and the intercepts are given by:

$$\text{intercept} = \frac{1}{V_{\max}} \left(1 + \frac{\alpha K_b}{[B]}\right) \quad (8)$$

A secondary plot of intercepts above plotted against the inverse of the concentration of B yields a straight line with:

$$\text{intercept} = \frac{1}{V_{\max}} \quad (9)$$

$$\text{slope} = \frac{\alpha K_b}{V_{\max}} \quad (10)$$

Similarly a plot of slopes from the primary plot plotted against the inverse of the concentration of B yields a straight line with:

$$\text{intercept} = \frac{\alpha K_a}{V_{\max}} \quad (11)$$

If α is less than unity, the binding of one ligand increases the affinity of the

$$\text{slope} = \frac{\alpha K_a K_b}{V_{\max}} \quad (12)$$

enzyme for the other ligand, the primary reciprocal plots intersect at a point above the horizontal axis. For $\alpha = 1$, one ligand has no effect on the binding of the other, the plots intersect on the horizontal axis at $-1/K_b$ or $-1/K_a$. If the binding of one ligand decreases the affinity for the second ligand ($\alpha > 1$), the family of reciprocal plots will intersect below the $1/[A]$ or $1/[B]$.

Figure 22 is a secondary plot of the slopes and vertical intercepts obtained from Figure 20 against the reciprocal of ATP concentration. Kinetic parameters are summarized in Table 4. The α , K_a and K_b as determined from the secondary plot are 1.6, 0.056 mM and 1.6 mM respectively.

Figure 20. Double reciprocal plots for GNK catalysis at different fixed concentration of [gluconate], *i.e.*, 0.3 mM ($\square$), 0.5 mM ($\diamond$), 0.7 mM (Δ), 1.5 mM ($\circ$), and 5.0 mM ($\blacksquare$). ATP concentrations were 0.03 mM, 0.05 mM, 0.1 mM, 0.2 mM and 1.0 mM. Data were taken from three determinations of three preparations with an experimental error of $\pm 10\%$.

Figure 21. Double reciprocal plots for GNK catalysis at different fixed concentration of [ATP], *i.e.* 0.03 mM (Δ), 0.05 mM ($\diamond$), 0.1 mM (+), 0.2 mM ($\square$), and 1.0 mM ($\blacktriangle$). Gluconate concentrations were 0.3 mM, 0.5 mM, 0.7 mM, and 1.5 mM. Data were taken from three determinations of three preparations with an experimental error of $\pm 10\%$.

Figure 22. Secondary plot of data taken from plots of $1/v$ versus $1/[ATP]$ at different fixed concentration of $[gluconate]$. The functions, slopes ($\circ$) and intercepts ($+$), are replotted against the corresponding $1/[gluconate]$.

Table 4. Kinetic parameters for the dependence of gluconokinase on gluconate, and ATP^a.

Parameter	Value
$V_{\max}/[E]_t$ (s ⁻¹)	1.5 ± 0.2
$K_{\text{gluconate}}$ (mM)	1.6 ± 0.4
K_{ATP} (mM)	0.056 ± 0.010
α	1.6 ± 0.5

^aData were obtained at pH 7.2, 25 mM Hepes, and room temperature as described under Material and Methods. Parameters estimates are from a graphical analysis. The total concentration of Mg²⁺ was kept constant at 10.0 mM. 6-Phosphogluconate dehydrogenase was used as the coupled enzyme in the assay of the gluconokinase activity.

3.5.2 Kinetic studies in the presence of ADP

The products of a reaction are formed at the catalytic site on the surface of the enzyme and are the substrates for the reverse reaction. Product inhibition studies can be quite useful in diagnosing kinetic mechanisms (Segel, 1975). Two ligands A (ATP) and B (gluconate) are cosubstrates which yield two products, P and Q where Q (ADP) is the "product" of A, and P (6-phosphogluconate) is the "product" of B. In the reverse reaction with P and Q as substrates, A would be the product of Q and B the product of P.

In the presence of a finite concentration of P, an EP complex can form. P excludes both A and B. Hence there are no EAP or EBP complexes. In the presence of a finite concentration of Q, an EQ complex can form. Q will not interfere with the binding of B. Consequently, a dead-end EBQ complex can form.

In the presence of ADP (Q):

The velocity equation is obtained from:

$$\frac{v}{[E]_t} = \frac{k_p [EAB]}{[E] + [EA] + [EB] + [EQ] + [EAB] + [EBQ]} \quad (13)$$

and:

$$\frac{v}{V_{\max}} = \frac{\frac{[A][B]}{\alpha K_a K_b}}{1 + \frac{[A]}{K_a} + \frac{[B]}{K_b} + \frac{[Q]}{K_q} + \frac{[A][B]}{\alpha K_a K_b} + \frac{[B][Q]}{\gamma K_b K_q}} \quad (14)$$

Rearranging velocity equation to show either A or B as the varied substrate, the above equations become:

$$\frac{v}{V_{\max}} = \frac{[A]}{\alpha K_a \left(1 + \frac{K_b}{[B]} + \frac{K_b [Q]}{[B] K_q} + \frac{[Q]}{\gamma K_q}\right) + [A] \left(1 + \frac{\alpha K_b}{[B]}\right)} \quad (15)$$

$$\frac{v}{V_{\max}} = \frac{[B]}{\alpha K_b \left(1 + \frac{K_a}{[A]} + \frac{K_a [Q]}{[A] K_q}\right) + [B] \left(1 + \frac{\alpha K_a}{[A]} + \frac{\alpha K_a [Q]}{\gamma K_q [A]}\right)} \quad (16)$$

Q effects only the slope of the $1/v$ versus $1/[A]$ plot. Q is a competitive inhibitor with respect to A at all B concentrations. Consequently, Q acts as a noncompetitive or mixed type inhibitor with respect to B.

In the case of Q as the competitive inhibitor with respect to A at a constant B concentration, the double reciprocal plot will yield a family of converging lines at the $(1/[A])$, each being obtained for a different fixed concentration of Q and the constant [B]. The slopes of each line are given:

$$\text{slope}_{(1/[A])} = \frac{\alpha K_a}{V_{\max}} \left(1 + \frac{K_b}{[B]} + \frac{K_b [Q]}{[B] K_q} + \frac{[Q]}{\gamma K_q}\right) \quad (17)$$

A secondary plot of $\text{slopes}_{(1/[A])}$ above plotted against the concentration of Q yields a plot with:

$$\text{slopes}_{[Q]} = \frac{\alpha K_a}{V_{\max}} \left(\frac{1}{\gamma K_q} + \frac{K_b}{[B] K_q}\right) \quad (18)$$

A replot of a family slopes_(Q) obtained at various concentration of B against the inverse of the concentration of B yield a plot with:

$$\text{slope}_{(1/[B])} = \frac{1}{V_{\max}} \frac{\alpha K_a K_b}{K_q} \quad (19)$$

and

$$\text{intercpet}_{(1/[B])} = \frac{1}{V_{\max}} \frac{\alpha K_a}{\gamma K_q} \quad (20)$$

Figure 23 shows the results of the experiments in which the inhibition by ADP was examined as a function of ATP concentrations. As predicted, ADP (Q) effects only the slope of the *1/v versus 1/[ATP]* plot. ADP is a competitive inhibitor with respect to ATP.

The results of the companion experiments in which the variable substrate is gluconate are shown in Figure 24. When gluconate is the varied substrate, ADP effects both slope and *1/v*-axis intercept. The ADP inhibition appears to be mixed-type with respect to gluconate ($\gamma = 0.73$).

A family of the secondary plot of slopes_(1/A) above plotted against the concentration of Q at various B concentration from 0.7 mM to 5.0 mM is shown in Figure 25. The replot from Figure 25 is shown in Figure 26. The γ and K_q were determined as 0.73 and 0.089 mM.

Figure 23. Reciprocal plots of $1/v$ versus $1/[ATP]$ at different fixed concentrations of product ADP and a constant [gluconate] which at (a) 0.7 mM, (b) 1.5 mM, and (c) 5.0 mM. ADP concentrations were 0.0 mM (+), 0.5 mM (•), 1.0 mM (Δ), and 2.0 mM ($\circ$). Data were taken from two determinations of two preparations with an experimental error of $\pm 10\%$.

Figure 23.a. [gluconate] = 0.7 mM

Figure 23.b. [gluconate] = 1.5 mM

Figure 23.c. [gluconate] = 5.0 mM

Figure 24. Reciprocal plots of $1/v$ versus $1/[\text{gluconate}]$ at different fixed concentrations of product ADP and a constant $[\text{ATP}]$ which at (a) 0.04 mM, (b) 0.05 mM, and (c) 0.1 mM. ADP concentrations were 0.0 mM (+), 0.5 mM ($\bullet$), 1.0 mM (Δ), and 2.0 mM ($\circ$). Data were taken from two determinations of two preparations with an experimental error of $\pm 10\%$.

Figure 24.a. [ATP] = 0.04 mM

Figure 24.b. [ATP] = 0.05 mM

Figure 24.c. [ATP] = 0.1 mM

Figure 25. $\text{Slope}_{(1/[ATP])}$ replot of data taken from plots of $1/v$ versus $1/[ATP]$ plot at different fixed concentrations of product ADP and a constant [gluconate], from 0.7 mM to 5.0 mM. Each line represents data from a family of reciprocal plots at a different concentration of gluconate, *i.e.*, 0.7 mM (+), 1.5 mM (Δ), and 5.0 mM (O).

Figure 26. Replot of $\text{slope}_{\text{ADP}}$ versus $[\text{gluconate}]$ (data taken from Figure 25).

3.5.3 Kinetic studies in the presence of 6-phosphogluconate (P)

The velocity equation is obtained from:

$$\frac{v}{[E]_c} = \frac{k_p [EAB]}{[E] + [EA] + [EB] + [EP] + [EAB]} \quad (21)$$

When the concentration of each species is expressed in terms of [E]:

(22)

Rearranging to express each of the substrates as the varied ligand:

$$\frac{v}{V_{\max}} = \frac{[A]}{\alpha K_a \left(1 + \frac{K_b}{[B]} + \frac{K_b [P]}{[B] K_p}\right) + [A] \left(1 + \frac{\alpha K_b}{[B]}\right)} \quad (23)$$

Only the slope factors are effected by P. The $1/v$ -axis intercept factor (*i.e.*, the

$$\frac{v}{V_{\max}} = \frac{[B]}{\alpha K_b \left(1 + \frac{K_a}{[A]} + \frac{K_a [P]}{[A] K_p}\right) + [B] \left(1 + \frac{\alpha K_a}{[A]}\right)} \quad (24)$$

factor multiplying the $[A]$ and $[B]$) is independent of P . Thus P acts as a competitive inhibitor with respect to both A and B .

A secondary plot of slope from the $1/v$ versus $1/[A]$ plot against different P concentration at a constant $[B]$ gives:

$$\text{slope} = \frac{1}{V_{\max}} \frac{K_b}{[B] K_p} \quad (25)$$

At low levels of 6-phosphogluconate (≤ 20 mM), it behaves as a competitive inhibitor with respect to gluconate at a constant $[ATP]$ (1.0 mM) is shown in Figure 27. Figure 28 shows the results of an experiment in which the inhibition by 6-phosphogluconate was examined as a function of ATP concentrations (at a constant $[gluconate]$, 1.7 mM). As predicted, 6-phosphogluconate acts as a competitive inhibitor with respect to ATP. The secondary plot of slope against different 6-phosphogluconate concentration is shown in Figure 29. The K_p was determined as 3.0 mM. Inhibition constants are summarized in Table 5.

Figure 27. Reciprocal plots of $1/v$ versus $1/[\text{gluconate}]$ at different fixed concentrations of product 6-phosphogluconate and a constant $[\text{ATP}]$, 1.0 mM. Gluconate concentrations were 0.3 mM, 0.5 mM, 1.0 mM, 1.7 mM and 5.0 mM. 6-Phosphogluconate concentrations were 0.0 mM (+), 10 mM ($\bullet$), 20 mM (Δ), and 30 mM ($\circ$). Data were taken from one determination with an experimental error of $\pm 15\%$.

Figure 28. Reciprocal plots of $1/v$ versus $1/[ATP]$ at different fixed concentrations of product 6-phosphogluconate and a constant [gluconate], 1.7 mM. ATP concentrations were 0.2 mM, 0.4 mM, 1.0 mM, 1.4 mM and 2.0 mM. 6-Phosphogluconate concentrations were 0.0 mM ($\circ$), 10 mM ($\bullet$), 20 mM (+), and 30 mM ($\blacksquare$). Data were taken from one determination with an experimental error of $\pm 15\%$.

Figure 29. $\text{Slope}_{(1/[ATP])}$ and $\text{slope}_{(1/[gluconate])}$ replot. Each line represents data from a family of reciprocal plots at a different concentration of the constant ligand gluconate (+) and ATP (○).

Table 5. Kinetic parameters of gluconokinase for the products

The total Mg^{2+} was kept constant at 10.0 mM. 6PGDH was used as the coupled enzyme in the studies of product ADP inhibition. Pyruvate kinase and lactate dehydrogenase were used as the coupled enzymes in the product inhibition of 6-phosphogluconate. Data were taken from two determinations of two preparations.

products	ADP	6-phosphogluconate
γ	0.73 ± 0.08	
$V_{\max}/[E]_t$ (s^{-1})	1.5 ± 0.2	1.1 ± 0.2
K_i (mM)	0.089 ± 0.030	3.0 ± 0.6

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3.6 Initial rate studies of hexokinases in the absence of products

Figures 30 and 31 show the results of the experiments for two hexokinases (HK-I and HK-II) from *S. pombe*. The dependence of the reaction rate on the concentration of glucose and ATP was examined with a set of combination of both substrates in the concentration ranges of 0.05 mM to 5.0 mM for glucose and 0.02 mM to 1.0 mM for ATP. The concentration of total magnesium was held constant at 10.0 mM. A secondary plot of the slopes and vertical intercepts is shown in Figure 32 for HK-I and HK-II. The Michaelis constants as determined from the secondary plot are for 0.071 and 0.092 mM ATP and 0.086 and 0.068 mM for glucose for HK-I and HK-II respectively. Kinetic parameters of hexokinases are summarized in Table 6.

Figure 30. Reciprocal plots of $1/v$ versus $1/[\text{glucose}]$ at different fixed concentration of ATP for hexokinase I. ATP concentrations were 0.01 mM ($\square$), 0.05 mM ($\circ$), 0.1 mM (Δ), and 0.2 mM ($+$). Glucose concentration were 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM. Data were taken from one determination with an experimental error $\pm 15\%$.

Figure 31. Reciprocal plots of $1/v$ versus $1/[\text{glucose}]$ at different fixed concentration of ATP for hexokinase II. ATP concentrations were 0.01 mM ($\square$), 0.05 mM ($\circ$), 0.1 mM (Δ), and 0.2 mM (+). Glucose concentration were 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM. Data were taken from one determination with an experimental error $\pm 15\%$.

Figure 32. Secondary plot of data taken from plots of $1/v$ versus $1/[\text{glucose}]$ at different fixed concentration of $[\text{ATP}]$. The functions, slopes (O) and intercepts (+), are replotted against the corresponding $1/[\text{ATP}]$. (a) hexokinase I, and (b) hexokinase II.

Figure 32.a. Hexokinase I

Figure 32.b. Hexokinase II

Table 6. Kinetic parameters of hexokinase for the substrates

Total concentration of Mg^{2+} were kept constant at 10.0 mM. The enzyme activity was assayed by the coupled reaction with glucose-6-phosphate dehydrogenase. Data were taken from one preparation. Experimental error is $\pm 15\%$.

	Hexokinase I	Hexokinase II
$V_{\max}/[E]_t \text{ (s}^{-1}\text{)}$	1.5 ± 0.2	1.7 ± 0.3
$K_{\text{ATP}} \text{ (mM)}$	0.071 ± 0.011	0.092 ± 0.014
$K_{\text{glucose}} \text{ (mM)}$	0.085 ± 0.013	0.068 ± 0.010
α	0.82 ± 0.12	0.62 ± 0.09

CHAPTER 4

DISCUSSION

4.1 Glucose metabolism in *S. pombe*

Both glycolytic and the pentose phosphate pathway enzymes are present in the cell-free extract of *S. pombe*. It indicates that both pathways contribute to D-glucose metabolism *in vivo*. The flow of carbon through glycolysis and the pentose phosphate pathway can generate ATP for energy requirement and NADPH as a major reductant in the biosynthesis.

Besides the classical oxidative branch of the pentose phosphate pathway, the gluconate route, which transfer D-glucose via $D\text{-glucose} \rightarrow D\text{-gluconate} \rightarrow D\text{-6-phosphogluconate}$, provides alternate path to produce 6-phosphogluconate as an intermediate for the pentose phosphate pathway. The *in vitro* steady state activities of glucose dehydrogenase and glucose-6-phosphate dehydrogenase has a ratio of 1:2.7 (see Figure 4). It may implicate the ratio of those two routes' capacity to produce 6-phosphogluconate *in vivo*.

The presence of gluconokinase as a constitutive enzyme of the fission yeast indicates that free gluconate is formed during the glucose metabolism. It has been shown that gluconate can be utilized by the organism as the sole carbon and energy source for the growth of *S. pombe* mutants (Hoever *et al.*, 1992).

By comparing the *in vitro* specific activities of glucose dehydrogenase and gluconokinase, the *in vivo* rate-limiting step of gluconate route seems to be the oxidation

of glucose. The specific activity of gluconokinase increases during the exponential phase, and reaches a steady state at the stationary phase when cell mass ceases to increase. By contrast, the glucose-6-phosphate dehydrogenase activity decreases its activity as gluconokinase increases its activity.

Carbon-13 NMR experiments using the cell-free extracts demonstrate that the ^{13}C -label from $[1\text{-}^{13}\text{C}]$, $[2\text{-}^{13}\text{C}]$ and $[\text{U-}^{13}\text{C}]$ glucose is incorporated in the intermediates of the glycolysis, the pentose phosphate pathway and the gluconate route. It confirms that the pentose phosphate pathway intermediate, 6-phosphogluconate, can be formed either *via* hexokinase and glucose-6-phosphate dehydrogenase or glucose dehydrogenase and gluconokinase.

The detection of glucose-6-phosphate in the ^{13}C NMR spectra can not be verified because the chemical shifts are very similar in glucose-6-phosphate and glucose, and the spectrum contained very intense glucose peaks. However there were the appearance of C-1 derived from α and β FDP at $\delta 67$ ppm. The broad peaks around $\delta 65$ to 66 ppm can be explained by the multiple resonances of ^{13}C -labels from both pentose phosphate and the gluconate. The sharp, triplet at $\delta 65.5$ ppm can be assigned to C-6 of gluconate with reference to the experiment with $[\text{U-}^{13}\text{C}]$ glucose-glucose oxidase reaction. The observed broad peaks at $\delta 65.3$ ppm, which can be assigned to C-6 of β -fructose-1,6-bisphosphate ambiguously, can occur as a consequence of scrambling of the label at the triosephosphate isomerase level coupled to back synthesis of fructose-1,6-bisphosphate from the trioses by pathway through fructose diphosphate aldolase and transaldolase. The formation of the peak at $\delta 100.8$ ppm as C-1 of ribose-5-phosphate came from the C-2 of glucose due

to CO₂ loss in the pentose phosphate pathway.

A direct effect of ATP on glucose catabolism is made evident by the appearance of ¹³C-labels in the phosphorylated intermediates of glycolysis and the pentose phosphate pathway. The addition of NADP⁺ to the cell-free extract of *S. pombe* sharpened the resonance at 65.5 ppm for [6-¹³C] and 76.8 ppm for [2-¹³C] of gluconate and/or 6-phosphogluconate. The resonances of other phosphorylated intermediates were not detected. The results suggest that gluconate route can also be initiated, along with the first half oxidative branch of the classical pentose phosphate pathway, to provide NADPH as the reducing equivalent in the biosynthesis. The relationship of those two routes *in vivo* is still unclear.

Although the gluconate route by-passes the glycolysis and the first half of the pentose phosphate pathway, the existence of this system suggests that they may be used as an alternative path of glucose utilization in *S. pombe*. The principle source of NADPH as the reducing equivalents in *Saccharomyces cerevisiae* is the pentose phosphate pathway (Lagunas and Gancedo, 1973). However, the pentose phosphate pathway does not appear to generate all the NADPH utilized for biosynthesis. The gluconate route also provide NADPH at the oxidative reaction catalysed by glucose dehydrogenase. The regulation of the oxidative branch of the pentose phosphate pathway at glucose-6-phosphate stage is mainly controlled by the ratio of NADPH/NADP⁺. The control of gluconate route could also be the oxidation of glucose, however the control could be at the gluconokinase step as well. The regulatory mechanisms for the two dehydrogenases activities, glucose and glucose-6-phosphate dehydrogenases by the ratio of NADPH/NADP⁺ need further investigation.

4.2 Purification of gluconokinase

Gluconokinase is the first enzyme in gluconate metabolism. It has been described in a number of bacteria in which the enzyme is induced by growth on gluconate (Cohen, 1950). The enzyme is also present as the first step of the metabolism of gluconate by animals (Zachariou and Scopes, 1985).

Gluconokinase from *S. pombe* was purified 410-fold with a specific activity of 140 units/mg protein. It has been purified to homogeneity with a subunit M.W. of 24,000 dal as evaluated from SDS-PAGE.

The first step in the purification procedure was ammonium sulfate fractionation at 45 - 70% saturation. In the purification procedure of gluconokinase from *E. coli*, the addition of $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation precipitates considerable protein, including about 30% of the ATPase and some of the glycolytic complex (Cohen, 1950). It has been confirmed that this fraction also contained most of the 6-phosphogluconate dehydrogenase activity (Cohen, 1950). The enzyme was purified 1.4-fold at this step. In contrast to 6-phosphogluconate dehydrogenase, which can be detected at high ionic strength such as in the presence of ammonium sulfate at 70% saturation, gluconokinase activity can be detected only after the removal of ammonium sulfate.

The P-6 gel column was used to remove the ammonium sulfate from the proteins. The Blue-A agarose column provided a 13-fold purification. This is due to the relatively specific binding of gluconokinase with Blue A ligand coupled to the column. 2',5'-Adenosine diphosphate column is used primarily to separate gluconokinase and 6-phosphogluconate dehydrogenase. This step is essential since 6-phosphogluconate

dehydrogenase is used as the coupled enzyme for assaying gluconokinase activity. Bio-gel P-100 column was another efficient step in this procedure due to the relatively small molecular weight of gluconokinase. It provided a single step with 84% recovery and a 94-fold purification. Based on the gel filtration elution profile, the molecular weight of gluconokinase is estimated to be 50,000 dal.

The last step in the purification procedure was ATP affinity chromatography. This column yields an overall 410-fold purification. The specific binding of the ATP ligand with the enzyme renders an efficient separation of gluconokinase from other proteins possible. The enzyme can be eluted by either 0.5 mM ATP, or 10 mM Mg^{2+} . The competition of Mg^{2+} at high concentrations with ATP for gluconokinase may explain the ability of Mg^{2+} to elute the enzyme. A broad gluconokinase activity peak for the elution profile of Mg^{2+} contrasts a sharp peak by (0.5 mM) ATP elution.

The molecular weight of the enzyme from *S. pombe* is different from other gluconokinases (see Table 2), such as 112 Kdal for *Pseudomonad* enzyme and 18 Kdal for *Zymomonas mobilis* enzyme (a monomer). It has been suggested that the *Pseudomonad* enzyme could exist in different molecular weight forms under various conditions, one of them could have a molecular weight of less than 70 Kdal (Coffee and Lu, 1970).

4.3.1 Kinetic study of gluconokinase

In the direction of phosphorylation of gluconate, the initial velocity pattern obtained by varying ATP at different fixed levels of gluconate intersects to the left of the

ordinate, suggesting a sequential kinetic mechanism. A fit of those data to the equation for a random bireactant mechanism indicates that the all denominator terms are consistent with a random mechanism that approximates rapid equilibrium. At fixed saturation concentration of Mg^{2+} , the terreactant mechanism reduces to bireactant mechanism.

The product inhibitions for ADP vs ATP, 6-phosphogluconate vs ATP, and 6-phosphogluconate vs gluconate, exhibited competitive patterns. ADP acted as a mixed-type inhibitor ($\gamma = 0.73$) with respect to gluconate. These data are clearly consistent with a random bi bi with dead-end E-gluconate-ADP complex mechanism involving an ternary complex of enzyme, ATP, and gluconate.

Gluconokinase from *S. pombe* displays much higher affinity for ATP ($K_{ATP} = 0.056$ mM), in comparison 1.04 mM with *Pseudomonas* enzyme and 1.5 mM for *Zymomonas mobilis* enzyme (see Table 2). The affinity of the *S. pombe* enzyme for ATP is also much greater than that for gluconate ($K_{gluconate} = 1.6$ mM). This may be an indication of the efficiency with which cell utilize available ATP. The relatively high $K_{gluconate}$ suggests that the uptake of gluconate by this enzyme will be very sensitive to gluconate concentration in the cell.

The enzyme exhibits inhibition by the product ADP by forming a dead-end E-gluconate-ADP complex ($K_{ADP} = 0.089$ mM), which may be an important factor in the regulation of gluconokinase activity. This regulation has been proposed for the yeast hexokinases as well (Middleton, 1990). The inhibition of ADP with respect to gluconate can be overcome when ATP is saturating (Equation 16). At high concentration of ADP, the role of the gluconate route may become subordinate to that of the pentose phosphate

pathway.

4.3.2 Kinetic study of hexokinases

The data of initial velocity studies in the absence of products suggested a sequential kinetic mechanism. The K_m for glucose from both hexokinases I and II were consistent with the K_m values for glucose in other sources (Colowick, 1973; Gerber *et al.*, 1974). In general, hexokinases display low K_m values in the range 0.1 - 0.001 mM for D-glucose, whereas glucokinase has a K_m of 5 mM. From the gel filtration elution profile of hexokinase II, the molecular weight of hexokinase II is estimated to be around 100 Kdal, because the enzyme was eluted around the void volume of the column. Both hexokinases have a slightly lower affinity for ATP than that of gluconokinase for ATP. The K_m for ATP in hexokinases I and II were 0.086 mM and 0.068 mM respectively, while the gluconokinase had a K_m of 0.056 mM for ATP.

4.4 Summary

The results of enzymic activities and ^{13}C -NMR experiments are consistent with the operation of the glycolysis, the pentose phosphate pathway, and the gluconate route in *S. pombe*. A six-step purification procedure for gluconokinase from *S. pombe* has been described.

Gluconokinase is a Mg^{2+} -activated enzyme, catalyzing the formation of 6-phosphogluconate from gluconate and ATP. The kinetic properties of gluconokinase have been examined. The catalytic reaction appears to follow a random addition of substrates

to the enzyme with a formation of the dead-end E-gluconate-ADP complex.

The gluconokinase from *S. pombe* possesses the molecular weight which differ with the enzymes from other sources. This enzyme also shows a much higher affinity for ATP. The regulation of its activity could effect the flow of carbon through the gluconate route as well as the pentose phosphate pathway. Together with a possible control at the glucose dehydrogenase step, it is proposed that gluconokinase may play an important role in the glucose metabolism of *S. pombe*.

APPENDIX I

INHIBITION STUDIES BY K_{ii} and K_{is}

For a single substrate reaction such as the one below, the inhibition Q is seen to have two possible sites of interaction with the enzyme.

The inverse rate equation below accommodates these two sites of inhibition by inclusion of inhibition factors as above.

$$\frac{1}{v} = \frac{K_s \left(1 + \frac{[Q]}{K_{is}}\right) + [A] \left(1 + \frac{[Q]}{K_{ii}}\right)}{V_{\max} [A]} \quad (1)$$

Double reciprocal plots yield:

$$intercepts = \frac{1}{V_{\max}} + \frac{[Q]}{K_{ii} V_{\max}} \quad (2)$$

from which a secondary plot of intercepts *versus* the concentration of Q can be used to obtain K_{ii} . Similarly, slopes obtained from the double reciprocal plots are:

from which K_{is} obtained from the slope of a secondary plot of double reciprocal plot

$$slopes = -\frac{K_a}{V_{max}} + \frac{K_a [Q]}{K_{is} V_{max}} \quad (3)$$

slopes *versus* concentrations of Q.

Competitive inhibition results when the inhibitor binds only to the free enzyme and therefore only a value for K_{ia} obtained. Uncompetitive inhibition results when the inhibitor interacts with the binary complex only. As a result only the intercepts term is affected and therefore only K_{ii} is obtained. Non-competitive or mixed-type inhibition results when both slope and intercept terms are affected. If $K_{ia} = K_{ii}$ then inhibition is considered pure non-competitive. Otherwise inhibition is considered mixed-type.

The product inhibition patterns of gluconokinase is shown in Table 7.

Table 7. Product inhibition patterns of Gluconokinase

Products		Varied substrate					
		ATP			Gluconate		
		K_{ii}	K_{is}	Figures ^a	K_{ii}	K_{is}	Figures ^a
ADP	i)	-	0.16	(25)	0.39	0.18	(24)
	ii)	-	0.11	(25)	0.20	0.29	(24)
	iii)	-	0.13	(25)	0.33	0.48	(24)
6PGA	iv)	-	87	(29)	-	98	(29)

Note: Inhibition constants are given in mM. When both constants are obtained, non-competitive inhibition is observed. When either K_{ii} or K_{is} only are obtained, uncompetitive and competitive are observed respectively. Average errors for inhibition constants were determined from correlation coefficients of linearly regressed data are $K_{ii} \pm 26\%$, $K_{is} \pm 39\%$.

a For specific plots refer to the Figure number stated.

- i) When ATP was varied over concentrated as indicated as indicated in figure legends, gluconate concentrated was fixed at 0.7 mM. For variation of gluconate as indicated in figure legends, ATP was fixed at 0.04 mM.
- ii) When ATP was varied over concentrated as indicated as indicated in figure legends, gluconate concentrated was fixed at 1.5 mM. For variation of gluconate as indicated in figure legends, ATP was fixed at 0.05 mM.
- iii) When ATP was varied over concentrated as indicated as indicated in figure legends, gluconate concentrated was fixed at 5.0 mM. For variation of gluconate as indicated in figure legends, ATP was fixed at 0.1 mM.
- iv) When ATP was varied over concentrated as indicated as indicated in figure legends, gluconate concentrated was fixed at 1.7 mM. For variation of gluconate as indicated in figure legends, ATP was fixed at 1.0 mM.
- No value observed.

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