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**PURIFICATION, CHARACTERIZATION AND KINETIC
STUDIES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM
*SCHIZOSACCHAROMYCES POMBE***

by

Qing Chen, B.Sc.

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

Master of Science

Department of Chemistry
Carleton University
Ottawa, Ontario
November, 1995

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**"PURIFICATION, CHARACTERIZATION AND KINETIC STUDIES OF
6-PHOSPHOGLUCONATE DEHYDROGENASE FROM
SCHIZOSACCHAROMYCES POMBE"**

submitted by Qing Chen. B. Sc.

in partial fulfilment of the requirements for
the degree of Master Science

Chairman, Chemistry Department

Thesis Supervisor

ABSTRACT

6-Phosphogluconate dehydrogenase from *Schizosaccharomyces pombe* has been purified to electrophoretic homogeneity with 463-fold purification (specific activity of 39.4 units/mg) and a total recovery of 27.5%. The purification procedure consisting of the combination of the Sephacryl S-300 gel filtration, anion-exchange (DE-52) chromatography, Matrex Red Gel chromatography, and affinity (AG-NADP⁺) chromatography, is similar to that employed to purify the gluconate pathway enzymes from *Schizosaccharomyces pombe*. A partial characterization is presented and a comparison of physical and catalytic properties is made with the enzymes from other sources. This enzyme exists as a tetramer of four identical subunits with a native molecular weight of approximately 152 KDa. The enzyme utilizes NADP⁺ as an obligatory coenzyme. The fission yeast enzyme does not require metal ions, however several divalent cations enhance its activity. The purified enzyme is stable at pH 4-10 and the pH optimum for catalytic activity is between 7.0 and 8.0.

Kinetic and product inhibition studies show that the oxidative decarboxylation of 6-phosphogluconate catalyzed by *S. pombe* 6PGDH proceeds via random Bi Ter mechanism. The kinetic mechanism of the 6PGDH catalysis is pH independent and does not change in the presence of Mn²⁺. The pattern of CO₂ as a product inhibiting the 6PGDH catalysis is first reported in this study. The results show CO₂ as the first product released in the direction of oxidative decarboxylation.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AG-NADP	Agarose-hexane NADP⁺ (type 3) affinity
AlcDH	Alcohol dehydrogenase
AldDH	Aldehyde dehydrogenase
APS	ammonium persulfate
Bis	N,N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
DTT	Dithiothreitol, Clelands Reagent
EDTA	Ethylenediamine-tetraacetate
EMM2	Edinburgh minimal medium No. 2
Et	Total enzyme concentration
g	Gram
G6PDH	Glucose 6-phosphate dehydrogenase
GSSG	Oxidized glutathione
hr.	Hour
L	Litre
M	Molar
mg	Milligram
ml	Millilitre

mM	Millimolar
min	minute
M.W.	Molecular weight
NAD(P) ⁺	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
6PG	6-phosphogluconate
6PGDH	6-phosphogluconate dehydrogenase
pI	Isoelectric point
PMP	Pentose monophosphate pathway
PMSF	Phenylmethylsulfonylfluoride
rpm	Revolutions per minute
Ru 5-P	D-Ribulose 5-phosphate
SDS	Sodium dodecyl sulphate
<i>S. (pombe)</i>	<i>Schizosaccharomyces (pombe)</i>
TCA	Tricarboxylic acid
TEMED	N,N,N',N',-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
U	Enzyme activity unit
V _{max}	Maximum velocity
V _{max} /Et	Turnover number

INTRODUCTION

The fission yeast, *Schizosaccharomyces (S) pombe*, has been extensively used as a model for studies of D-glucose metabolism (Fiechter, *et al.*, 1981; Tsai, *et al.*, 1987; 1995). This yeast was first isolated from the east African millet beer by Lindner in 1893 (Mitchison, 1970) and is one of the most thoroughly studied eukaryotic microorganisms. Its cell cycle, physiology, and common growth characteristics are well known (Fantes, 1984; Mitchison, 1989).

Recent studies concerning D-glucose catabolism have shown that *S. pombe* possesses capabilities for operation of the glycolytic pathway, the pentose phosphate pathway and the tricarboxylate cycle (Tsai, *et al.*, 1992). *S. pombe* commonly utilizes D-glucose for growth and energy metabolism and is unable to grow in media with acetate or ethanol as the sole carbon source (Tsai, *et al.*, 1987). An alternative growth substrate, D-gluconate, can be used as the sole carbon and energy source (Hoever, *et al.*, 1992). *S. pombe* grows diauxically in the D-glucose batch media and can utilize acetate or ethanol only in the presence of D-glucose (Tsai, *et al.*, 1987).

Two kinds of cell cultures are generally used to investigate D-glucose metabolism in yeast, i.e. batch culture and continuous culture. Batch cultures are normally used to determine enzymic activities during the yeast growth, which were also used in this study. Batch culture is a closed system in which the growth environment changes progressively and cell quantities increase until limiting nutrient is exhausted or limiting metabolite accumulates to toxic concentrations. The growth of population passes through a series of

distinct phases, namely, lag phase, accelerating phase, exponential phase, decelerating phase, stationary phase and decreasing phase (Pirt, 1975).

Continuous cultures allow microorganisms to be grown in a time-independent dimension. The growth conditions of cells are maintained continuously under steady state, where the factors such as temperature, pH, dissolved oxygen concentration, the growth rate of cells, nutrients and metabolic products are maintained at fixed levels until they are deliberately changed. The technique can be used to investigate the changes in the physiology, metabolism, cell morphology, enzymic activities when a single environmental condition is varied. In the previous studies, continuous cultures were used to control the growth rate of the yeast, which gave the correlation of the growth rates with enzymic activities from different pathways of D-glucose metabolism with varied metabolic activities (Tsai, *et al.*, 1992).

D-glucose is catabolized in yeast cells mainly *via* glycolytic pathway which involves at least ten steps each catalyzed by a different enzyme. All intermediates in the glycolytic pathway are phosphorylated derivatives having three to six carbons (Axelrod, 1976). This pathway is initiated by phosphorylation of D-glucose followed by oxidation. The phosphorylation of D-glucose is catalyzed by hexokinase (EC 2.7.1.2). The product, glucose-6-P can be converted to fructose-6-P catalyzed by phosphoglucose isomerase (EC 5.3.1.9). Alternatively, glucose-6-P can be oxidized to gluconolactone-6-P catalyzed by glucose-6-phosphate dehydrogenase. The latter route leads to an alternate pathway in parallel with the glycolytic pathway, i.e. pentose phosphate pathway (Fig.1). D-glucose can also be oxidized to D-gluconate followed by phosphorylation. This leads to gluconate

pathway which has been recently demonstrated in *S. pombe* (Tsai, *et al.*, 1995). The phosphorylation of D-gluconate is catalyzed by gluconokinase (EC 2.7.1.12). Previous studies showed that about 12% of D-glucose was catabolized by the pentose phosphate pathway and the rest *via* the glycolytic pathway. This alternate pathway is responsible for the generation of NADPH required for the reductive biosynthetic processes (Wang *et al.*, 1956, 1958; Chen, 1959). The glycolytic and the pentose phosphate pathways are the two central and constitutive routes of intermediary carbohydrate metabolism. A third route, the Entner-Doudoroff pathway is inducible (in Fig.1). All enzymes of these pathways as listed in Table 1 have been characterized from various biological sources.

The glycolysis is the best understood carbohydrate catabolic pathway which has been the subject of intensive research by many investigators for several decades. The overall reaction of this pathway can be summarized as follows:

The overall reaction of glycolysis can be simply considered as a chain of linked reactions (see Fig. 1). The conversion of glucose to the final product, pyruvate is energetically favoured. But the glycolytic pathway is not a simple, straightforward operation and is controlled by several regulatory enzymes. The main function of the Embden-Meyerhof pathway(glycolysis), in conjunction with the tricarboxylic acid cycle, is to supply the energy in the form of ATP. An additional function, usually of a minor importance, is to supply the carbon skeletons for the synthesis of cell constituents, such as glutamate, alanine, aspartate, galactose, glucosamine and acetyl CoA (see Table 2).

Fig. 1. Glycolysis, the pentose pathway, and the Entner-Doudoroff pathway. The reactions are schematized, and cofactors and cosubstrates(ADP, P_i, NAD, etc.) are not shown.

Table 1. Enzymes of D-glucose Metabolic Pathways

Reaction	References
Glycolytic pathway	
Hexokinase(EC 2.7.1.2)	Fukuda et al., 1984
Phosphoglucose isomerase(EC 5.3.1.9)	Schreyer & Bock, 1980
Phosphofructokinase(EC 2.7.1.11)	Blangy et al., 1968
Fructose-1,6-bisphosphatase(EC 3.1.3.11)	Babui & Guixé, 1933
Fructose-1,6-bisphosphate aldolase(EC 4.1.2.13)	Baldwin & Perham, 1978
Triose-phosphate isomerase(EC 5.3.1.1)	Anderson & Cooper, 1969
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	Hillman, 1979
Phosphoglycerate kinase(EC 2.7.2.3)	D'Alessio & Josse, 1971
Phosphoglycerate mutase(EC 2.7.5.3)	D'Alessio & Josse, 1971
Enolase(EC 4.2.1.11)	Spring & Wold, 1971
Pyruvate kinase(EC 2.7.1.40)	Garcia-Olalla et al. 1982
Pentose phosphate pathway	
Glucose-6-phosphate dehydrogenase(EC 1.1.1.49)	Banerjee & Fraenkel, 1972
6-phosphogluconolactonase(EC 3.1.1.31)	Kupor & Fraenkel, 1969
6-phosphogluconate dehydrogenase(EC 1.1.1.44)	Wolf et al. 1979
Ribose-5-phosphate isomerase(EC 5.3.1.6)	Skinner & Cooper, 1974
Transketolase(EC 2.2.1.1)	Josephson & Fraenkel, 1969
Entner-Duodoroff pathway	
6-phosphogluconate dehydrase(EC 4.2.1.12)	Fraenkel & Banerjee, 1972
2-Keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14)	Pouyssegur & Stoeber, 1971

The first step in glycolysis is the phosphorylation of glucose catalyzed by hexokinase with first ATP as the phosphate donor. The second phosphorylation in glycolytic pathway is catalyzed by phosphofructokinase (PFK) converting fructose-6-phosphate to fructose-1,6-*bis*phosphate with the expenditure of second ATP. Phosphofructokinase is shown to be a crucial regulatory enzyme in glycolysis and its activity is influenced by many effectors including adenine nucleotides (Cori, 1942; Uyeda, 1979). Another regulatory enzyme in the glycolytic pathway is fructose diphosphatase which catalyzes the conversion of fructose diphosphate to fructose 6-phosphate. These two enzymes constitute the futile cycle as the first regulatory site in the glycolysis. The direction of glycolysis is under the control of these two enzymes (Axelrod, 1967). It has been noted that 5'-AMP accelerates the reaction catalyzed by phosphofructokinase and inhibits that catalyzed by fructose diphosphatase (Atkinson & Walton, 1965; Taketa & Pogell, 1963). It has also been observed that citrate inhibition of the phosphofructokinase reaction makes glycolysis sensitive to regulation by the TCA cycle.

The pentose phosphate pathway is another central route of glucose metabolism occurring in many bacteria and in most eukaryotic organisms. The main function of this pathway is to supply NADPH for the reductive anabolism, and ribose phosphate for the biosynthesis of nucleic acids (Table 2). The pentose phosphate pathway can be divided into two branches: the oxidative branch and nonoxidative branch. All the individual enzymic steps in the pentose phosphate pathway are thoroughly characterized and all of the intermediates are known (Fig. 2). The flow diagram (Fig.3) showing the passage

Table 2. Functions of different pathways of glucose degradation

Embden-Meyerhof-Parnas pathway plus associated reactions(TCA cycle)	Pentose phosphate pathway
<p>supplies</p> <p>ATP</p> <p>Carbon skeletons for biosynthesis of</p> <p>non-essential amino acids</p> <p>acetyl-CoA(fat, steroids,</p> <p>acetylations)</p> <p>glycerophosphate(fat)</p>	<p>supplies</p> <p>Ribose-5-P</p> <p>NADPH for</p> <p>reductive syntheses</p> <p>hydroxylation of steroids</p> <p>reduction of Met-Hb</p> <p>Reduction of GSSG</p> <p>H₂O₂ production in phagocytes</p>

Taken from Krebs & Eggleston. 1974.

of six molecules of hexose phosphate through the oxidative pentose phosphate cycle illustrates the fate of the individual carbon atoms of the original hexose (Axelrod, 1976).

Pentose phosphate is generated in the initial two steps of reactions catalyzed by the dehydrogenases of the pathway, which comprise the oxidative branch. The subsequent interconversions of sugar phosphates catalyzed by transketolase and transaldolase comprise the nonoxidative branch which equilibrates the pentose phosphates with hexose phosphates of glycolysis. Pentose phosphate formed by the oxidative branch is converted back to hexose phosphate by nonoxidative branch.

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 (G6PDH), the first enzyme in the pathway, is considered to be the regulatory enzyme of the oxidative branch. The flux of the reaction catalyzed by glucose-6-phosphate dehydrogenase is controlled by the enzymic activity and by the NADPH/NADP⁺ ratio (Krebs & Eggleston, 1974). This enzyme is strongly inhibited by NADPH. When the ratio of [NADPH]/[NADP⁺] is above 8 the inhibition is almost complete (Gumaa & Mclean, 1971; Kather *et al*, 1972). Transaldolase is known to be the rate-limiting enzyme for nonoxidative 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).

The Entner-Doudoroff pathway has two reactions, the conversion of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate by 6-phosphogluconate

Fig. 2. Enzymes of the pentose phosphate pathway

(1) Glucose-6-phosphate dehydrogenase,	(2) 6-Phosphogluconate dehydrogenase,
(3) Phosphoriboisomerase,	(4) D-Xylulose-5-phosphate epimerase,
(5) Transketolase,	(6) Transaldolase,
(7) Aldolase,	(8) Fructose diphosphatase,
(9) Phosphoglucoisomerase,	(10) Triosephosphate isomerase

Fig. 3. Schematic representation of the passage of six molecules of hexose phosphate through the pentose phosphate cycle (numbers refer to the positions of the carbons in the original hexose). (Taken from Axelrod, 1976).

dehydratase and cleavage of the phosphogluconate to glyceraldehyde-3-phosphate and pyruvate by 2-keto-3-deoxyphosphogluconate aldolase. The pathway is induced by gluconate, which is converted to 6-phosphogluconate by gluconokinase (Eisenberg & Doborogosz, 1967; Fraenkel & Horecker, 1964; Fraenkel & Levisohn, 1967). The product, 6-phosphogluconate, is further metabolized through the pentose phosphate pathway by 6-phosphogluconate dehydrogenase and/or the Entner-Doudoroff pathway by 6-phosphogluconate dehydrase. Unlike the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway, this pathway is less widely used by microorganisms, but operates in a number of *Pseudomonas* species and a few other Gram-negative bacteria. The two enzymes of this pathway have been purified and characterized (Fraenkel & Banerjee, 1972; Grazi, *et al.*, 1963).

Glucose metabolism in *S. pombe*, having been studied recently, is mainly *via* the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway, in conjunction with the tricarboxylic cycle (Tsai, *et al.* 1992). The Entner-Doudoroff pathway for glucose metabolism in *S. pombe* still requires further investigation. A new alternative route, the gluconate pathway which connects the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway, has been recently demonstrated in *S. pombe* (Tsai, *et al.* 1995). This gluconate pathway consists of two steps: oxidation of D-glucose to D-gluconate by NADP⁺-dependent glucose dehydrogenase and phosphorylation of D-gluconate to 6-phosphogluconate by gluconate kinase. The two enzymes of the gluconate pathway have been purified from *S. pombe* and characterized. This alternative route provides a bridge to connect the two central pathways of glucose metabolism namely the Embden-

Meyerhof-Parnas pathway and the pentose phosphate pathway (see Fig. 1).

6-Phosphogluconate dehydrogenase (EC.1.1.1.44)(6PGDH) was first isolated from a mammalian source by Villet and Dalziel (Villet and Dalziel, 1969), who obtained an apparently homogeneous preparation from sheep liver. Their isolation procedure was modified to purify the enzyme from various sources (Procsal & Holten, 1972; Silverberg & Dalziel, 1975; Stournaras, *et al.* 1982). Since this enzyme plays a key role in the pentose phosphate pathway, its properties and the kinetic mechanism of the reaction have been well studied by several groups (Table 3).

This enzyme is relatively stable over a period of months at neutral pH (pH 5-10) in the cold. It was reported by Veronese *et al.*(Veronese, *et al.*, 1982) that the enzyme from *Bacillus stearothermophilus* retains full activity for several months when stored at 4 °C as an ammonium sulphate suspension or in acetate buffer, pH 6.0, in the presence of a crystal of thymol to prevent bacterial growth. The enzyme shows no loss of activity after heating for 15 min at 60°(the growth temperature of the organism). In addition, the *Bacillus stearothermophilus* 6PGDH shows high stability toward other protein denaturants, such as urea, organic solvents and proteolytic enzymes (Veronese, *et al.* 1982).

The enzyme requires either NADP⁺ or NAD⁺ as the coenzyme depending on its biological sources. The dehydrogenase from yeast and mammals requires NADP⁺ specifically, whereas the enzyme from *Gluconobacter* species reduces both NADP⁺ and NAD⁺ at the same rate (Adachi & Ameyama, 1982). It was also reported that the enzyme from sheep liver does not utilize NAD⁺ as the coenzyme (Silverberg & Dalziel, 1975), whereas 6PGDH from *Pseudomonas fluorescence* prefers NAD⁺ rather than NADP⁺, with

Table 3. Characterizations of 6-Phosphogluconate Dehydrogenase

Molecular weight/ Subunits	Michaelis Constants		$E_{1\text{cm}}^{1\%}$	References
	NADP ⁺	6PG		
94,000/2	6.8	6.9 ¹	11.4	Silverberg & Dalziel, 1975
104,000/2	30	20	12.5	Pearse & Rosemeyer, 1975
108,000/2	15	24	N/A	Bridges, <i>et al.</i> 1975
100,000/2	25	20 ²	10.5	Veronese, <i>et al.</i> 1982
175,000/4	100	70	10.8	Adachi & Ameyama, 1982
230,000/4	6.4	63.7	N/A	Holden & Storey, 1994

1. In 0.13 M triethanolamine buffer, pH 7.0, at 25 °C.

2. In 0.1 M Tris-HCl buffer, pH 8.0, at 43 °C.

a rate ratio of 5.26 (Stouranaras, et al. 1982). The enzyme activity varies with ionic strength, being maximal in the range of 0.1-0.2. In this range, V is a maximum and the K_m toward 6-phosphogluconate is a minimum. Phosphate ions show competitive inhibition with respect to 6-phosphogluconate. NADPH inhibits competitively with respect to NADP⁺ (Pearse & Rosemeyer, 1975). The enzyme from *Bacillus stearothermophilus* is activated by MgCl₂ at low concentrations, whereas it is inhibitory at high concentrations. The enzyme from *Streptococcus faecalis* is strongly inhibited by certain heavy metal ions (zinc, cadmium, copper) and by p-chloromercuribenzenate. It was also found that fructose 1,6-bisphosphate is a potent and specific physiological inhibitor of the enzyme (Bridges & Wittenberger, 1975). Neither KCl nor NaCl shows any effect on the enzymic activity of the enzyme from most sources. However, it was reported that KCl inhibited the enzyme from a freeze tolerant insect. Inhibition of the enzyme by KCl increases with a decrease in temperature (Holden & Storey, 1994).

The molecular weight of this enzyme has been determined to be about 100,000 with two identical subunits in *Bacillus stearothermophilus* (Veronese, et al. 1982), or about 175,000 with four identical subunits in *Gluconobacter suboxydans* (Adachi & Ameyama, 1982). The complete amino acid composition of 6PGDH has been reported for the enzyme from *Bacillus stearothermophilus* (Veronese, et al. 1982), sheep liver (Silverberg & Dalziel, 1975), human erythrocyte (Pearse & Rosemeyer, 1975), *Streptococcus faecalis* (Bridges & Wittenberger, 1975), *Candida utilis* (Rippa, et al., 1967). The complete N-terminal or C-terminal amino acid sequences of the enzyme from several sources have also been reported (Somers, et al., 1992; Harbitz, et al., 1990;

Barrett & Le Page, 1993). The amino acid residues which include lysine, cysteine, tyrosine, and histidine have been detected to be present at the active site of the enzyme (Rippa & Signorini, 1975). Two types of 6PGDH were reported from *Candida utilis* (Rippa, *et al.*, 1967). The two forms of the enzyme have identical pH optimum, same K_m for substrates and same specificity. They differ in amino acid composition, molecular weight, electrophoretic mobility, stability, sensitivity to chlorodinitrobenzene and proteolytic treatment. The type I enzyme can be obtained in the crystalline form, but the type II enzyme does not crystallize under the same conditions. The enzymatic properties of the two forms are essentially the same, and some differences in the observed properties can be attributed to different structural forms. Furthermore, it is interesting to note that two forms of fumarase and at least two forms of transaldolase exist in *Candida utilis* (Rippa, *et al.* 1967).

6-Phosphogluconate dehydrogenase catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate (Ru 5-P) and carbon dioxide with the concomitant generation of NADPH.

This reaction is similar in nature to those catalyzed by isocitrate dehydrogenase and malic enzyme in that all three reactions yield a ketone, CO_2 , and NAD(P)H as products. However, unlike the other two enzymes, 6PGDH does not require divalent metal ions for the activity (Pontremoli *et al.*, 1961; Siebert *et al.*, 1957; Rutter & Lardy, 1958).

Numerous kinetic studies have been performed on 6PGDH from various sources. The kinetic parameters for the enzyme in the direction of oxidative decarboxylation have

been determined by several groups using enzymes from rat liver (Holten & Procsal, 1972), sheep liver (Hansen *et al.*, 1973), and *Leuconostoc mesenteroides* (Rendina *et al.*, 1984). Villet and Dalziel (1972) used the sheep liver enzyme to determine the kinetic parameters in the direction of reductive carboxylation, while the kinetic parameters for the enzyme in both directions were determined by Berdis and Cook (1993) using *Candida utilis* 6PGDH.

The kinetic mechanism for 6PGDH has been proposed by several groups to be random on the basis of product and dead-end inhibition studies. Bellini *et al.* (1985) using human erythrocyte 6PGDH and Dalziel *et al.* (1986) using the enzyme from sheep liver have shown that NADPH is competitive with respect to NADP^+ but noncompetitive with respect to 6-phosphogluconate. Berdis and Cook (1993) using *Candida utilis* 6PGDH have shown that NADPH is competitive and noncompetitive with respect to both NADP^+ and 6PG, whereas Ru 5-P is competitive versus 6PG but noncompetitive with respect to NADP^+ . Product inhibition studies using CO_2 have not been previously reported.

The chemical mechanism (see Appendix 2) of 6-phosphogluconate dehydrogenase has also been proposed by Berdis and Cook (1993) using *Candida utilis* 6PGDH, in which an active site general base accepts the proton from the 3-hydroxyl group of 6-phosphogluconate in concomitant with the hydride transfer at C-3. The resulting 3-keto intermediate is decarboxylated to give the enol form of ribulose-5-phosphate. This is followed by tautomerization of the enol structure to the keto product with the assistance of a second residue acting as a general acid. The maximum velocity of the 6PGDH reaction is pH dependent, decreasing at low and high pH. However, the kinetic

mechanism is pH independent by comparing initial velocity patterns in the absence of products at different pH values.

Berdis and Cook (1993) also reported that the 2'-phosphate of NADP⁺ is critical for optimum productive binding to *Candida utilis* 6PGDH by carrying out pH kinetic studies using different dinucleotide substrates. All dinucleotide substrates having 2'-phosphate display a sequential mechanism. But NAD⁺ and 3'-NADP⁺ which do not have 2'-phosphate exhibit an apparently parallel initial velocity pattern. The very low V/K values measured with NAD and 3'-NADP must reflect a decreasing efficiency of the dinucleotide, for it, thus, in triggering the conformational change of the enzyme needed to be catalytically active.

The action mechanism has been recently proposed for lamb liver 6PGDH by Hunau, *et al.* (1993). The proposal is similar to that of Miller which is based on the following observations: (1) each active site of the enzyme is composed of amino acid residues from both subunits, and (2) the binding of an effector to one active site induces conformational changes involving both subunits co-operatively (Miller, *et al.* 1991). The two active sites of the enzyme, which is a dimer of equal subunits each containing one binding site for 6-phosphogluconate and NADP⁺, are on the opposite sides of the dimer molecule, whereas the 6PG binding site is made up of residues from both subunits. In the presence of 6PG, the covalent binding of one molecule of a NADP⁺ analogue to only one of the subunits hinders binding of adenylylate derivative to the other subunit. This indicates that the simultaneous binding of 6PG and a coenzyme analogue, through an intersubunit communication, induces asymmetry in the dimeric structure with the enzyme

exhibiting the half-site reactivity. The half-site reactivity of this enzyme is further confirmed by stopped-flow experiments (Topham, *et al.* 1986) which indicate the formation of only one NADPH molecule per enzymic dimer during the first turnover. The 6-phospho-3-keto-2-deoxygluconate (3kd6PG), formed from 6PG in the subunit which catalyzes the redox reaction, must be decarboxylated. A simpler (and likely) assumption is that decarboxylation is catalyzed by the same subunit, while 6PG is bound and oxidized by the other subunit. According to this proposal, the two equal subunits reverse their role during each turnover of oxidative decarboxylation. Each subunit alternately catalyses the redox and decarboxylation reactions during catalysis. There is a fluid functional asymmetry triggered by intersubunit communications and the enzyme exhibits positive co-operativity. The validity of this hypothesis could be tested by determining whether the release of NADPH precedes or follows the decarboxylation. An alternative, less likely, hypothesis foresees the dimer with one permanently catalytic subunit and the other with only a regulatory role: in this case the turnover number would be lower.

Materials and Methods

Materials:

D-glucose was obtained from Caledon Laboratories Ltd. Malt Extract Broth was from Oxiod. Acrylamide, N,N'-methylene-bis-acrylamide, dithiothreitol(DTT), glycerol and glycine were from Aldrich Chemical Company, Inc. Milwaukee, USA. Oxidized form of β -nicotinamide adenine dinucleotide phosphate (NADP⁺), 6-phosphogluconic acid (6PG), D-ribulose-5-phosphate(Ru 5-P), phenylmethyl sulphonyl fluoride(PMSF), SDS molecular weight markers, N,N,N,'N'-tetramethylethylenediamine(TEMED), glass beads (0.45-0.50 mm) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Chemical Company, St Louis. Vitamins (d-biotin, myo-inositol, nicotinic acid, and D-pantothenic acid) were also from Sigma. Reduced form of β -nicotinamide adenine dinucleotide phosphate (NADPH) was from United States Biochemical Corporation. Ohio, USA. Sephacryl S-300 (high resolution) and Agarose-NADP⁺ (type 3)(AG-NADP) were from Pharmacia Biotech. DE-52 was from Whatman. Matrex Red Gel A was from Amicon Corp. Sodium dodecyl sulphate(SDS), ammonium sulphate and sodium dihydrogen orthophosphate were obtained from BDH Chemicals. Crystalline bovine albumin was from Miles Laboratories, Inc. Bromophenol blue, potassium phosphate were from Fisher Scientific Company and Coomassie brilliant blue R-250 was supplied by Eastman Kodak Co. Ammonium persulfate was purchased from Anachemia. Other reagent grade chemicals and biochemicals used in this study were purchased from Aldrich Chemical Company, Inc. or Sigma Chemical Company.

Yeast culture:

The *S. pombe* yeast, NCYC 132 S₂-2, was maintained by periodic transfer to 2.0% malt extract broth at room temperature (Tsai, *et al.*, 1987). The minimal salt medium EMM2 (Mitchison 1970) was modified to contain 0.2% (w/v) D-glucose as only carbon source at pH 5-6. Batch cultures (500 ml) of the fission yeast were grown for 36 hours in 2-litre Erlenmeyer flasks with constant shaking at 150 rpm, 30 °C (Tsai, *et al.*, 1989), in a G-25 environment shaker (New Brunswick scientific Co., New Brunswick, NJ). Cells were harvested by centrifugation carried out at 0 °C, 5000 rpm for 10 min.

Preparation of cell-free extract:

One gram of cells in 3.0 ml of 20 mM Tris-phosphate buffer, pH 7.0, containing 0.20 mM PMSF, 5.0 mM DTT and 1.0 mM EDTA, was mixed with approximate 5 g of acid-washed glass beads (0.45-0.50 mm) and homogenized by a prechilled cell homogenizer MSK (Braun) at 4000 rpm for 2 min with intermittent CO₂ gas as a coolant. The homogenate was extracted by using minimal volume of the same buffer and then centrifuged (Sorval RC2-B) at 10000 rpm for 20 min twice to remove the cell debris. The supernatant was used as cell-free extract.

Purification of 6-phosphogluconate dehydrogenase:

Ammonium sulphate fractionation. Ammonium sulphate was added slowly to the yeast extract whilst stirring to 30% saturation. The precipitate was removed by centrifugation at 10000 rpm for 30 min. The supernatant then was saturated by addition

of ammonium sulphate up to 70% followed by gently stirring for 1 hour to precipitate the desired proteins. After centrifugation at 10000 rpm for 30 min., the supernatant was discarded and the 6PGDH-containing pellet was resuspended in 15 ml of 20 mM phosphate buffer, pH 7.0.

Sephacryl S-300 chromatography. The protein solution was loaded onto a Sephacryl S-300 column (2.6x30 cm) equilibrated with 20 mM phosphate buffer, pH 7.0. The enzyme was eluted with same buffer at a flow rate of 40 ml/hour. The fractions (5 ml/fraction) were collected and assayed for 6PGDH activity. The peak fractions were pooled and concentrated in dialysis bag subject to polyethylene glycol(PEG). The enzyme solution then was dialysed against 1000 ml of 20 mM phosphate buffer for 2 hours.

Anionic exchange chromatography. The dialysed solution was applied to a DE-52 column (2.6x25 cm) equilibrated with 20 mM phosphate buffer and the enzyme was eluted with a linear gradient of 0.02-1 M phosphate buffer, pH 7.0, at a flow rate of 25 ml/hour. Fractions containing the enzyme were collected and pooled. The pooled enzyme solution was dialysed against 500 ml of 20 mM phosphate buffer, pH 7.0, for 2 hours.

Matrex Red gel chromatography. The enzyme solution from DE-52 column was loaded onto the Matrex Red gel column(0.9x12 cm) equilibrated with 20 mM phosphate buffer, pH 7.0. The column was washed with two 10 ml aliquots of the same buffer and then the enzyme was eluted with a linear gradient of 0-1.0 M KCl in 20 mM phosphate buffer, pH 7.0. Fractions (2 ml) were collected at a flow rate of 20 ml/hr. and the tubes containing highest 6PGDH activity were combined and dialysed for two hours against 500 ml of 20 mM phosphate buffer, pH 7.0.

Agarose-NADP⁺(type 3) chromatography. The active enzyme solution from last step after dialysing was applied onto an AG-NADP⁺ column (0.9x4.7 cm). The column was thoroughly washed with the buffer solution and the absorbed 6PGDH was eluted with 50 ml of 20 mM phosphate buffer, pH 7.0, containing a linear gradient (0-3 mM) of NADP⁺. Fractions(1 ml) were collected at a flow rate of 10-12 ml/hour and the enzymically active fractions were pooled to be dialysed against the same buffer. NADP⁺ was removed by 24 hour dialysis with three changes of 1.0 L buffer solution. The purified enzyme was used for structural characterization and kinetic studies.

Enzyme assays:

All enzymic assays (Tsai, *et al.* 1992) were carried out in a Varian Cary (model 3E) spectrophotometer at room temperature. The reaction was started by the addition of 10 μ l enzyme preparation to the reaction mixture (1 ml) consisting of 50 mM tris-HCl buffer pH 7.0, 1.0 mM NADP⁺ and 2.5 mM 6-phosphogluconate. Rates of NADP⁺ reduction were followed at 340 nm ($\epsilon_{340}=6.22 \text{ cm}^{-1}\text{mM}^{-1}$). One unit of enzyme activity (U) is defined as that amount of enzyme which yields 1 μ mole of NADPH per minute at 25 °C. The specific activity is expressed as units of enzyme activity per mg. protein.

Protein assay:

Protein concentrations were determined by using a rapid and reproducible method described by Bradford(Bradford, 1976). Coomassie Brilliant Blue-R was used as a dye ligand for proteins. The binding of the dye to protein causes a shift in the absorption

maximum of the dye from 465 to 595 nm. The absorbance of protein-dye complex at 595 nm was measured. Crystalline bovine albumin was used as the standard and the standard curve was constructed by using bovine albumin in the concentration range of 10 µg to 100 µg. All measurements were taken 2 minutes after the mixing and before 30 minutes of complexation.

Absorption spectrum of the purified 6PGDH was recorded using a Varian Cary (model 3E) spectrophotometer at room temperature. The extinction coefficient at 280 nm was calculated according to the equation:

$$\epsilon_{280 \text{ nm}} = \text{concentration}/A_{280}$$

Isoelectric focusing of 6PGDH:

Isoelectric focusing was carried out by using 110 ml of Ampholine 8100 column (LKB). The solutions were made as following:

Cathode solution: 0.6 ml monoethanolamine, 21 ml distilled water, 18 g sucrose

Anode solution : 0.1 ml phosphoric acid, 9.9 ml distilled water

Ampholine working solution: 2.5 ml of ampholines pH 3.5-10,

7.5 ml distilled water

Heavy solution: 7.5 ml ampholine working solution, 34.5 ml distilled water,

28 g sucrose

Light solution: 2.5 ml ampholine working solution, 57.5 ml distilled water

The cathode solution was introduced at the bottom and anode solution at the top of the column. The purified 6PGDH was added in the middle of the column. The pH

gradient was established in 0-60% sucrose gradient with 1% ampholines ranging from pH 3.5-10. The preparation was focused for 14 hours at 4°C using the Ortic power supply at 450 volts. At the end of the run, the power was switched off and the cathode solution was drained first (about 15 ml). The dense electrode solution was eluted out of the column at a flow rate of 1 ml/min. The fractions (1 ml) were collected for the determination of pH and the enzymic activity.

Molecular weight determination of 6PGDH:

The native molecular weight of 6PGDH was determined *via* Sephacryl S-300 gel filtration with a column of length 45 cm and diameter 2.5 cm. The elution buffer (pH 7.0) consists of 20 mM KH_2PO_4 , 100 mM KCl, 1 mM EDTA and 1 mM DTT. An 1 ml aliquot of the purified 6PGDH (2 units/ml) was loaded onto the column and 2 ml fractions were collected at a flow rate of 40 ml/hr. The enzymic activity was determined under optimal assay conditions. The standard proteins used are G6PDH (258,000 Da) from torula yeast, bakers yeast AldDH (200,000 Da), bakers yeast AlcDH (150,000 Da) and hemoglobin (62,000 Da) from beef blood. Their elution peaks were determined by either dehydrogenase assays at 340 nm, or absorbance at 405 nm for hemoglobin. The native molecular weight of 6PGDH was determined from a plot of elution volume vs. log. mol. wt. of the standard proteins.

Sodium dodecyl sulfate (SDS) gel electrophoresis:

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) in

0.1% (w/v) SDS was performed in a 1.0 slab gel electrophoresis apparatus (Bio-Rad Laboratories) using 10% separating gel and 5% stacking gel (Laemmli, U.K. 1970). The subunits molecular weight of the purified 6PGDH was determined from a plot of relative mobility (Rf) vs. molecular weight of the standard proteins.

1. Preparation of stock solutions

A. 30% acrylamide mix solution:

29.2 g acrylamide in 100 ml deionized water

0.8 g bisacrylamide

B. Separating (lower) gel buffer 100 ml:

18.2 g Tris, pH 8.8 (1.5 M Tris-HCl)

0.4% SDS, (0.4 g/100 ml)

C. Stacking (upper) gel buffer 100 ml:

6 g Tris, pH 6.8 (0.5 M Tris-HCl)

0.4% SDS, (0.4 g/100 ml)

D. Running Buffer, pH 8.3:

3 g/l Tris base

14.4 g/l Glycine

5 g/l SDS

E. 10% SDS: 10 g/100 ml

Other solutions were freshly prepared as follows:

10% ammonium persulfate

sample buffer (5 ml):

Glycerol:	0.75 ml
10% SDS:	2.25 ml
Tris base (0.5 M, pH 6.8):	1.24 ml
β -mercaptoethanol:	0.37 ml
3.8% bromophenol blue:	0.39 ml

TEMED (N,N,N',N',-Tetramethyl-ethylenediamine): commercial stock.

2. Preparation of 10% separating (lower) gel

The composition of the separating gel is shown below:

H ₂ O	15.9 ml
30% acrylamide mix:	13.3 ml
1.5 M Tris (pH 8.8):	10.0 ml
10% SDS:	0.4 ml
10% APS:	0.4 ml
TEMED:	0.016 ml

The above solution (40 ml) was poured into the gap between the glass plates by touching the side of the Erlenmeyer flask to the back gel plate. Then water saturated n-butanol was layered on the top of lower gel before it polymerizes. The polymerization of the lower gel approximately took one hour (until two layers were observed above the polymerized gel). The butanol and water layers were washed away using deionized water and the glass was carefully dried using a paper towel.

3. Preparation of 5% stacking (upper) gel.

The composition of upper gel is:

H ₂ O	5.5 ml
30% acrylamide mix:	1.3 ml
0.5 M Tris (ph 6.8):	1.0 ml
10% SDS:	0.08 ml
10% APS:	0.08 ml
TEMED:	0.008 ml

The solution (total 8 ml) was added on the top of the running gel and the sample comb was placed into the upper gel. After polymerization of the upper gel (about 1 hour), the sample comb was removed and the wells were immediately washed with deionized water to remove any unpolymerized acrylamide.

4. Sample preparation

The protein standards and the purified enzyme (about 10 µg for each lane) were mixed with equal volume of the sample buffer. All samples were placed in Eppendorf tubes and heated for 4 minutes in a boiling water bath. After heating, 50 µl of each of the samples was loaded into the bottom of the wells by using a Hamilton microliter syringe.

5. Running the gel

The gel was mounted in the electrophoresis apparatus and the Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. The gel was run under constant current conditions (16 mA for stacking gel and 25 mA for separating gel) by using an Ortec 4100 power supply at room temperature. Run time was between 4-5 hours.

6. Fixing, staining and destaining the gel

After electrophoresis, the gel was carefully removed from the electrophoresis

apparatus and fixed in 10% acetic acid (v/v), 30% (v/v) methanol for 2 hours, followed by washing with 10% methanol and 5% acetic acid three times. The gel was then stained for 2 hours in the staining solution consisting of 0.25% Coomassie Brilliant Blue R, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was then thoroughly destained in fixing solution (10% acetic acid (v/v), 30% (v/v) methanol) for overnight by changing the destaining solution several times until a good contrast between bands and background was observed.

Effects of metal ions on the activity of 6PGDH:

Effects of metal ions on the 6PGDH activity were investigated by measuring the initial rates of the reactions at various metal ion concentrations, from 0-10 mM. The reaction mixture (1.0 ml) contained 2.5 mM 6-phosphogluconate, 1 mM NADP⁺, varied concentrations of metal ions and 20 µg/ml 6PGDH in 50 mM tris-HCl buffer, pH 7.0.

pH-Rate profile of 6PGDH:

The pH profile of 6PGDH was obtained by measuring the rates of NADPH formation at 340 nm in the pH range of 4-10. The initial rates of the reaction were measured in the presence or absence of metal ions. The reaction mixture contained 2.5 mM 6-phosphogluconate, 1 mM NADP⁺ and 2 µg purified 6PGDH in tris-HCl buffer solution. All buffers were prepared with ultrapure deionized water from a Millipore Milli-Q water purification system. The pH values were measured using a Fisher Accumet model 825 MP pH meter standardized with Anachemia buffers.

Kinetic studies:

Initial rates of NADP⁺ reduction were followed at 340 nm ($\epsilon_{340}=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) in a Varian Cary (model 3E) spectrophotometer at room temperature. The reaction mixtures (1.0 ml) contained 2 μg of the purified enzyme, a varied concentration of 6PG (0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2.5 mM), NADP⁺ (0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 1 mM) in 50 mM tris-HCl buffer pH 7.0. All kinetic assays were performed in three different preparations and two determinations were carried out for each test from each enzyme preparation. All data are the mean of two determination of different preparations.

The rates (v) of NADPH formation were analyzed according to the bisubstrate equation (Cleland, 1963):

$$v = \frac{V[A][B]}{[A][B] + K_b[A] + K_a[B] + K_{ia}K_b} \quad (1)$$

where V is the maximum velocity, K_a and K_b are Michaelis Menten constants for NADP⁺ (A) and 6PG (B) respectively. K_{ia} is the inhibition constant for NADP⁺.

The inverse of the equation (1) gives:

$$\frac{1}{v} = \left[1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ia}K_b}{[A][B]} \right] \frac{1}{V} \quad (2)$$

The plots $1/v$ against $1/[NADP^+]$ at a fixed concentration of 6PG will be linear with

$$\text{slope} = \frac{1}{V} \left(K_a + \frac{K_{ia}K_b}{B} \right) \quad (3)$$

and

$$intercept = \frac{1}{V} \left(1 + \frac{K_b}{[B]} \right) \quad (4)$$

The secondary plots of the slopes and intercepts against $1/[6PG]$ will yield V , K_{ia} , K_a , and K_b .

pH kinetic studies:

Kinetic studies on pH effects were performed in order to obtain information concerning the participation of ionizable groups of 6PGDH from the fission yeast in the catalysis. All kinetic assays were carried out using a Varian Cary (model 3E) spectrophotometer equipped with a strip-chart recorder to measure the appearance of NADPH at 340 nm. Reaction cuvettes were 1 cm in path length and 1.0 ml in volume. The temperature was maintained at room temperature and pH range was 5-8 in 50 mM tris-HCl buffer solutions. Initial velocity data were obtained as a function of pH under conditions in which one substrate was varied while the other was maintained at a fixed unsaturating concentration.

In order to obtain information concerning the effects of metal ion on the chemical mechanism of *S. pombe* 6PGDH, the pH dependence of kinetic parameters was also determined in the presence of Mn^{2+} . The assay conditions were same as the above (in the absence of Mn^{2+}). The pH range was from 6 to 8 using 50 mM tris-HCl buffer solutions and the concentration of Mn^{2+} was 1 mM.

All assay data were processed according to the bisubstrate equations described by Cleland (1963). Reciprocal initial velocities were plotted against reciprocal substrate concentrations.

Product inhibition:

The product inhibition studies were carried out to investigate the kinetic mechanism of the fission yeast 6PGDH. All assays were performed by using a Varian Cary (model 3E) spectrophotometer equipped with a strip-chart recorder to measure the appearance of NADPH at 340 nm in 50 mM tris-HCl buffer, pH 7.0. The temperature was maintained at room temperature (25 °C). The experimental protocol for a bisubstrate enzyme involves holding one substrate constant at nonsaturating level and varying concentrations of the other substrate. The product was added at several fixed concentrations (including zero). The concentrations of NADPH were 0, 0.05, 0.1, 0.2 mM. The concentrations of 6PG were 0, 0.25, 0.5, 1 mM and CO₂ (CO₃²⁻) concentrations were 0, 30, 45, 60 mM. The product, CO₂, was added as bicarbonate with CO₂ concentration calculated according to the Henderson-Hasselbach equation using a pK value of 6.4.

The inhibition patterns can be obtained constructing Double-reciprocal plots of initial velocity (*v*) versus substrate concentrations and may be further analyzed by replots of the slopes and intercepts of the reciprocal plots versus the inhibitor concentrations. The product inhibition patterns described by Alberty (1958) can be used to differentiate bisubstrate sequential mechanisms. The Ordered mechanism should exhibit one competitive and three noncompetitive patterns, and Random mechanism should have two, three, or four competitive patterns depending on whether the abortive complexes form. The reciprocal rate equations of product inhibition for four major bisubstrate mechanisms were described by Rudolph (1979).

In Random mechanism with abortive complex formation, the rate equation in the

presence of product P becomes:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_a}{V_{\max}A} + \frac{K_b}{V_{\max}B} \left(1 + \frac{P}{K_{ip}}\right) + \frac{K_{ia}K_b}{V_{\max}AB} \left(1 + \frac{P}{K_{ip}}\right) \quad (5)$$

This equation predicts that P will be competitive and noncompetitive with substrate B and substrate A. Thus at a nonsaturating concentration of B, the product P will act as a noncompetitive inhibitor with respect to substrate A as both the slope and the intercept are affected. There will be a series of converging lines intersecting to the left of the ordinate on a double reciprocal plot. In the secondary plots of slopes and intercepts taken from the primary plot against the P concentrations, the intercepts on the [P]-axis will give $K_{i_{\text{slope}}}$ and $K_{i_{\text{intercept}}}$. When substrate A is held at a nonsaturating level and substrate B is varied, the product P will act as a competitive inhibitor with respect to substrate B as only the slope is affected. There will be a series of converging lines intersecting to the 1/v-axis on a double reciprocal plot. In the secondary plot of slopes taken from primary plot against P concentrations, the intercept on the [P]-axis will give $K_{i_{\text{slope}}}$.

In the presence of Q, the rate equation is given:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_a}{V_{\max}A} \left(1 + \frac{Q}{K_{iq}}\right) + \frac{K_b}{V_{\max}B} + \frac{K_{ia}K_b}{V_{\max}AB} \left(1 + \frac{Q}{K_{iq}}\right) \quad (6)$$

The Eq.(6) predicts that the product Q will be competitive and noncompetitive versus substrate A and substrate B respectively. When B is fixed at a nonsaturating concentration and A is varied, Q acts as a competitive inhibitor with respect to A as only the slope is

affected. $K_{i_{\text{slope}}}$ can be obtained by same treatment as above. When A is fixed and B is varied, Q becomes noncompetitive against B as both the slope and the intercept are affected. $K_{i_{\text{slope}}}$ and $K_{i_{\text{intercept}}}$ can be obtained.

The analogous equation is obtained in the presence of the third product R:

$$\frac{1}{v} = \frac{1}{V_{\max}} + \frac{K_a}{V_{\max}A} + \frac{K_b}{V_{\max}B} \left(1 + \frac{R}{K_{ir}}\right) + \frac{K_{ia}K_b}{V_{\max}AB} \left(1 + \frac{R}{K_{ir}}\right) \quad (7)$$

In analogy to the product P, R behaves as a mixed-type inhibitor with respect to substrates. At nonsaturating concentrations of the other substrate, R is competitive and noncompetitive against substrate B and A respectively. Product inhibition constants can be obtained for R using same method described above.

RESULTS

Purification of 6PGDH. 6-Phosphogluconate dehydrogenase was purified to homogeneity from *S. pombe* with 463-fold purification (specific activity of 39.4 units/mg) and a total recovery of 27.5%. The purification procedure described above is similar to that which was used to purify the gluconate pathway enzymes from *S. pombe* by Tsai, *et al.*(1995). The purification steps are summarized in Table 4. It represents a considerable modification of Tsai's method, mainly by replacement of carboxymethyl cellulose ion-exchangers(CM-52) with anionic exchanger (DE-52). The procedure started with ammonium sulphate fractionation at 30-70% saturation, including Sephacryl S-300 gel filtration, anion-exchange (DE-52) chromatography, dye ligand(Matrix Red gel) chromatography, and affinity (AG-NADP⁺) chromatography. Fig.4 to Fig.7 show the elution profiles of the fission yeast enzyme from Sephacryl S-300 gel chromatography, anion-exchange chromatography, Matrix Red gel chromatography, and affinity chromatography respectively. The Sephacryl S-300 gel filtration was used to separate the enzyme from the majority of the cell proteins. Reasonable recovery (78.1%) and resolution (4.9-fold) were achieved at this step (Fig.4). Since the isoelectric pH of the yeast 6PGDH is about 5.6, the enzyme was retained by anionic exchanger at pH 7.0 and then eluted by the linear gradient (0.02-1.0 M) phosphate buffer (Fig. 5). Red A matrix gel chromatography is an effective step for purifying the yeast 6PGDH giving 8-fold purification. The enzyme retaining on the matrix gel was eluted with a linear gradient of 0-1.0 M KCl in 20 mM phosphate buffer(Fig. 6). The last trace of contaminant proteins was removed by an affinity chromatography. The enzyme was bound to the AG-NADP⁺

Table 4. Summary of purification of 6PGDH from *S. pombe*. The enzyme was purified from yeast cells grown in 0.2% D-glucose batch culture for 36 hours.

Purification steps	Volume (ml)	Total enzyme (unit)	Protein (mg/ml)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Cell extract	70.0	47.6	7.99	-	0.0851	-
(NH ₄) ₂ SO ₄ fractionation	15	43.6	26.2	91.5	0.111	1.3
Sephacryl S-300	20	37.2	4.46	78.1	0.417	4.9
DE-52	15	28.9	0.750	52.3	2.57	30.2
Matric Red A	10	19.3	0.0951	40.6	20.3	238.4
AG-NADP ⁺ (type 3)	5	13.1	0.0665	27.5	39.4	463.2

Fig. 4. Sephacryl S-300 filtration chromatography in 20 mM phosphate buffer pH 7.0. Fractions (5 ml/fraction) were collected at a flow rate of 40 ml/hr. and the enzymic activity was measured by the standard method.

— enzyme activity (u/ml),

..... A₂₈₀

Fig.5. Elution profile of 6PGDH from DE-52 anion exchanger with a linear gradient of 0.02-1.0 M phosphate buffer, pH 7.0. Fractions (5 ml/fraction) were collected at a flow rate of 25 ml/hr. and the 6PGDH activity was measured by the standard method.

—— enzyme activity (u/ml),

..... phosphate (M)

Fig.6. Elution profile of 6PGDH from Matrex Gel Red A column chromatography with a linear gradient of 0-1.0 M KCl in 20 mM phosphate buffer, pH 7.0. Fractions (5 ml/fraction) were collected at a flow rate of 20 ml/hr. and the 6PGDH activity was measured by standard method.

—— enzyme activity (u/ml).

..... KCl (M)

Fig.7. Elution profile of 6PGDH from AG-NADP⁺ affinity column with a linear gradient of 0-3 mM NADP⁺ in 20 mM phosphate buffer, pH 7.0. Fractions (2 ml/fraction) were collected at a flow rate of 10 ml/hour.

———— enzyme activity (u/ml).

..... NADP (mM)

Fig. 8. SDS-polyacrylamide gel electrophoresis of 6PGDH from the fission yeast. Lane 1, protein standards; lane 2, the enzyme from Matrix Red gel chromatography; lane 3 and 4, the enzyme from AG-NADP⁺ affinity chromatography with 20 μ g/ml and 10 μ g/ml, respectively.

Fig. 9. Isoelectric Focusing of purified 6PGDH. A pH gradient was established with Ampholines in sucrose and run as described in the method section. The enzyme activity was measured for the peak fractions.

—— enzyme activity (u/ml),

..... pH

column and subsequently released by the use of the NADP⁺ gradient (0-3 mM NADP⁺) (Fig. 7). The purified enzyme is electrophoretically homogenous. This is confirmed by a single band in SDS-polyacrylamide gel electrophoresis (Fig. 8). The isoelectric point of the purified 6PGDH from *S. pombe* was determined by isoelectric focusing to be 5.6 (Fig. 9).

Molecular weight determination. The native molecular weight of *S. pombe* 6PGDH was determined using Sephacryl S-300 gel filtration. By plotting the log molecular weight of four standard proteins against their elution volumes, the mean value was measured to be $152,000 \pm 12,000$ Da (Fig. 10). The relative mobility values (Rf) of the standard proteins from SDS-polyacrylamide gel electrophoresis were plotted vs their molecular weights. The subunit molecular weight of the purified 6PGDH was measured from the plot to be $38,000 \pm 1,900$ Da (Fig. 11). From the two estimations it can be deduced that the fission yeast 6PGDH consists of 4 subunits.

Effects of metal ions and pH on 6PGDH. The effects of metal ions on the enzyme activity were investigated by measuring the initial rates of the reaction in the presence of metal ions. Five metal ions, Mg²⁺, Mn²⁺, Co²⁺, Ni²⁺ and Zn²⁺, were tested for their effects on the enzyme at the concentrations ranging from 0.2 to 10 mM. All assays were carried out by the standard method and 20 µg/ml of the enzyme was used (Fig. 12).

The pH profile of 6PGDH was obtained by monitoring the initial rates of the enzymic reaction at pH values ranging from 4 to 10, without additions of metal ions. The optimum pH for 6PGDH in the absence of metal ions is 7.0, and about 15% of enzyme activity was detected below pH 4 or above pH 10. The pH profile of 6PGDH in the

Fig.10. Determination of the molecular weight of purified 6PGDH by Sephacryl S-300 gel filtration. Standard proteins are: G-6PDH(258,000), AldDH(200,000), AlcDH(150,000) and hemoglobin(62,000). The elution buffer consists of 20 mM phosphate, 100 mM KCl, 1 mM EDTA and 1 mM DTT at pH 7.0. The constant flow rate was maintained to be 40 ml/hr.

The eluting volume of 6PGDH from *S.pombe* was 40 ml.

Fig. 11. Determination of subunit molecular weight of the yeast 6PGDH by SDS-polyacryamide gel electrophoresis. The standard proteins are: egg albumin(45,000), pepsin(34,7000), carbonic anhydrase(29,000) and egg lysozyme(14,300). The relative mobility of the purified 6PGDH corresponds to 0.38.

Fig. 12. The effects of metal ions on 6PGDH activity. The reaction rates were measured at 340 nm, pH 7.0. The purified 6PGDH(20 μ g/ml) was used. Concentrations of NADP⁺ and 6PG are 1 mM and 2.5 mM, respectively.

Fig.13. pH profiles of 6PGDH. The enzyme activity was measured at pH 4.0-10 in the absence of metal ions (— without Mn²⁺). The enzyme activity in the presence of 1.0 mM Mn²⁺ (.... with Mn²⁺) was assayed at pH 6.0-10.0. Concentrations of NADP⁺ and 6PG are 1 mM and 2.5 mM respectively.

presence of Mn^{2+} was also determined at 1.0 mM Mn^{2+} which shifts the optimum pH of the 6PGDH reaction from 7.0 to 8.0. Fig. 13 shows the pH profiles of 6PGDH.

Kinetic studies of the yeast 6PGDH. Kinetic studies with two substrate reactions were carried out to evaluate the kinetic parameters of 6PGDH from *S. pombe*. These data are listed in Tables 5 and 6. In order to obtain information concerning the ionizable residues involved in the catalysis by the fission yeast 6PGDH, the pH dependence of kinetic parameters was determined. The effect of metal ions on the kinetic parameters of the enzyme was also investigated in the presence of Mn^{2+} . Initial velocity patterns were obtained at pH 5-8 under conditions in which $NADP^+$ was varied at different fixed levels of 6PG. The initial velocity patterns intersect to the left of the ordinate, suggesting a sequential mechanism. All patterns obtained at different pH values in the presence of Mn^{2+} are similar to those in the absence of Mn^{2+} . This indicates that Mn^{2+} does not change the kinetic mechanism of the 6PGDH catalysis but exert an enhancing effect on the activity of the enzyme. The maximum velocity (V_{max}) and Michaelis constant (K_m) are pH dependent. All kinetic parameters at various pH values are also listed in Table 5 (in the absence of Mn^{2+}) and Table 6 (in the presence of Mn^{2+}).

Table 5. The kinetic parameters for 6-phosphogluconate dehydrogenase from *S. pombe* in the absence of Mn^{2+} at different pH.

pH	V_{max} ($\mu M/min$)	K_a (μM)	K_b (μM)	K_{ia} (μM)	V_{max}/E_t (S^{-1})
5	0.494	39.5	28.0	27.6	0.623
6	0.526	32.5	30.5	21.9	0.664
6.5	0.524	26.0	29.3	22.3	0.662
7	0.595	24.3	32.6	30.7	0.751
7.5	0.627	24.5	57.0	25.0	0.792
8	0.587	18.0	179	21.2	0.741

Kinetic parameters were estimated from double reciprocal plots of 6PGDH catalysis at varied concentrations of $NADP^+$ and several different fixed concentrations of 6PG in the absence of Mn^{2+} . V_{max} , K_a , K_b , K_{ia} and V_{max}/E_t are the maximum velocity, Michaelis constants for $NADP^+$ and 6-phosphogluconate, inhibition constant for $NADP^+$ and turnover number respectively.

Table 6. The kinetic parameters for 6-phosphogluconate dehydrogenase from *S. pombe* in the presence of Mn^{2+} at different pH.

pH	V_{max} ($\mu M/min$)	K_a (μM)	K_b (μM)	K_{ia} (μM)	V_{max}/E_t (S^{-1})
6.0	0.472	24.5	24.2	82.4	0.598
6.5	0.537	26.6	29.4	35.8	0.680
7.0	0.633	20.5	25.3	60.2	0.802
7.5	0.924	28.2	39.1	38.4	1.171
8.0	0.608	14.5	111.3	29.5	0.770

Kinetic parameters were estimated from double reciprocal plots of 6PGDH catalysis at varied concentrations of $NADP^+$ and several different fixed concentrations of 6PG in the presence of Mn^{2+} . V_{max} , K_a , K_b , K_{ia} and V_{max}/E_t are the maximum velocity, Michaelis constants for $NADP^+$ and 6-phosphogluconate, inhibition constant for $NADP^+$ and turnover number respectively.

Table 7. Kinetic pK values for 6-phosphogluconate dehydrogenase from *S. pombe* in the absence of Mn^{2+} .

parameter	acid side (pK $\pm$ SE)	basic side (pK $\pm$ SE)
V/Et	6.2 $\pm$ 0.2	
V/K _{NADP}	6.5 $\pm$ 0.1	
V/K _{6PG}		8.0 $\pm$ 0.2

Note: The pK values are calculated from the plots of logarithms of kinetic constants against pH described by Tipton and Dixon (1979). The plot of logV gives the dissociation constants for the enzyme-substrate complex, while the plots of logV/K_m give the dissociation constants for the free enzyme.

Fig.14a. Double reciprocal plot for 6PGDH catalysis. The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 5.0. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentrations are 0.1, 0.2, 0.5, 1.0 mM from top to the bottom.

- $y = 2.838 + 0.0778x,$
- ◆ $y = 2.367 + 0.0695x,$
- $y = 2.251 + 0.0615x,$
- ▲ $y = 2.072 + 0.0574x.$

Fig. 14b. Secondary plots of slopes and intercepts vs 1/6PG, pH 5.0.
The enzyme concentration is 2 $\mu\text{g/ml}$.

- intercepts vs 1/6PG: $y = 2.023 + 0.0799x$.
- slopes vs 1/6PG: $y = 0.0566 + 0.0220x$,

Fig.15a. Double reciprocal plots for 6PGDH catalysis. The enzyme concentration was 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 6.0. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentrations are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

• $y = 2.471 + 0.0749x,$

◆ $y = 2.204 + 0.0673x,$

■ $y = 2.047 + 0.0647x,$

▲ $y = 1.924 + 0.0633x.$

Fig. 15b. Secondary plots of slopes and intercepts vs $1/6PG$. pH 6.0.
The enzyme concentration is $2 \mu g/ml$.

- intercepts vs $1/6PG$: $y = 1.901 + 0.0580x$,
- slopes vs $1/6PG$: $y = 0.0618 + 0.00127x$.

Fig. 16a. Double reciprocal plots for 6PGDH catalysis. The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer pH 6.5. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentrations are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

• $y = 2.536 + 0.0639x,$

◆ $y = 2.294 + 0.0588x.$

■ $y = 2.132 + 0.0576x.$

▲ $y = 1.980 + 0.0521x.$

Fig. 16b. Secondary plots of slopes and intercepts vs 1/6PG, pH 6.5.
The enzyme concentration is 2 $\mu\text{g/ml}$.

- intercepts vs 1/6PG: $y = 1.975 + 0.0578x$.
- slopes vs 1/6PG: $y = 0.0515 + 0.00129x$.

Fig. 17a. Double reciprocal plots for 6PGDH catalysis. The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 7.0. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

- $y = 2.244 + 0.0569x$,
- ◆ $y = 1.901 + 0.0504x$,
- $y = 1.852 + 0.0456x$,
- ▲ $y = 1.708 + 0.0404x$.

Fig. 17b. Secondary plots of slopes and intercepts vs $1/6PG$, pH 7.0.
The enzyme concentration is $2\ \mu g/ml$.

- intercepts vs $1/6PG$: $y = 1.680 + 0.0548x$,
- slopes vs $1/6PG$: $y = 0.0407 + 0.00168x$.

Fig. 18a. Double reciprocal plots for 6PGDH catalysis. The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 7.5. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

• $y = 2.467 + 0.0607x,$

◆ $y = 2.114 + 0.0517x,$

■ $y = 1.834 + 0.0467x,$

▲ $y = 1.597 + 0.0377x.$

Fig. 18b. Secondary plots of slopes and intercepts vs 1/6PG, pH 7.5.
The enzyme concentration is 2 μg/ml.

- intercepts vs 1/6PG: $y = 1.594 + 0.0908x$.
- slopes vs 1/6PG: $y = 0.0390 + 0.00227x$.

Fig. 19a. Double reciprocal plots for 6PGDH catalysis. The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 8.0. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

- $y = 4.481 + 0.1043x$,
- ◆ $y = 3.365 + 0.0642x$,
- $y = 2.364 + 0.0447x$,
- ▲ $y = 1.885 + 0.0353x$.

Fig. 19b. Secondary plots of intercepts and slopes vs 1/6PG, pH 8.0.
The enzyme concentration is 2 $\mu\text{g/ml}$.

- intercepts vs 1/6PG: $y = 1.704 + 0.304x$,
- slopes vs 1/6PG: $y = 0.0306 + 0.00646x$.

Figure 20. pH Dependence of kinetic parameter V for the 6PGDH catalysis from *S. pombe*. The data were obtained at room temperature.

Figure 21. pH Dependence of kinetic parameter V/K_{NADP} for the 6PGDH catalysis from *S. pombe*. The data were obtained at room temperature.

Figure 22. pH Dependence of kinetic parameter V/K_{6PG} for the 6PGDH catalysis from *S. pombe*. The data were obtained at room temperature.

Fig 23a. Double reciprocal plots for 6PGDH catalysis in the presence of Mn^{2+} . The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 6.0. The concentration of Mn^{2+} was 1 mM. Initial velocities were measured at varied $NADP^+$ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

• $y = 2.589 + 0.0926x,$

◆ $y = 2.448 + 0.0758x,$

■ $y = 2.284 + 0.0616x,$

▲ $y = 2.069 + 0.0532x.$

Fig. 23b. Secondary plots of intercepts and slopes vs 1/6PG, pH 6.0.

The enzyme concentration is 2 $\mu\text{g/ml}$. The concentration of Mn^{2+} is 1 mM.

• intercepts vs 1/6PG: $y = 2.117 + 0.0512x$,

■ slopes vs 1/6PG: $y = 0.0518 + 0.00422x$.

Fig 24a. Double reciprocal plots for 6PGDH catalysis in the presence of Mn^{2+} . The enzyme concentration is 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 6.5. The concentration of Mn^{2+} was 1 mM. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

• $y = 2.397 + 0.0692x,$

◆ $y = 2.170 + 0.0592x,$

■ $y = 1.979 + 0.0541x,$

▲ $y = 1.895 + 0.0511x.$

Fig. 24b. Secondary plots of intercepts and slopes vs 1/6PG, pH 6.5.
The enzyme concentration is 2 $\mu\text{g/ml}$. The concentration of Mn^{2+} is 1 mM

- intercepts vs 1/6PG: $y = 1.864 + 0.0548x$,
- slopes vs 1/6PG: $y = 0.0496 + 0.00196x$.

Fig 25a. Double reciprocal plots for 6PGDH catalysis in the presence of Mn^{2+} . The enzyme concentration was 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 7.0. The concentration of Mn^{2+} was 1 mM. Initial velocities were measured at varied NADP⁺ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

- $y = 1.964 + 0.0564x$,
- ◆ $y = 1.814 + 0.0446x$,
- $y = 1.663 + 0.0366x$,
- ▲ $y = 1.598 + 0.0352x$.

Fig. 25b. Secondary plots of intercepts and slopes vs 1/6PG, pH 7.0.
The enzyme concentration is 2 $\mu\text{g/ml}$. The concentration of Mn^{2+} is 1 mM.

- intercepts vs 1/6PG: $y = 1.580 + 0.0399x$,
- slopes vs 1/6PG: $y = 0.034 + 0.00240x$.

Fig 26a. Double reciprocal plots for 6PGDH catalysis in the presence of Mn^{2+} . The enzyme concentration was 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 7.5. The concentration of Mn^{2+} was 1 mM. Initial velocities were measured at varied NADP^+ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

- $y = 1.508 + 0.0452x$.
- ◆ $y = 1.286 + 0.0416x$.
- $y = 1.167 + 0.0355x$.
- ▲ $y = 1.128 + 0.0289x$.

Fig. 26b. Secondary plots of intercepts and slopes vs 1/6PG, pH 7.5.
The enzyme concentration is 2 $\mu\text{g/ml}$. The concentration of Mn^{2+} is 1 mM.

- intercepts vs 1/6PG: $y = 1.082 + 0.0423x$,
- slopes vs 1/6PG: $y = 0.0305 + 0.00162x$.

Fig 27a. Double reciprocal plots for 6PGDH catalysis in the presence of Mn^{2+} . The enzyme concentration was 2 $\mu\text{g/ml}$ in 50 mM tris-HCl buffer, pH 8.0. The concentration of Mn^{2+} was 1 mM. Initial velocities were measured at varied NADP⁺ concentration at fixed 6PG concentration. The 6PG concentration are 0.1, 0.2, 0.5, 1.0 mM from the top to the bottom.

- $y = 3.387 + 0.0785x$,
- ◆ $y = 2.752 + 0.0492x$,
- $y = 2.067 + 0.0370x$,
- ▲ $y = 1.675 + 0.0282x$.

Fig. 27b. Secondary plots of intercepts and slopes vs 1/6PG, pH 8.0.

The enzyme concentration is 2 $\mu\text{g/ml}$. The concentration of Mn^{2+} is 1 mM.

- intercepts vs 1/6PG: $y = 1.646 + 0.183x$,
- slopes vs 1/6PG: $y = 0.0239 + 0.00541x$.

Product inhibition studies. The product inhibition patterns for 6PG catalysis were obtained in the direction of oxidative decarboxylation. All patterns were linear at nonsaturating concentration of the fixed substrate. With NADP⁺ as the variable substrate, NADPH is a competitive inhibitor and Ru 5-P is noncompetitive inhibitor to the 6PGDH catalysis at nonsaturating 6PG. With 6PG as the variable substrate, Ru 5-P is competitive inhibitor and NADPH is a noncompetitive inhibitor to the 6PGDH catalysis at nonsaturating NADP⁺. The pattern of CO₂ as a product inhibiting the 6PGDH catalysis was also obtained, in which CO₂ is competitive with respect to 6PG but noncompetitive with NADP. Fig. 28a, 32a, and 33a show these competitive inhibition patterns. Fig. 29a, 30a, and 31a show those noncompetitive inhibition patterns. All Fig.bs are the second plots of the inhibition patterns. Product inhibition constants calculated from those second plots are listed in Table 8.

Fig. 28a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of NADP^+ were varied in the presence of NADPH as an inhibitor at a fixed concentration of 6PG (1 mM). Each line corresponds to different NADPH concentration and these are (shown from top to bottom) 0.2, 0.1, 0.05, 0 mM. Enzyme concentration was 2 $\mu\text{g/ml}$. All assays at room temperature, pH 7.0.

Fig. 28b. Secondary plot for product inhibition of 6PGDH. The slopes obtained from the primary plot (Fig. 28a) were plotted against NADPH concentrations.

Fig. 29a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of NADP^+ were varied in the presence of Ru 5-P as an inhibitor at a fixed concentration of 6PG (1 mM). Each line corresponds to different Ru 5-P concentration and these are (shown from top to bottom) 1, 0.5, 0.25, 0 mM. Enzyme concentration was 2 $\mu\text{g/ml}$. All assays at room temperature, pH 7.0.

Fig. 29b. Secondary plots for product inhibition of 6PGDH. The slopes and intercepts obtained from the primary plot (Fig. 29a) were plotted against Ru 5-P concentrations.

- intercepts vs Ru 5-P
- slopes vs Ru 5-P

Fig. 30a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of NADP^+ were varied in the presence of CO_2 as an inhibitor at a fixed concentration of 6PG (1 mM). Each line corresponds to a different CO_2 concentration and these are (shown from top to bottom) 60, 45, 30, 0 mM. Enzyme concentration was 2 $\mu\text{g/ml}$. All assays at room temperature, pH 7.0.

Fig. 30b. Secondary plots for product inhibition of 6PGDH. The slopes and intercepts obtained from the primary plot (Fig. 30a) were plotted against CO_2 concentrations.

- intercepts vs CO_2
- slopes vs CO_2

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Fig. 31a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of 6PG were varied in the presence of NADPH as an inhibitor at a fixed concentration of NADP^+ (0.5 mM). Each line corresponds to a different NADPH concentration and these are (shown from top to bottom) 0.2, 0.1, 0.05, 0 mM. Enzyme concentration was 2 $\mu\text{g/ml}$. All assays at room temperature, pH 7.0.

Fig. 31b. Secondary plots for product inhibition of 6PGDH. The slopes and intercepts obtained from the primary plot (Fig. 31a) were plotted against NADPH concentrations.

- intercepts vs NADPH
- slopes vs NADPH

Fig. 32a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of 6PG were varied in the presence of Ru 5-P as an inhibitor at a fixed concentration of NADP^+ (0.5 mM). Each line corresponds to a different NADPH concentration and these are (shown from top to bottom) 1, 0.5, 0.25, 0 mM. Enzyme concentration was 2 $\mu\text{g}/\text{ml}$. All assays at room temperature, pH 7.0.

Fig. 32b. Secondary plots for product inhibition of 6PGDH. The slopes obtained from the primary plot (Fig. 32a) were plotted against Ru 5-P concentrations.

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Fig. 33a. Double reciprocal plot for product inhibition of 6PGDH catalysis. The concentrations of 6PG were varied in the presence of CO₂ as an inhibitor at a fixed concentration of NADP⁺ (0.5 mM). Each line corresponds to a different CO₂ concentration and these are (shown from top to bottom) 60, 45, 30, 0 mM. Enzyme concentration was 2 μg/ml. All assays at room temperature, pH 7.0.

donated by a general acid ($pK \sim 8$) from the enzyme to C-1, while the general base ($pK \sim 7.5$) again abstracts the proton from C-2.

Product inhibition. All product inhibition patterns are obtained at nonsaturating fixed levels of one substrate while varying concentrations of the other. The patterns predicted by the equations listed in the method section are linear inhibition. The competitive product inhibition is exhibited by products vs the reactants (NADPH vs NADP, Ru 5-P vs 6PG, and CO_2 vs 6PG). NADPH as the product is competitive with respect to NADP⁺ (Fig. 28a), but noncompetitive versus 6PG (Fig. 31a). The inhibition by the product Ru 5-P is competitive with respect to 6PG (Fig. 32a), but noncompetitive against NADP⁺ (Fig. 29a). This competitive inhibition suggests a rapid equilibrium mechanism at nonsaturating concentrations of the fixed substrate. It was found that CO_2 as the product also is competitive with respect to 6PG (Fig. 33a), but noncompetitive versus NADP⁺ (Fig. 30a). Results from the product inhibition studies show that the oxidative decarboxylation of 6-phosphogluconate catalyzed by 6PGDH from *S. pombe* proceeds via the random Bi-Ter mechanism with formation of abortive ternary complexes (EAR and EBQ) (see Scheme 1). Product inhibition patterns for bireactant mechanism were described by Rudolph (1979). No abortive complex is formed if the products are all competitive with respect to both substrates in random mechanism. The competitive patterns for NADPH against NADP⁺ and Ru 5-P versus 6PG indicate the abortive complex formation in the random mechanism. The pattern of CO_2 as a product inhibiting the 6PGDH catalysis has not been reported. This study suggests that in the direction of oxidative decarboxylation, CO_2 may be the first product released from the reaction. This

Fig. 34. Absorption spectrum of purified 6PGDH from *S. pombe* in 20 mM phosphate buffer at 25° C. Enzyme concentration was 50 $\mu\text{g/ml}$.

Table 8. Product inhibition constants for NADP⁺ dependent activity of 6-phosphogluconate dehydrogenase from *S.pombe*.

Product ^a	Varied Substrate ^b	K _i _{intercept} (mM)	K _i _{slope} (mM)
NADPH	NADP ⁺	-	9.0 x 10 ⁻³
	6PG	4.68 x 10 ⁻¹	8.71 x 10 ⁻²
Ru 5-P	NADP ⁺	2.57	1.20
	6PG	-	3.53 x 10 ⁻²
CO ₂	NADP ⁺	221	21.6
	6PG	-	7.35

Note: Inhibition constants are given in mM. When both constants are obtained, the noncompetitive pattern of product inhibition is observed. When either K_i_{ii} or K_i_s only is obtained, the uncompetitive or competitive pattern of inhibition is observed. Average errors for inhibition constants were determined from correlation coefficients of linearly regressed data to be K_i_{ii} ± 12%, K_i_s ± 10%.

- a Product concentrations were varied for NADPH: 0.2, 0.1, 0.05, 0 mM.
Ru 5-P: 1, 0.5, 0.25, 0 mM. CO₂: 60, 45, 30, 0 mM.
- b A fixed concentration of NADP⁺ is 0.5 mM, 6PG: 1mM.
- No value observed.

DISCUSSION

Purification of the fission yeast 6PGDH. 6-Phosphogluconate dehydrogenase is a key enzyme in the pentose phosphate pathway of D-glucose metabolism. This enzyme has been purified to various degree according to the different purpose by several groups. They are Silverberg and Dalziel from sheep liver(1975), Ripa and Signorini from *Candida utilis* (1975), Barbara, *et al* from human erythrocytes(1975), and Holden and Storey from tolerant insect (1993). In this study, the fission yeast 6PGDH was purified to homogeneity by an efficient purification scheme which gave a good yield of 27.5% and a final specific activity of 39.4 units/mg protein. The purification procedure for the enzyme is summarised in Table 4. It represents a considerable modification of Tsai's method for the purification of glucose dehydrogenase from *S. pombe* (Tsai, et al. 1995), mainly by replacement of cation exchangers (CE-52) with anion exchangers (DE-52). The first step employed is the ammonium sulfate fractionation which precipitated 6PGDH in the range of 35-70% ammonium sulfate saturation and reduced the crude extract to a manageable volume for subsequent gel filtration. It was reported that in this range of ammonium sulfate saturation, the fraction contained other enzymes of gluconate pathway, such as glucose dehydrogenase and gluconokinase (Tsai, et al. 1995). The elution profiles of the fission yeast 6PGDH (Fig. 4-Fig.7) show that each column chromatography is necessary to remove contaminant proteins. Matrex Red gel chromatography which is the most effective method for purifying the fission yeast enzyme yields about 8-fold purification. *S. pombe* 6PGDH requires NADP⁺ as an obligatory coenzyme. It was reported that the immobilized Cibacron F3G-A retarded NAD⁺-dependent dehydrogenases more effectively

than NADP⁺-dependent dehydrogenases, whilst Procion Red HE-3B retarded NADP⁺-dependent dehydrogenases more effectively than NAD⁺-dependent dehydrogenases (Dean & Watson, 1979). Indeed, the present experiment demonstrates that Matrex Gel Red was more effective than Cibacron Blue F3G-A for purifying the fission yeast enzyme. Affinity chromatography is an efficient and convenient mean of generating pure proteins. Agarose-NADP⁺ (type 3) in which NADP⁺ is bound to the agarose *via* the adenine C₈ with six carbon spacer (hexane) was used as the affinity column to purify the fission yeast 6PGDH, since the ribose hydroxyles of NADP⁺ could contribute to the enzyme binding. NADP⁺ formed a reversible complex with the enzyme to be purified and the complex was resistant to the feedstream of solvent and washing buffers. The enzyme retained on the column could easily be dissociated when 0.3 mM NADP⁺ gradient was used (Fig. 7). The last step gave about 2-fold purification of 6PGDH and removed all the contaminant proteins.

Characterization of S. pombe 6PGDH. The purified *S. pombe* 6PGDH was electrophoretically homogeneous. This is confirmed by a single band in SDS-polyacrylamide gel electrophoresis (Fig. 8). The final specific activity was 39.4 U/mg protein. This value compares favourably with the final specific activities calculated for purified 6PGDH from other sources. These include *Candida utilis* enzyme at 41.2 U/mg (Rippa & Signorini, 1975); sheep liver enzyme at 18.7 U/mg (Silverberg & Dalziel, 1975); and *Neurospora crassa* enzyme at 30.8 U/mg (Scott & Abramsky, 1975). The native molecular weight determined from Sephacryl S-300 gel chromatography was 152,000 ± 12,000 Da. The subunit molecular weight calculated from SDS-polyacrylamide

gel electrophoresis was $38,000 \pm 1,900$ Da. These results suggest that the enzyme from *S. pombe* is a tetramer. Most 6PGDH purified from other sources were reported to be dimers with native molecular weights varied from 94,000 to 108,000 (Silverberg & Dalziel, 1975; Pearse & Rosemeyer, 1975; Bridges & Wittenberger, 1975). The tetrameric enzymes were also reported for *Gluconobacter suboxydans* with native molecular weight of 175,000 (Adachi & Ameyama, 1982) and for tolerant insect with native molecular weight of 230,000 (Holden & Storey, 1994).

The purified *S. pombe* 6PGDH does not utilize NAD^+ as coenzyme. It does not contain detectable amounts of Zn^{2+} , Mn^{2+} or Mg^{2+} ; nor are these added bivalent metal ions essential for the activity. This result agrees with those reported from other sources. Further investigations on the effect of metal ions on the enzyme activity suggest that the enzymic activity was enhanced (40-50%) in the presence of Mn^{2+} at 1.0 mM. High concentrations (about 10 mM) of Mn^{2+} inhibit the enzyme (40-50% decrease) in the activity (Fig.12). The enzyme is indifferent to Mg^{2+} . Complete inhibition of the enzyme was achieved by zinc ion. It was reported that the enzyme was strongly inhibited by certain heavy metal ions (Zn^{2+} , Cd^{2+} , Cu^{2+}) (Bridges & Wittenberger, 1975; Niehaus & Richardson, 1993). It is interesting to note that the activity of the enzyme was enhanced in the presence of Co^{2+} at 4-5 mM but completely suppressed at high concentrations of Co^{2+} (about 10 mM). However, addition of Co^{2+} to the enzymic reaction mixture caused precipitation. A decrease in the enzymic activity was also observed in the presence of Ni^{2+} . These results suggest that *S. pombe* 6PGDH does not require metal ions for the activity, but some divalent cations exert enhancing effects on the enzyme.

The fission yeast enzyme is also strongly inhibited by iodoacetamide and phenyl glyoxal. This result implicates that the cysteine and arginine residues may be involved in the catalysis. Chemical modifications of the enzyme were carried out to determine the active amino acid residues which affect the enzymic activity (Appendix).

The pH optimum for catalytic activity based on single assays was found to be between 7 and 8, which was similar to those reported for the enzymes from other sources (Bridges & Wittenberger, 1975; Rippa & Signorini, 1975). The broad pH range for the activity indicates that the enzyme can be stable from mild acidic to mild basic conditions. This result is similar to that found for *Pseudomonas fluorescens* enzyme (Stournaras, et al. 1982). The pH profile of the enzymic activity was obtained for the purified 6PGDH in the absence and the presence of 1.0 mM Mn^{2+} . Very low activities were observed under pH 4 and above pH 10. The pH optimum was shifted from pH 7.0 in the absence of Mn^{2+} to pH 8.0 in the presence of Mn^{2+} (Fig.13). The fission yeast enzyme shows no significant loss of activity in a period of two weeks when stored at 4 °C in 20 mM phosphate buffer (pH 7.0). It was reported that the purified enzyme from *Pseudomonas fluorescens* was stable for a period of months when stored under a solution of 3.2 M $(NH_4)_2SO_4$ in the neutral pH range (Stournaras, et al. 1982).

Absorption spectrum for the purified 6PGDH was obtained in 20 mM phosphate buffer, pH 7.0 at 25 °C. The enzyme exhibits an absorption maximum around 280 nm (Fig. 34). This result suggests that the purified enzyme has a high content of the aromatic amino acids, such as phenylalanine, tryptophan, and tyrosine, since aromatic rings in these residues give a maximum absorption at 280 nm. Complete analyses of amino acid

composition will confirm this result. The extinction coefficient at 280 nm ($\epsilon_{280\text{ nm}}$) of 11.6 ± 1.2 is comparable to those calculated for the purified enzymes from other sources (see Table 3).

Kinetic studies of the yeast 6PGDH. The kinetic mechanism can be classified into two classes as suggested by Alberty (1953) namely Sequential and Ping Pong mechanisms. These two classes of kinetic mechanisms can be differentiated by inspection of the double-reciprocal plots of initial-velocity versus substrate concentrations. These in turn may be divided into Random and Ordered types. The reaction which is catalyzed by the enzyme *via* sequential mechanism involves a ternary complex. The cardinal feature of these pathways of enzyme and substrate interaction is that the family of lines in double-reciprocal plots intersect at a common point, either to the left of the $1/v$ axis or on the axis. This implicates that all substrates must be present simultaneously at the enzyme's active-site before product formation can occur. The cardinal feature of the Ping Pong mechanism is that the enzyme reacts with one of the substrate to form a Michaelis-type complex, which then breaks down to yield a modified enzyme by dissociating the first product before second substrate binds to the enzyme. Initial velocity studies for the reaction catalyzed by 6PGDH from *S. pombe* are in agreement with sequential kinetic mechanism in the direction of oxidative decarboxylation of 6-phosphogluconate since the initial velocity pattern obtained by varying NADP^+ at different fixed levels of 6PG intersects to the left of the $1/v$ axis. A systematic study of the initial velocity pattern for *S. pombe* 6PGDH was carried out at different pH in the absence and in the presence of Mn^{2+} (Fig.14-Fig.19: pH 5-8, in the absence of Mn^{2+} ; Fig.23-Fig.27: pH 6-8, in the

presence of Mn^{2+}). All initial velocity patterns at different pH in the absence of Mn^{2+} have the same trend, i.e. intersecting to the left of the ordinate. This suggests that the kinetic mechanism of the fission yeast 6PGDH catalysis is not affected by a variation in pH. Similar initial velocity patterns obtained at different pH in the presence of Mn^{2+} indicate that the metal ion does not change the kinetic mechanism of the fission yeast 6PGDH catalysis, but it enhances the initial velocity of the reaction.

The kinetic parameters for 6PGDH at all pH values are listed in Table 5 (in the absence of Mn^{2+}) and Table 6 (in the presence of Mn^{2+}). The maximum velocity, V_{max} , has an optimum value at pH 7.5 in both the absence and the presence of Mn^{2+} . Michaelis constant, K_m for $NADP^+$ decreases with increasing pH values, while that for 6PG increases with increasing pH values. All pH profiles are obtained at nonsaturating fixed levels of one substrate while varying concentrations of the other. The predominant enzymic form is E:6PG in the case of the V/K_{NADP^+} profile while E: $NADP^+$ is the predominant enzymic form in the case of the V/K_{6PG} . The dissociation constants in both acidic and basic groups are difficult to be evaluated from a narrow pH range. Further studies are required to investigate pH dependence of the kinetic mechanism in a broad range. Recently Berdis and Cook (1993) proposed a chemical mechanism in which a general base ($pK \sim 7.5$) from the enzyme accepts the proton from the 3-hydroxyl group of 6PG concomitantly with the hydride transfer to $NADP^+$ to form the β -keto-acid intermediate and NADPH. The β -keto-6-phosphogluconate is then decarboxylated to form the 1,2-enediol intermediate with the general base ($pK \sim 7.5$) donating the proton abstracted in the first step to the keto oxygen. The enediol tautomerizes to give Ru 5-P with a proton

donated by a general acid ($pK \sim 8$) from the enzyme to C-1, while the general base ($pK \sim 7.5$) again abstracts the proton from C-2.

Product inhibition. All product inhibition patterns are obtained at nonsaturating fixed levels of one substrate while varying concentrations of the other. The patterns predicted by the equations listed in the method section are linear inhibition. The competitive product inhibition is exhibited by products vs the reactants (NADPH vs NADP, Ru 5-P vs 6PG, and CO_2 vs 6PG). NADPH as the product is competitive with respect to NADP⁺ (Fig. 28a), but noncompetitive versus 6PG (Fig. 31a). The inhibition by the product Ru 5-P is competitive with respect to 6PG (Fig. 32a), but noncompetitive against NADP⁺ (Fig. 29a). This competitive inhibition suggests a rapid equilibrium mechanism at nonsaturating concentrations of the fixed substrate. It was found that CO_2 as the product also is competitive with respect to 6PG (Fig. 33a), but noncompetitive versus NADP⁺ (Fig. 30a). Results from the product inhibition studies show that the oxidative decarboxylation of 6-phosphogluconate catalyzed by 6PGDH from *S. pombe* proceeds *via* the random Bi-Ter mechanism with formation of abortive ternary complexes (EAR and EBQ) (see Scheme 1). Product inhibition patterns for bireactant mechanism were described by Rudolph (1979). No abortive complex is formed if the products are all competitive with respect to both substrates in random mechanism. The competitive patterns for NADPH against NADP⁺ and Ru 5-P versus 6PG indicate the abortive complex formation in the random mechanism. The pattern of CO_2 as a product inhibiting the 6PGDH catalysis has not been reported. This study suggests that in the direction of oxidative decarboxylation, CO_2 may be the first product released from the reaction. This

is followed by the release of NADPH after it accepts the proton from the 3-hydroxyl group of 6PG. Finally, the enediol intermediate from 6PG tautomerizes to form Ru 5-P. This result is consistent with the chemical mechanism recently proposed by Berdis and Cook (1993). It can be concluded that the oxidative decarboxylation of 6PG catalyzed by 6PGDH *in vivo* actually is irreversible due to the loss of CO₂. Reasons for the competitive inhibition pattern of CO₂ with respect to 6PG are not clear. Product inhibition constants for the fission yeast 6PGDH calculated from the secondary plots of product inhibition are listed in Table 8.

6PGDH, NAD⁺-malic enzyme, and isocitrate dehydrogenase all catalyze similar reactions namely oxidative decarboxylation of a β -hydroxyl acid using NAD(P)⁺ as a hydride acceptor to yield a ketone, CO₂, and NAD(P)H as products. 6PGDH differs from the aforementioned enzymes in that it requires no metal ions for the activity. pH Studies (Kiick, *et al.*, 1986) and multiple isotope effects (Wiess, *et al.*, 1991) for the NAD⁺-malic enzyme from the *Ascaris suum* suggest the requirement for a general base (pK~4.9) and a general acid (pK~9) with the same general function as those proposed for the 6PGDH reaction. NAD⁺-Malic enzyme is reported to display a pseudo-Ter-Quad random kinetic mechanism in which Mg²⁺ acts as a pseudoreactant (Mallic, *et al.* 1991) while *S. pombe* 6PGDH proceeds *via* a Bi-Ter random kinetic mechanism.

The kinetic mechanism of *S. pombe* 6PGDH is depicted in Scheme 1. Further studies are required to confirm whether this proposal is correct.

Scheme 1. Kinetic mechanism of *S. pombe* 6PGDH catalysis. The random Bi Ter mechanism with the formation of the abortive complexes, EAR and EBQ, is shown where A, B, P, Q, and R represent NADP⁺, 6PG, CO₂, NADPH, and Ru-5-P, respectively. Each double arrow line reflects reversible step while the single arrow lines indicate that there may be no binding site for CO₂.

APPENDIX 1

CHEMICAL MODIFICATIONS

Chemical modifications of specific amino acid in an enzyme can provide insight into the mechanism of its action. This is accomplished by a measurement of the enzyme activity during modifications of specific residues to correlate the susceptibility of the enzymic activity with the specific modification. If those residues essential to the enzymic activity are chemically modified, a concomitant eradication of the activity is likely. Monitoring the enzymic activity during chemical modifications of specific amino acid residues can be useful in identifying these essential residues. In practice however, this technique often suffers from a lack of specificity of the reagents towards a specific amino acid residue. Quantitation of the change in the number of a specific residue affected by a chemical modification is not always essential.

Several chemical modifications have been performed on 6-phosphogluconate dehydrogenase from *S. pombe*. Conditions were chosen which affected the predominate changes within a time span of up to 2 hours. For each modification, the activity of the modified enzyme was measured every 10 min. after the reagent was added to the enzyme system. A comparison of the activity of the modified enzyme with a control assay may provide the information of the specific amino acid residue involving in the enzyme catalysis. The control assay follows the enzymic activity of the unmodified enzyme which was subjected to identical conditions over an identical time span as the modified enzyme. The results of chemical modifications of 6PGDH from *S. pombe* are listed in Table 9. The specific chemical modifications are described in the following section.

Table 9. Summary of Effects of Chemical Modifications on the Activity of 6PGDH from *S. pombe*.

Residue	Reagent	pH	Reaction time(min)	% residual activity	% activity of the control
Arginine	Phenyl Glyoxal	8.5	60	24	100
Cysteine	Iodoacetamide	7.5	120	70	100
Histidine	ethoxyformic anhydride	6.5	120	78	95

Note: 1. The enzymic activity was measured as the standard method described in Method section.

2. The reaction mixture for arginine residue: 1.5 μ M 6PGDH in 0.1 M borate buffer, pH 8.5 and 1.5 mM phenyl glyoxal.

The reaction mixture for cysteine residue: 1.5 μ M 6PGDH in 0.1 M phosphate buffer, pH 7.5 and 2.7 mM iodoacetamide.

The reaction mixture for histidine residue: 1.5 μ M 6PGDH in 0.1 M phosphate buffer , pH 6.5 and 2.5 mM ethoxyformic anhydride.

3. Modification results are average of at least two experiments.

Carboxymethylation of cysteine. Cysteine residues are particularly sensitive to carboxymethylation by haloacetates as alkylating agents. The nucleophilic displacement of iodine by an enzymatic sulfhydryl species is shown below:

Modification of 6-phosphogluconate dehydrogenase with iodoacetamide resulted in a 30% decrease in the enzymic activity after 2 hours of the reaction (see Table 9), a 70% decrease after 4 hours of the reaction. Iodoacetic acid employed as the alkylating reagent can eliminate the enzymic activity faster than iodoacetamide. The result suggests that the cysteine residue may involve in the 6PGDH catalysis.

Phenylglyoxalation of arginine. Arginine residues have been shown to be essential for the binding of coenzymes through the pyrophosphate moiety. Studies show this binding proceed *via* the attractive interaction between an arginine and the 2'-phosphate of the cofactor (Matthews, *et al.*, 1978; Dalziel & Dickinson, 1965). The reaction of arginine residues is relatively specific with respect to α -dicarbonyls which react with the guanidino functionality of the arginine residue as described by the following mechanism:

Phenyl glyoxal was employed as the α -dicarbonyl reagent (Hebert & Fackrell, 1987; Kantrowitz & Lipscomb, 1976) in an effort to identify arginine residue(s) at the nucleotide binding site of 6-phosphogluconate dehydrogenase from *S. pombe*. The result shows a loss of 64% enzymic activity after 30 min. reaction, a loss of 76% enzymic activity after 60 min. reaction (see Table 9). This indicates that the arginine residue(s) is essential for the activity of 6PGDH from *S. pombe*.

Ethoxyformylation of histidine. Ethoxyformylation and photooxidation are two methods generally employed to study the function of histidine residues in enzymes (Tsurushiin, et al., 1975; Meyer & Cromartie, 1980). Ethoxyformic acid anhydride at neutral pH reacts with histidine residues in the enzymes. After 2 hours of the reaction, the enzymic activity remained 78%. This implicates that the histidine residues may not be essential for the activity of 6PGDH from *S. pombe*. If the histidine residues are involved in the 6PGDH catalysis, an incomplete inactivation of the enzyme may be due to insufficient reagent used. Further studies are required to confirm whether the histidine residues are essential for the enzymic activity. Although it has been shown to react preferentially with histidine residues, ethoxyformic acid anhydride is also known to react with the free N-terminal amino group of a protein and with a variety of amino acid side chains, including lysine, tyrosine, serine, arginine, and cysteine (Melchior & Fahrney, 1970; Miles, 1977). Photooxidation will be performed on the yeast 6PGDH in the further studies to investigate the function of histidine residues. Hydroxylamine has been reported to cleave N-ethoxyformyl-histidine residues in proteins and to reactivate enzymes inactivated by ethoxyformic acid anhydride (Sjoberg, 1971).

APPENDIX 2

Chemical mechanism of 6PGDH

The general acid/general base chemical mechanism was proposed as shown above. An enzyme general base (pK ~ 7.5) accepts the proton from the 3-hydroxyl group of 6-phosphogluconate concomitant with hydride transfer to NADP⁺ to form a β -keto-acid intermediate (3-keto-6-phosphogluconate) and NADPH. The resulting 3-keto intermediate is then decarboxylated to form the 1,2-enediol intermediate with the general base donating the proton abstracted in the first step to the keto oxygen. The enediol tautomerizes to give ribulose-5-phosphate. This is followed by tautomerization of the enol structure to the keto product with the assistance of a second residue acting as a general acid.

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