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MECHANISTIC STUDIES ON

GLUCOSE-FRUCTOSE OXIDOREDUCTASE

FROM Zymomonas mobilis

A thesis presented in partial fulfilment of the requirements
for the degree of

Master of Science
in Biochemistry
at
Massey University
New Zealand

Marlene Tsao
1990
in memory of my father...
ABSTRACT

The reaction mechanism catalysed by glucose-fructose oxidoreductase was studied in detail by comparing the presteady kinetic results from absorbance and fluorescence experiments using the stopped-flow apparatus. The oxidation of enzyme-NADPH by gluconolactone was a biphasic reaction, with a fluorescence decrease within the dead time, followed by a slower decrease which corresponds to the absorbance change. This behaviour was due to quenching of the fluorescence of enzyme-NADPH by bound gluconolactone. In both absorbance and fluorescence experiments, reduction of enzyme-NADP$^+$ by glucose appeared to be a single first order process. At high glucose concentrations the rate constants for absorbance were higher than those for fluorescence, with limiting values of $2100 \pm 130$ s$^{-1}$ and $373 \pm 14$ s$^{-1}$, respectively, under similar conditions. The fluorescence change for the glucose/gluconolactone half reaction occurs during dissociation of gluconolactone, while the absorbance change represents hydrogen transfer from glucose to enzyme-NADP$^+$. 

The oxidation of enzyme-NADPH by fructose was shown to involve one phase in both absorbance and fluorescence. The reduction of enzyme-NADP$^+$ by sorbitol was shown to be biphasic in both absorbance and fluorescence. However, the two phases in the sorbitol reaction are not well separated, because the difference between the two rate constants is small, and the rate constants were difficult to determine.

Glucose-fructose oxidoreductase was unusually stable to temperature changes and unaffected by reactive dye binding, but unstable during dialysis. The NADP$^+$ was shown to be essential for the integrity of the enzyme, since GFOR was denatured upon removal of NADP$^+$ from this enzyme and this denaturation was irreversible. Preliminary inhibitor studies with diethyl pyrocarbonate suggested that histidine residues may be important in the catalytic reaction of GFOR.
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CHAPTER 1
INTRODUCTION

1.1 *Zymomonas mobilis*

*Zymomonas mobilis* is a Gram negative, non-sporing, polarly flagellated rod-shaped bacterium (Swings and De Ley, 1984). This bacterium naturally ferments sugar cane, honey, fruit juice and palm sap. It is used to make palm wine in the tropics. Secondary fermentation by *Z. mobilis* was found to be responsible for beer spoilage and cider-sickness (Swings and De Ley, 1984).

*Zymomonas mobilis* is an unusual bacterium because it produces a high percentage of ethanol. Most ethanol-producing microorganisms are yeasts or other fungi, with *Saccharomyces cerevisiae* being the most commonly used. However *Z. mobilis* is a widely used ethanol-producing bacterium in most tropical areas of the world. It produces ethanol much faster than yeasts: the ethanol productivity rate for yeast was 0.67 g (ethanol) g\(^{-1}\) (cells) h\(^{-1}\) and for *Z. mobilis* was 5.67 g (ethanol) g\(^{-1}\) (cells) h\(^{-1}\) (Buchholz et al., 1987). Unlike yeast which ferments glucose to ethanol via the glycolytic pathway, *Zymomonas mobilis* uses an unusual carbohydrate metabolic pathway, the Entner-Doudoroff pathway (EDP) (Fig. 1.1). The EDP only occurs in strictly aerobic microorganisms with tricarboxylic acid cycle for energy production (Doelle and Doelle, 1989). Since EDP produces the least energy of all biological carbohydrate metabolizing pathways, this forces the microorganism to have higher sugar monomer uptake and conversion to maintain growth (Doelle and Doelle, 1989).

Another advantage *Z. mobilis* has over yeast in ethanol production is that it could produce ethanol with quality close to pharmaceutical grade. The usual impurities found in
Fig. 1.1: Entner-Doudoroff pathway (EDP)
(based on Bringer-Meyer and Sahm, 1988)

glucokinase (1), glucose-6-P-dehydrogenase (2), 6-P-gluconolactonase (3), 6-P-gluconate dehydratase (4), KDPG aldolase (5),
glyceraldehyde-P-dehydrogenase (6), phosphoglycerate kinase (7),
phosphoglycerate mutase (8), enolase (9), pyruvate kinase (10), pyruvate decarboxylase (11), alcohol dehydrogenase (12)
ethanol produced by yeasts such as glycerol and higher alcohols (fusel oil) are absent in ethanol produced by Z. mobilis (Doelle and Doelle, 1989).

The two enzymes: pyruvate decarboxylase and alcohol dehydrogenase are important for ethanol production. The presence of pyruvate decarboxylase in bacteria is uncommon and this is why they do not normally produce ethanol. But there have been recent attempts to insert the gene encoding for pyruvate decarboxylase into other bacteria, so that their existing pathways can be modified to ethanol-producing pathways (Buchholz et al., 1987).

When Z. mobilis is grown on glucose alone, only ethanol is produced. However, fermentation of sugar cane, honey, fruit juice and palm sap by this bacterium involves metabolism of sucrose or a glucose-fructose mixture. Then sorbitol is produced as well as ethanol (Barrow et al., 1984).

The carbon skeleton of sorbitol was shown to be derived from fructose only. The formation of sorbitol and gluconolactone from fructose and glucose is catalysed by a single enzyme, glucose-fructose oxidoreductase (GFOR). Breakdown of gluconolactone is catalysed by gluconolactonase (Zachariou and Scopes, 1986). Therefore, the carbon skeleton of glucose is converted to ethanol via gluconate and the Entner-Doudoroff Pathway (Fig. 1.2).

Glucose-fructose oxidoreductase was first discovered by Leigh et al., 1984. The accumulation of sorbitol in Zymomonas mobilis led to the suggestion of two tightly coupled dehydrogenases, which were believed to catalyse the oxidation of glucose to gluconate and fructose to sorbitol (Leigh et al., 1984). But Zachariou and Scopes (1986) showed that these reactions were catalysed by only one enzyme, glucose-fructose oxidoreductase, and they isolated and characterised this enzyme.
Fig. 1.2: Mechanism of production of sorbitol and gluconate
(based on Zachariou and Scopes, 1986, and Rogers and Chun, 1987)

GFOR = glucose-fructose oxidoreductase
The reaction is virtually irreversible in vivo, because the reverse reaction of GFOR is very slow compared to the forward reaction and gluconolactonase is constantly active in removing gluconolactone to gluconate. Therefore, half of the carbon skeleton is removed to sorbitol instead of to ethanol. This results in lowering the yield of ethanol and sorbitol accumulates to a significant level.

Two compounds of this pathway are of commercial interest: sorbitol and gluconate. Sorbitol is used as a low calorie sweetener in food for diabetics, and sorbitol can also be converted to L-sorbose which is an intermediate in vitamin C production. Gluconic acid is used in making cleaning agents and used as food additives, which function as food coagulants.

1.2 Purification of Glucose-fructose Oxidoreductase

Glucose-fructose oxidoreductase (GFOR) has been purified by a combination of three techniques: dye-ligand chromatography; ion exchange chromatography, and gel filtration (Zachariou and Scopes, 1986). This original method has now been revised.

Dye-ligand chromatography has been shown to be an invaluable tool for protein purification. It is inexpensive, simple and reliable. The coupling process is more rapid and less toxic than for conventional affinity chromatography (Atkinson et al., 1981). Many enzymes as well as glucose-fructose oxidoreductase have been purified using dye-ligand chromatography as the only step or the main step of purification. Hey and Dean (1983) used dye-ligand chromatography to purify glucose-6-phosphate dehydrogenase. It gave enzyme with specific activity of 228 units/mg and 73% yield. In comparison to the methods used by Olive and Levy (1967), gave a specific activity of glucose-6-phosphate dehydrogenase of 250 units/mg, but only 18% yield.
The dye-ligand chromatography method of purification usually involves two types of dye columns arranged in tandem (Hey and Dean, 1983). The first column contains the "negative" adsorbent, which does not bind the desired enzyme, but does bind most other proteins. The desired protein is then partly purified, since it is separated from most of other proteins. The "purified" enzyme passes down to the second column. This contains a "positive" adsorbent, which binds the desired enzyme but not many other proteins.

For GFOR, the negative column was Procion Green H-4G, and the positive column was Remazol Brilliant Blue R. When all of the glucose-fructose oxidoreductase bound to the blue column, the columns were disconnected. Then, GFOR was eluted from the blue column with increasing ionic strength and pH (Zachariou and Scopes, 1986).

The disadvantage of dye-ligand chromatography is the long time spent in selecting a negative and a positive dye, which involves trial and error. One of the methods of selecting the suitable dyes has been described by Scopes (1986). There are five main groups of dyes: group 1 binds the least protein; group 5 binds the most protein; and the groups in between have average affinity for proteins (Scopes, 1986). The "negative" column should be in groups 4 or 5, where it binds to most of the proteins, but not the desired protein. Then, the "positive" dye is usually from groups 1 or 2, where it binds little of the other proteins, but all of the desired protein (Scopes, 1986). Therefore, instead of screening about 70 dyes randomly, it is only necessary to screen one or two from each of those five groups.

Ion exchange chromatography was the next step of purification for glucose-fructose oxidoreductase. A carboxymethyl Trisacryl column was used, and the enzyme was eluted by increasing salt concentration (Zachariou and Scopes, 1986). The oxidoreductase from this column contained traces of gluconolactonase. Therefore, gel filtration was used as the final step of purification.
1.3 Structural Properties of Glucose-Fructose Oxidoreductase

The oxidoreductase has a subunit molecular weight of about 40,000 daltons, and exists as a tetramer in solution at pH 5.0 (Zachariou and Scopes, 1986). A recent protein sequence of GFOR sent by R.K. Scopes from La Trobe University, Australia, showed that the enzyme has a molecular weight of 42,685 daltons (personal comm., 1990). The isoelectric point was estimated from the amino acid sequence to be approximately 8.0.

Glucose-fructose oxidoreductase has been shown to contain bound nicotinamide nucleotide, because an absorbance band at 340 nm was observed after addition of glucose. When the enzyme was deproteinized with perchloric acid and then neutralised, there was no absorbance at 340nm; but when glucose-6-phosphate dehydrogenase (an NADP+-requiring enzyme) and glucose-6-phosphate were added to the deproteinized enzyme solution, an absorbance band at 340 nm appeared. When an NAD⁺-dependent enzyme, alcohol dehydrogenase, and ethanol were added to the deproteinized solution, no change in absorbance was observed. This indicated that the cofactor was NADP⁺, not NAD⁺ (Zachariou and Scopes, 1986). There were no external sulphydryl groups detected and no metal ion has been shown to be associated with GFOR.

1.4 Kinetic Studies of Glucose-fructose Oxidoreductase

Glucose-fructose oxidoreductase has a high specificity, but low affinity for its substrates, glucose and fructose. This enzyme does not act on either substrate alone, and has no, or very little, activity towards other sugars, e.g. D-xylose, D-mannose, galactose and other common sugars (Zachariou and Scopes, 1986).

Glucose-fructose oxidoreductase was shown to be specific for β-D-glucose not α-D-glucose. The reduction of E-NADP⁺ by α-D-glucose was much slower than by the equilibrium mixture of glucose at the same concentration. Reduction by β-D-glucose was
much faster than by the equilibrium mixture where the same concentration of glucose was used (Hardman and Scopes, 1988).

The reaction mechanism of glucose-fructose oxidoreductase has been studied by Zachariou and Scopes (1986), and Hardman and Scopes (1988). Zachariou and Scopes (1986) showed that the oxidoreductase was active between pH 4.5 and 7.5, and the optimum activity was at pH 6.2. They also showed that high concentrations of glucose and sorbitol inhibited GFOR activity. Ethanol also inhibits GFOR activity, but only at high concentrations, for example, 2 M ethanol inhibited 16% of the enzyme activity.

The proposed overall scheme from kinetic studies using absorbance results is outlined in Fig. 1.3. It shows a classic ping-pong mechanism: a substrate, glucose, binds to the oxidoreductase and a product, gluconolactone, is released before another substrate, fructose, binds. Then the second product, sorbitol, is released. The product inhibition pattern was also typical of a classic ping-pong mechanism. For instance, sorbitol competitively inhibited the reduction of E-NADP+ by glucose and oxidation of E-NADPH by fructose was competitively inhibited by gluconolactone (Hardman and Scopes, 1988).

The reaction was shown to be reversible in vitro; therefore, all four sugars can serve as substrates. The rate determining steps for the overall reaction are probably the dissociation of gluconolactone from E-NADPH-gluconolactone in the forward direction, and formation of E-NADPH-fructose in the reverse reaction (the rate constants are shown in Fig. 1.3). The $k_{cat}$ value for the forward direction is 200 s$^{-1}$ and for the reverse direction, 0.8 s$^{-1}$ (Hardman and Scopes, 1988).

Since the ping-pong model requires that only one reactant can bind at a time, it implies that the enzyme contains a single binding site for all substrates. If the oxidoreductase has two binding sites, one for the ring forms and the other for acyclic forms, they must
Fig 1.3 The kinetic mechanism for glucose-fructose oxidoreductase

(Hardman and Scopes, 1988)

\( G = \text{glucose}, \quad L = \text{gluconolactone}, \quad E = \text{enzyme}, \)

\( F_{\text{open}}, \text{ring} = \text{fructose in acyclic and ring form respectively} \)
overlap to represent a single overall binding site. Glucose and gluconolactone probably bind to the enzyme in the pyranose ring form, while sorbitol and fructose bind in an acyclic form (Hardman and Scopes, 1988).

The overall reaction catalysed by glucose-fructose oxidoreductase consists of two half reactions (equations 1.1 and 1.2)

$$\text{glucose} + \text{E-NADP}^+ \rightleftharpoons \text{gluconolactone} + \text{E-NADPH} + \text{H}^+$$ \hspace{1cm} (1.1)

$$\text{fructose} + \text{E-NADPH} + \text{H}^+ \rightleftharpoons \text{sorbitol} + \text{E-NADP}^+$$ \hspace{1cm} (1.2)

$$\text{glucose} + \text{fructose} \rightleftharpoons \text{gluconolactone} + \text{sorbitol}$$ \hspace{1cm} (1.3)

Reduction of enzyme-NADP$^+$ by glucose, and oxidation of enzyme-NADPH by gluconolactone (equation 1.1) involve single first-order processes (Hardman and Scopes, 1988). Oxidation of enzyme-NADPH by fructose obeys first order kinetics, with a very fast rate constant of greater than 430 s$^{-1}$ (refer to rate constants in Fig. 1.3), and reduction of enzyme-NADP$^+$ by sorbitol (equation 1.2) is a biphasic process. The rate constants for the sorbitol (500 mM) reaction were: $0.74 \pm 0.05$ s$^{-1}$ and $0.303 \pm 0.028$ s$^{-1}$ with the fast phase comprised of 55% of the total amplitude.

1.5 Aims of This Project

The aims of this project were to study the reaction mechanism in more detail mainly by presteady kinetic studies using the stopped-flow apparatus. Nucleotide fluorescence and protein fluorescence experiments were conducted. Also, preliminary experiments on
structural studies were carried out. These involve removal of NADP+ from GFOR to find out the nature of the bond between and enzyme in the complex and inhibitor studies with diethyl pyrocarbonate to investigate the amino acid residues which are important in the reaction mechanism. Dye-enzyme binding spectral studies were undertaken to study the nature of the dye-enzyme complex and the surface charge of GFOR.