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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

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in Biochemistry
at
Massey University
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Marlene Tsao

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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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TABLE OF CONTENTS

PAGE

ABSTRACT i

ACKNOWLEDGEMENTS ii

TABLE OF CONTENTS iii

LIST OF FIGURES viii

LIST OF TABLES x

CHAPTER 1

INTRODUCTION

1.1 *Zymomonas mobilis* 1

1.2 Purification of Glucose-fructose Oxidoreductase 5

1.3 Structural Properties of Glucose-fructose Oxidoreductase 7

1.4 Kinetic Studies of Glucose-fructose Oxidoreductase 7

1.5 Aims of This Project 10

CHAPTER 2

ENZYME PURIFICATION

2.1 Introduction 12

2.2 Materials and Methods 13

2.2.1 Materials 13

2.2.2 Methods 13

2.2.2.1 MES Stock Buffer 13

2.2.2.2 Enzyme Assay 14

2.2.2.3 Protein Assay 14

2.2.2.4 Culturing *Zymomonas mobilis* 15

2.2.2.5 Extraction of GFOR 16

2.2.2.6 Enzyme Purification 16

2.2.2.7 SDS Gel Electrophoresis 18

2.3 Results 19

2.3.1 Culturing *Zymomonas mobilis* 19

2.3.2 Efficiency of GFOR Extraction 19

2.3.3 Enzyme Stability and Storage 21

2.3.3.1 Buffer and pH Dialysis 21

2.3.3.2 Storage Stability of GFOR 22

2.3.4 Enzyme Purification 24

2.3.4.1 Dye-ligand Chromatrography 24

2.3.4.2 Hydrophobic Chromatography 28

2.3.4.3 Summary of GFOR Purification 28

2.3.4.4 Ion Exchange Chromatography 31

2.3.5 SDS Gel of Purified enzyme 31

2.4 Summary 31

CHAPTER 3

PRESTEADY STATE KINETIC STUDIES ON GLUCOSE-FRUCTOSE OXIDOREDUCTASE

3.1 Introduction 35

3.2 Methods 37

3.2.1 Operation of the Stopped-flow Apparatus 37

3.2.1.1 Data Collection 40

3.2.1.2 Data Processing 40

3.2.2 Preparation of Enzyme Samples for Stopped-flow 43

3.2.3 Determination of GFOR Active Site 43

3.2.4 Stopped-flow Experiments 43

3.2.4.1 Nucleotide Absorbance 43

3.2.4.2 Nucleotide Fluorescence 44

3.2.4.3 Protein Fluorescence 44

3.3 Results and Discussion 44

3.3.1 The Oxidation of E-NADPH by Gluconolactone 44

3.3.2 Reduction of the E-$NADP^+$ Complex by Glucose 50

3.3.3 Oxidation of E-NADPH by Fructose 59

3.3.4 Reduction of E-$NADP^+$ by Sorbitol 63

3.3.4.1 Differences Between Fluorescence and Absorbance 63

3.3.4.2 Effect of Temperature 66

3.3.4.3 Effect of pH 66

3.3.4.4 Effect of Enzyme Concentration 66

3.3.4.5 Effect of Differences in the Methods of GFOR Purification 66

3.3.4.6 Differences in Data Processing 70

3.3.4.7 Time Scale for Recording the Sorbitol Reaction 70

3.3.5 Protein Fluorescence 76

3.4 Summary 80

CHAPTER 4

DYE-PROTEIN BINDING STUDIES

4.1 Introduction 82

4.2 Methods 83

4.2.1 Measurement of Spectral Shift of Dye-protein Binding 83

4.3 Results and Discussion 83

4.3.1 Spectral Shift of Dye-Protein Binding 83

4.3.2 Active Site of Glucose-fructose Oxidoreductase 87

4.3.3 Reversibility of Dye-Protein Binding 87

4.4 Conclusion 88

CHAPTER 5

CHEMICAL MODIFICATION OF GLUCOSE-FRUCTOSE OXIDOREDUCTASE

5.1 Introduction 90

5.2 Methods 90

5.2.1 Measurement of DEPC 90

5.2.2 Modification of GFOR with DEPC 91

5.2.3 UV Absorbance Spectrum of Modified GFOR 91

5.3 Results and Discussion 91

5.3.1 The Enzymatic Activity of Modified GFOR 91

5.3.2 UV Absorbance Spectrum of GFOR Modified by DEPC 93

5.3.3 Protection of GFOR by Substrates against DEPC Inactivation 101

5.4 Conclusion 102

CHAPTER 6

REMOVAL OF COENZYME FROM GFOR

6.1 Introduction 104

6.2 Methods 105

6.2.1 Acidic Ammonium Sulphate Precipitation of GFOR 105

6.2.2 Removal of $NADP^+$ from GFOR by Dialysis 106

6.2.3 Removal of $NADP^+$ from GFOR by Charcoal 106

6.3 Results and Discussion 107

6.3.1 Acidic Ammonium Sulphate Precipitation of GFOR 107

6.3.2 Removal of $NADP^+$ from GFOR by Dialysis 107

6.3.3 Removal of $NADP^+$ from GFOR by Charcoal 108

6.4 Conclusion 108

CHAPTER 7

SUMMARY

7.1 Summary of Results 110

7.2 Future Work 113

REFERENCES 115

List of Figures

FIGURE	TITLE	PAGE
1.1	Entner-Doudoroff pathway	2
1.2	Mechanism of production of sorbitol and gluconate	4
1.3	The kinetic mechanism for GFOR	9
2.1	Purification scheme for GFOR	17
2.2	Growth curve of *Z. mobilis*	20
2.3	The stability of GFOR extract over a period of time	25
2.4	The stability of purified GFOR over a period of time	26
2.5	Elution profile of GFOR from Remazol Brilliant Blue R8001 column	27
2.6	Elution profile of GFOR from the Thio C_4-Sepharose column	29
2.7	Elution profile of GFOR from CM-Sepharose CL-6B	32
2.8	SDS gel of purified GFOR	33
3.1	Schematic Diagram of the stopped-flow apparatus	38
3.2	A flowchart showing how results were obtained from the stopped-flow	39
3.3	The printout of the results produced from a sample reaction by EXPFIT	41
3.4	Comparison of the original and fitted curves for a sample reaction	42
3.5	Time course of the fluorescence change on mixing E-NADPH and gluconolactone	45
3.6	Dependence of the amplitude of fluorescence change on gluconolactone concentration	47
3.7	Mechanism of gluconolactone carbonyl group quenching E-NADPH fluorescence	51
3.8	Time course of fluorescence change during reduction of E-$NADP^+$ by glucose	53
3.9	Rate constants for absorbance and fluorescence change at various glucose concentration under the same conditions	54

3.10	Rate constants for fluorescence change at various glucose concentrations at different temperatures	55
3.11	Dependence of fluorescence rate constants on fructose concentration	61
3.12	Structures of GFOR substrates	62
3.13	Change in absorbance for the reaction of E-NADP$^+$ with 50 mM sorbitol on different time scales	74
3.14	Protein fluorescence traces of oxidation and reduction of GFOR by substrates	77
4.1	Spectrum of Procion Green H-4G dye plus GFOR	84
4.2	Spectrum of Procion Yellow H-E4R dye plus GFOR	85
4.3	Sprectum of Remazol Brillian Blue R8001 dye plus GFOR	86
5.1	Activation of GFOR with DEPC	92
5.2	Effect of DEPC on GFOR during 60 min incubation with various DEPC concentrations	94
5.3	Effect of different DEPC concentrations on GFOR	95
5.4	UV spectrum of the effect of 20.9 μM DEPC on GFOR	96
5.5	UV spectum of the effect of 281 μM DEPC on GFOR	97
5.6	The absorbance change at 242 nm over 60 min	98
5.7	The graph of GFOR activity and absorbance at 242 nm of 20.9 μM DEPC against time	99

List of Tables

TABLE	TITLE	PAGE
2.1	Extraction of GFOR from *Z. mobilis* under different conditions	22a
2.2	Stability of GFOR during dialysis under different conditons	23
2.3	Summary of GFOR purification for 67 g of wet cells	30
3.1	Initial results from this work and those of Hardman and Scopes (1988) for rate constants plus percentage of the fast amplitude	64
3.2	Rate constants plus percentage of total amplitude in the faster phase for the reduction of E-NADP+ by sorbitol	65
3.3	Fluorescence results for rate constants and percentage of total amplitude in the faster phase for the reduction of E-NADP+ by sorbitol at different temperatures	67
3.4	Fluorescence results for rate constants and percentage of total amplitude in the faster phase for the reduction of E-NADP+ by sorbitol at different pH	68
3.5	The absorbance results for the reduction for the reduction of E-NADP+ at different concentrations by sorbitol (100 mM)	69
3.6	Absorbance results of reduction of E-NADP+ by sorbitol after GFOR was purified by CM-Sepharose column	71
3.7	Absorbance results of reduction of E-NADP+ by sorbitol after GFOR was purified from the Remazol Brilliant Blue column	71
3.8	The rate constants and % fast phase for the reduction of E-NADP+ by 50 mM sorbitol when different points were fitted	72
3.9	The absorbance results for the reduction of E-NADP+ by 50 mM sorbitol at different time scales	75
3.10	The protein fluorescence rate constants of oxidation of E-NADPH by fructose	78

3.11 Protein fluorescence rate constants of oxidation of E-NADPH by 50 mM fructose at different enzyme concentrations 79

4.1 Effect of dye binding on GFOR activity 87

4.2 Absorbance maxima of dye-GFOR after overnight dialysis 88

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

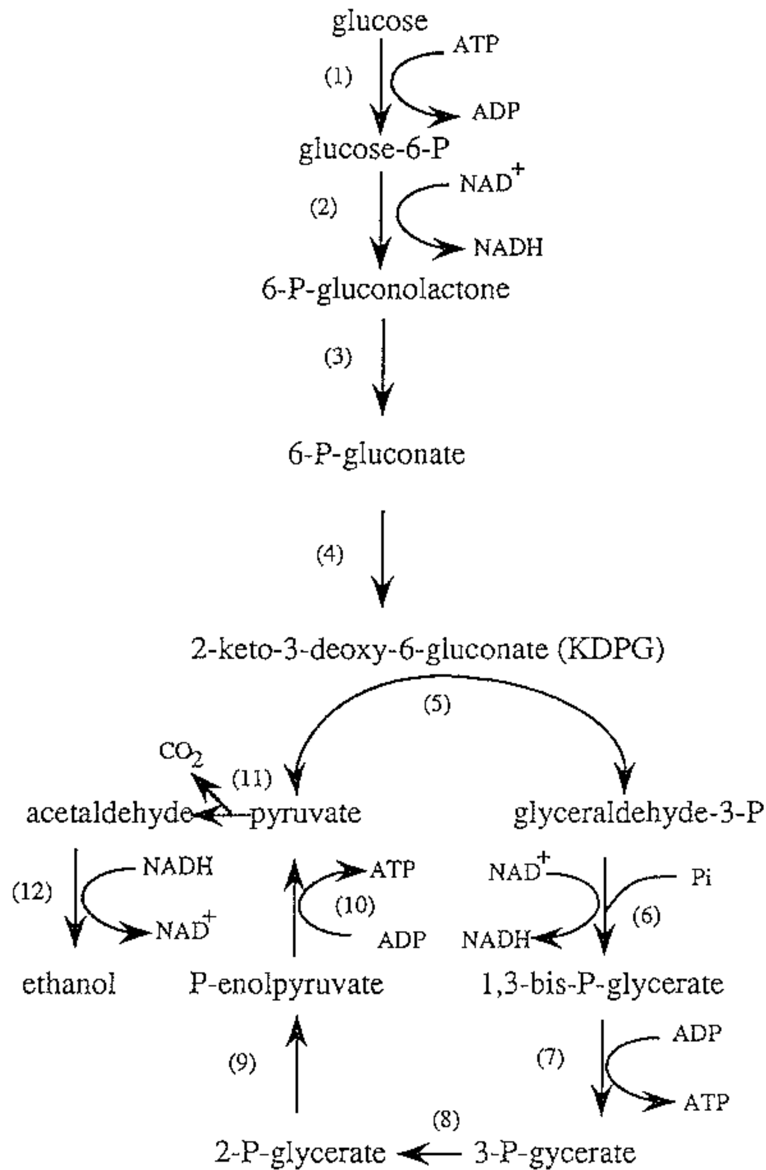


Fig. 1.1: Entner-Doudoroff pathway (EDP)

(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.

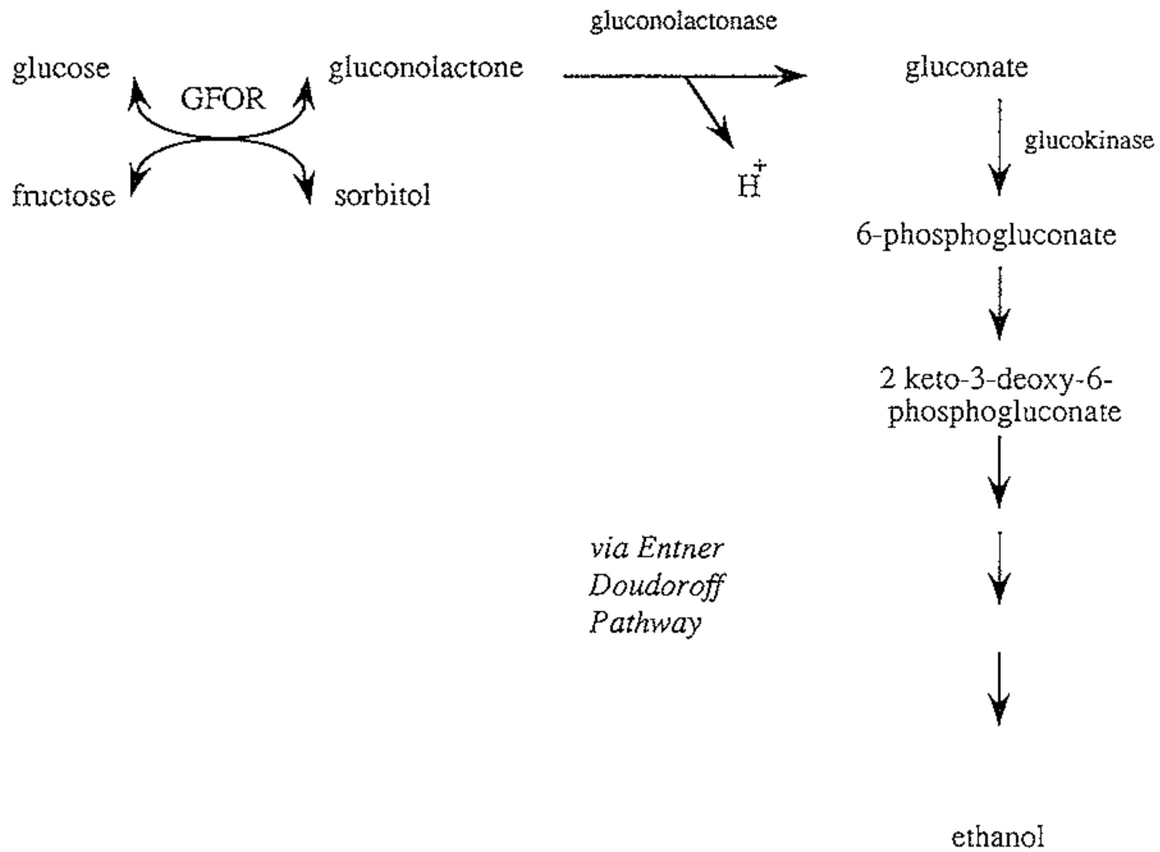


Fig. 1.2: Mechanism of production of sorbitol and gluconate

(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 oxiddoreductase (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 ß-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 ß-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 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

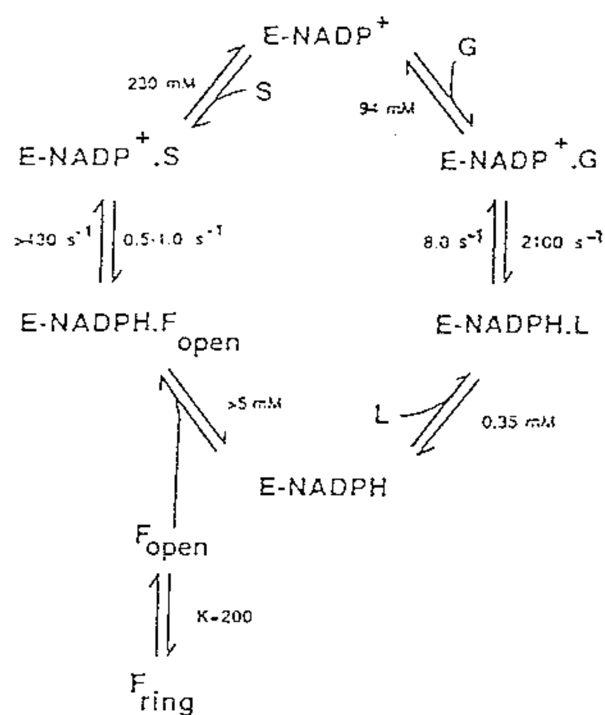


Fig 1.3 The kinetic mechanism for glucose-fructose oxidoreductase

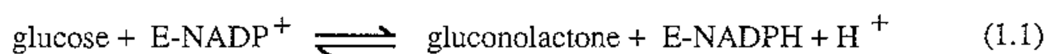
(Hardman and Scopes, 1988)

G = glucose, L = gluconolactone, E = enzyme,

$F_{open,\ ring}$ = fructose in acyclic and ring form respectively

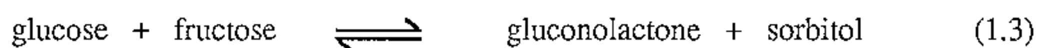
overlap to represent a single overall binding site. Glucose and gluconolactone probably bind to the enzyme in the pyranose ring form, while sorbitol and fructose bind in an acyclic form (Hardman and Scopes, 1988).

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



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

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



$$\text{glucose} + \text{fructose} \rightleftharpoons \text{gluconolactone} + \text{sorbitol} \qquad (1.3)$$

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 ± 0.05 s^{-1} and 0.303 ± 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.