MECHANISTIC STUDIES ON

GLUCOSE-FRUCTOSE OXIDOREDUCTASE

FROM *Zymomonas mobilis*

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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.

ACKNOWLEDGEMENTS

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Finally, I wish to thank my family and all my friends for making my life enjoyable.

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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

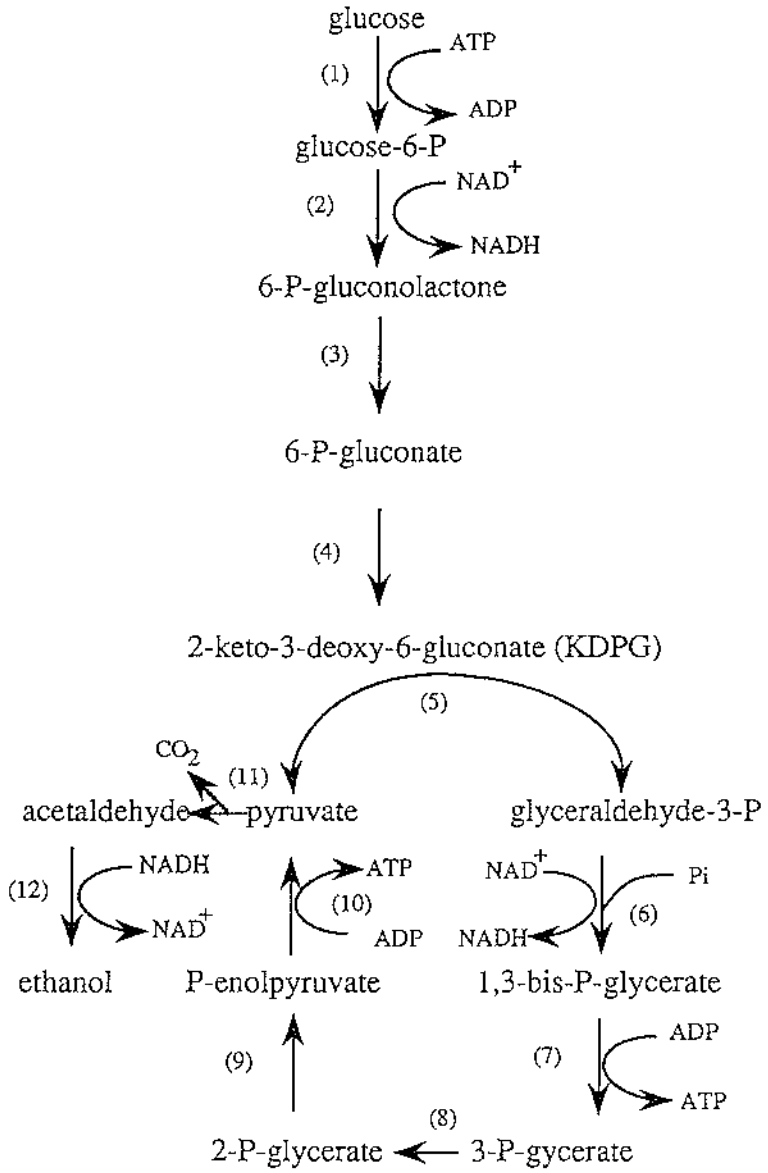

Fig. 1.1: Entner-Doudoroff pathway (EDP)

(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.

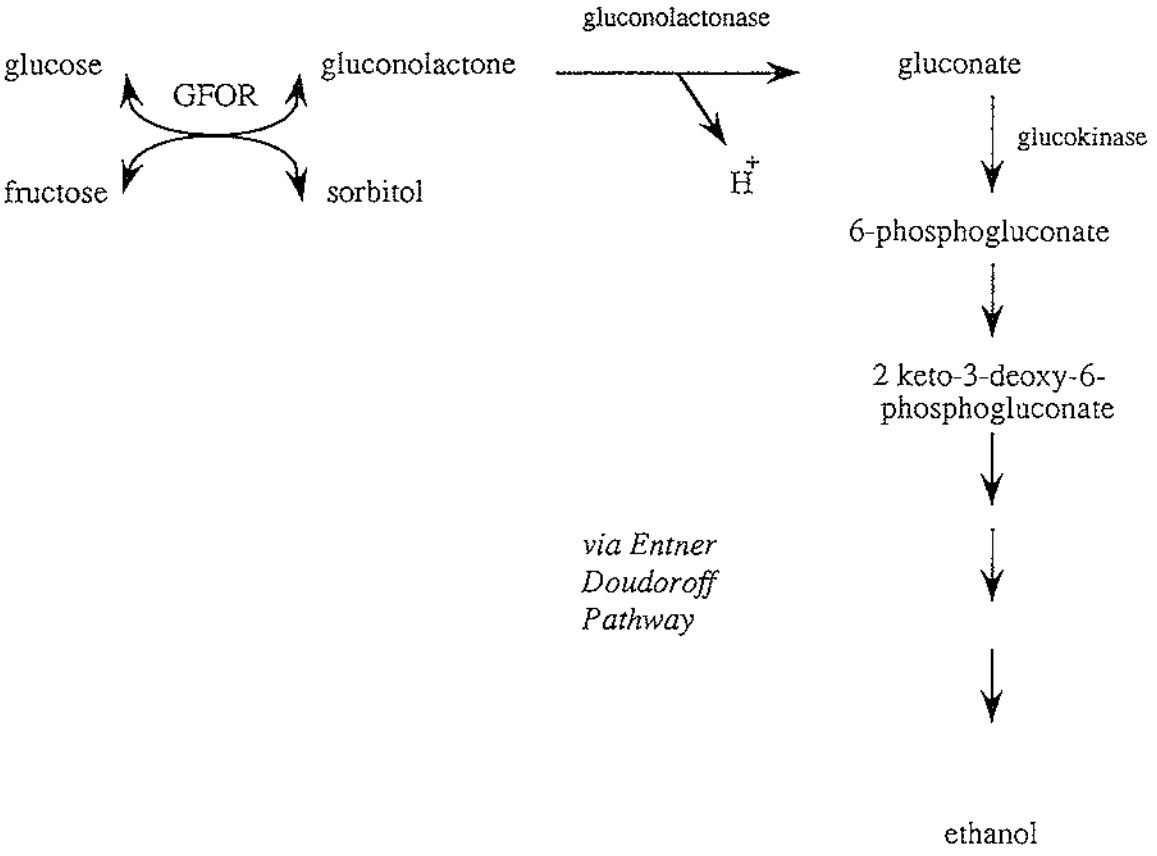

Fig. 1.2: Mechanism of production of sorbitol and gluconate

(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 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

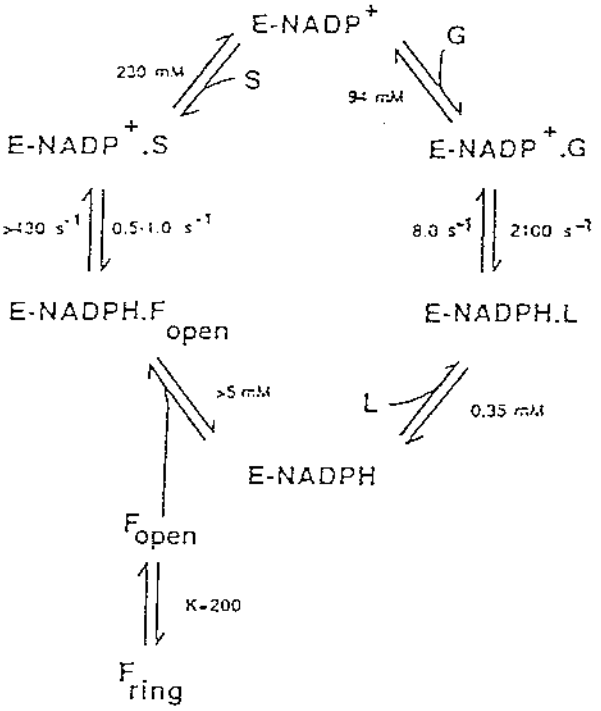

Fig 1.3 The kinetic mechanism for glucose-fructose oxidoreductase

(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.

CHAPTER 2
ENZYME PURIFICATION

2.1 Introduction

Glucose-fructose oxidoreductase was purified using a modification of the method of Zachariou and Scopes (1986). The original method produced low glucose-fructose oxidoreductase activity when I did it. The modifications involved enzyme extraction, purification and storage.

Glucose-fructose oxidoreductase (GFOR) was purified by two steps: dye-ligand chromatography, followed by hydrophobic chromatography, which replaced ion-exchange chromatography. The principle of dye-ligand chromatography has already been discussed in Chapter 1. Hydrophobic chromatography is similar to salting-out precipitation of proteins. Both methods involve using high salt concentrations, particularly of larger salts, such as ammonium sulphate. These salts increase the hydrophobic interaction between protein molecules. Hydrophobic columns consist of long linear aliphatic chains with various lengths (C_4 , C_6, C_8, and C_{10}) attached to Sepharose (Scopes, 1987). The spacer arm may contain various substituents, such as phenyl, amino, hydroxyl and even thiol groups.

A differential chromatography (tandem column) system for protein purification using hydrophobic columns, similar to that for dye ligands, has been developed by Scopes and Porath (1990). There are a range of hydrophobic adsorbents with subtly different structures, which will selectively bind different proteins. However, the hydrophobic adsorbents will bind different proteins from those which will bind to the dye ligands. In this project, only one hydrophobic column was used.

Ion exchange chromatography was employed instead of hydrophobic chromatography for purifying GFOR for one sorbitol stopped-flow experiment only (refer to Chapter 3).

2.2 Materials and Methods

2.2.1 Materials

Initial *Zymomonas mobilis* innoculum, Procion Green H-4G dye, and Thio C_4 hydrophobic column adsorbent were from Dr. Robert K. Scopes, La Trobe University, Australia. Thio C_4 column was made by synthesizing thiolated agarose from epichlorohydrin-activated agarose and dithioerythritol, then coupling the alkyl group ligand to the thio-activated matrix (Scopes and Porath, 1990). The carboxyl methyl-Sepharose CL-6B was from Pharmacia Ltd, Sweden.

Some gluconolactonase samples were from Dr. R.K. Scopes, and the rest of the gluconolactonase used in this project was from a side step during glucose-fructose oxidoreductase purification.

D-Glucose was from United States Biochemical Corporation, Cleveland, Ohio. D-sorbitol, D-gluconic acid lactone (gluconolactone), and D-fructose were from Sigma Chemical Company. MES (N-Morpholino ethanesulphonic acid) was from Sigma Chemical Company. All other reagents were of analytical grade.

2.2.2 Methods

2.2 2.1 MES Stock Buffer

The 100 mM stock buffer was prepared by first dissolving 6 g of KOH, 25 g of MES and

22 g of KCl in about 800 ml of water. Then the pH was adjusted to 6.5 with more MES or 5M acetic acid. Before the volume was made up to 1 litre, 0.5 g of azide and 4.07 g of $MgCl_2.6H_20$ were added. This concentrated stock buffer was diluted ten fold before use. The pH of the buffer was adjusted to pH 6.0 or 5.5 with 5 M.acetic acid.

2.2.2.2 Enzyme Assay

The enzyme activity of glucose-fructose oxidoreductase was measured by the decrease in absorbance of p-nitrophenol at 400 nm on a Cecil spectrophotometer. p-Nitrophenol was an indicator for the proton released when gluconolactone was converted to gluconate by gluconolactonase (Fig. 1.2). Excess amounts of gluconolactonase were added to ensure the proton releasing step was not rate limiting. The enzyme assay was carried out at 25°C and pH 6.5 (Zachariou and Scopes, 1986). Gluconolactone was formed by the action of GFOR which converted glucose (0.4 M) and fructose (0.8 M) to gluconolactone and sorbitol.

2.2.2.3 Protein Assay

Two methods were used to measure the protein concentration.

(a) Absorbance at 280nm:

This was a fast method of measuring protein concentration used for following the protein profile when eluting enzymes from a column. When the absorbance of the protein solution was greater than 1.0, the solution was diluted to give an appropriate reading.

(b) Coomassie Blue Method:

This method was used to measure the protein concentration accurately. Coomassie Blue solution was prepared by dissolving 600 mg of Coomassie Brilliant Blue G-250 in 1 litre of 2% perchloric acid (Sedmak and Grossberg, 1977; Scopes 1987). The Coomassie solution could be stored indefinitely at room temperature. The protein concentration of the sample was measured by adding 1.5 ml of protein sample to 1.5 ml of Coomassie Blue solution and the absorbance at 595 nm was measured. A standard curve for 0 - 50 μg of bovine serum albumin was used to standardise protein concentration. The curve was linear from 10 μg to 50 μg.

2.2.2.4 Culturing *Zymomonas mobilis*

The method was based on that of Zachariou and Scopes (1986). *Zymomonas mobilis* was grown at 30°C, pH 5.0 in 6 litre medium for 24 hours. The medium contained: 21% glucose (w/v), 0.5% yeast extract, 0.05% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2(SO_4).6H_2O$ (Swings and DeLey, 1984).

The bacterium was grown in a 12 litre steel bucket under nonsterile conditions. The medium was aerated by an overhead stirrer and the pH was monitored with a pH titrator.

The innoculum size was 5 -10 g wet cells or 400 ml of broth from the previous batch of *Z.mobilis*.. The cell growth stage was monitored in terms of the optical density change at 660 nm (Koch, 1981). Cells were harvested before they reached the stationary phase (Scopes, 1987). The cells were washed with pH 6.5 MES buffer and stored at -18 °C until required.

2.2.2.5 Extraction of GFOR

This was the final method used after various trials to find the best method for extracting the highest GFOR activity. The frozen *Z.mobilis* cells were thawed and added to the extraction medium (5 ml/g). The extraction medium contained 10 mM Tris base, 2 mM $MgCl_2$, 0.1% Nonidet, 0.5% toluene, one part of DNAase solution (1mg/ml) per 200 part of extraction medium and one part of lysozyme solution (50 mg/ml) per 200 part of extration medium. The enzymes were added after the extraction volume was adjusted to 1 g/ 5 ml.

The extraction medium was heated up to 40 $^{\circ}$C for 30 minutes, then cooled and the pH was titrated to pH 6.0 with solid MES before centrifugation at 9000 x g for 30 minutes. The *Z.mobilis* cells were resuspended in the supernatant before they were put through the French press. The cells were centrifuged for 30 minutes at 9000 x g to obtain the cell-free extract. The cell debris was discarded.

Extraction of gluconolactonase was similar to that of GFOR, except the heating at 40°C during detergent extraction was omitted. Instead of 30 minutes extraction, the detergent extraction was carried out overnight, followed by the French press.

2.2.2.6 Enzyme Purification

Enzyme purification was carried out at room temperature. The general scheme of glucose-fructose oxidoreductase purification is shown in Fig. 2.1. The two main GFOR purification steps were dye-ligand chromatography and hydrophobic chromatography. The enzyme extract was loaded onto two 10 cm^2 x 15 cm tandemly arranged dye columns at pH 6.0 in MES buffer. The Procion Green H4G column preceded the Remazol Brilliant Blue R8001 column. Then the columns were disconnected, and gluconolactonase was eluted with 30 mM potassium phosphate in pH 6.5 MES buffer.

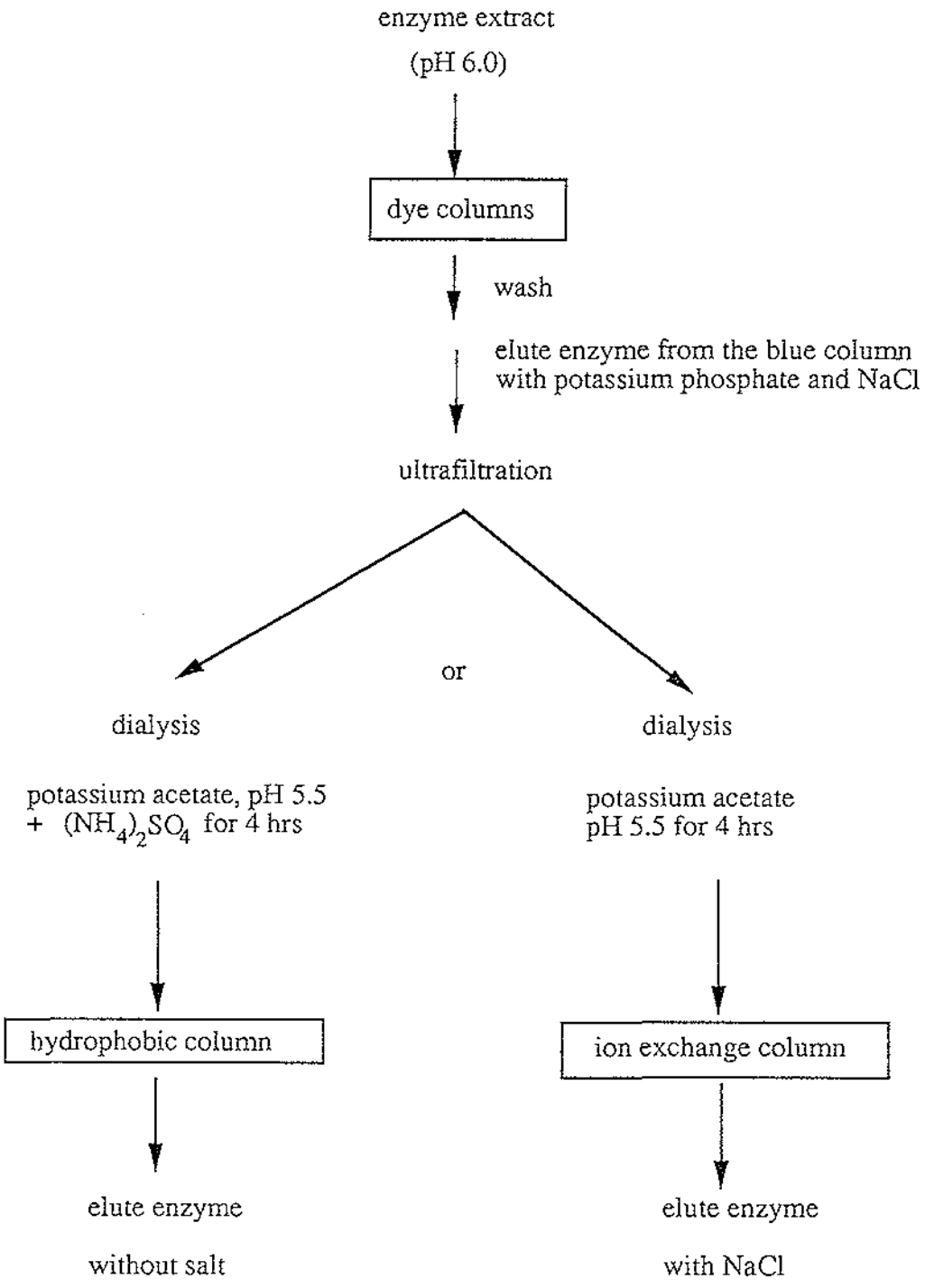

Fig. 2.1 Purification scheme for glucose-fructose oxidoreductase

The blue column was eluted with 30 mM potassium phosphate and 50 mM NaCl in pH 6.5 MES buffer, follow by the same buffer containing 100 mM NaCl. Glucose-fructose oxidoreductase was eluted with 100 mM NaCl(Zachariou and Scopes, 1986). The pooled enzyme from the Remazol Brilliant Blue R was ultrafiltered to 20 ml, then it was dialysed for 4 hours against 2 litres of 20 mM potassium acetate, 1M $(NH_4)_2SO_4$, pH 5.5 MES buffer.

The dialysed GFOR solution was loaded onto a 2.25 cm^2 x 8 cm thio C_4 -Sepharose hydrophobic column. The column was washed until no protein could be detected. Then GFOR was eluted by the same buffer, but without $(NH_4)_2SO_4$. The purified glucose-fructose oxidoreductase was pooled and stored at 5°C or -18°C until use.

Ion exchange was used instead of hydrophobic chromatography for the preparation of purified GFOR for one of the sorbitol stopped-flow experiments. This method was based on that of Zachariou and Scopes (1986). The ultrafiltered GFOR (from the blue column) was dialysed for 4 hours against 20 mM potassium acetate (pH 5.5) at room temperature before GFOR was loaded on the ion exchange column. Glucose-fructose oxidoreductase was eluted with 0.2 M NaCl in 20 mM potassium acetate (pH 5.5).

2.2.2.7. SDS Gel Electrophoresis

The method was based on that of Laemmli (1970). The protein was dissociated to subunits by heating with 6 M urea, 1% sodium dodecyl sulphate (SDS), 5% 2-ß-mercaptoethanol at 100°C for 10 minutes. Bromophenol blue was added to the incubation mixture as a dye marker. The digested samples were loaded onto an 8% acrylamide slab gel . The molecular markers were: catalase (58 kD), ß-galactosidase (130 kD), malate dehydrogenase 37 kD), human transferrin (80 kD), bovine albumin (60 kD), egg albumin (45 kD).

The proteins were stained with Coomassie Brilliant Blue R-25 dye, methanol and acetic acid overnight at room temperature or for 1 hour at 60°C. The gel was destained with methanol and acetic acid.

2.3 Results

2.3.1 Culturing *Zymomonas mobilis*:

The bacterium grew for 24 hours before it reached the stationary phase (Fig.2.2). The cell yield was usually between 100 g and 120 g per 6 L. The lag phase depended on the innoculum size, being shorter with a larger innoculum.

2.3.2 Efficiency of Glucose-fructose Oxidoreductase Extraction

I found that the usual detergent method of GFOR extraction used by Zachariou and Scopes (1986) gave low glucose-fructose oxidoreductase (GFOR) activity. Therefore, various conditions of detergent enzyme extraction were tested:

(a) The usual detergent method as described in the method section (section 2.2.2.5). But it does not involve French press.

(b) The same as in (a), except the extraction medium was titrated with concentrated acetic acid instead of MES.

(c) The same as in (a), except the bacterial cells were added to the extraction volume before the exact extraction volume was made up to 1 g/ 5 ml.

(d) The same as in (a), except the DNAase and lysozyme were added before the Nonidet and toluene.

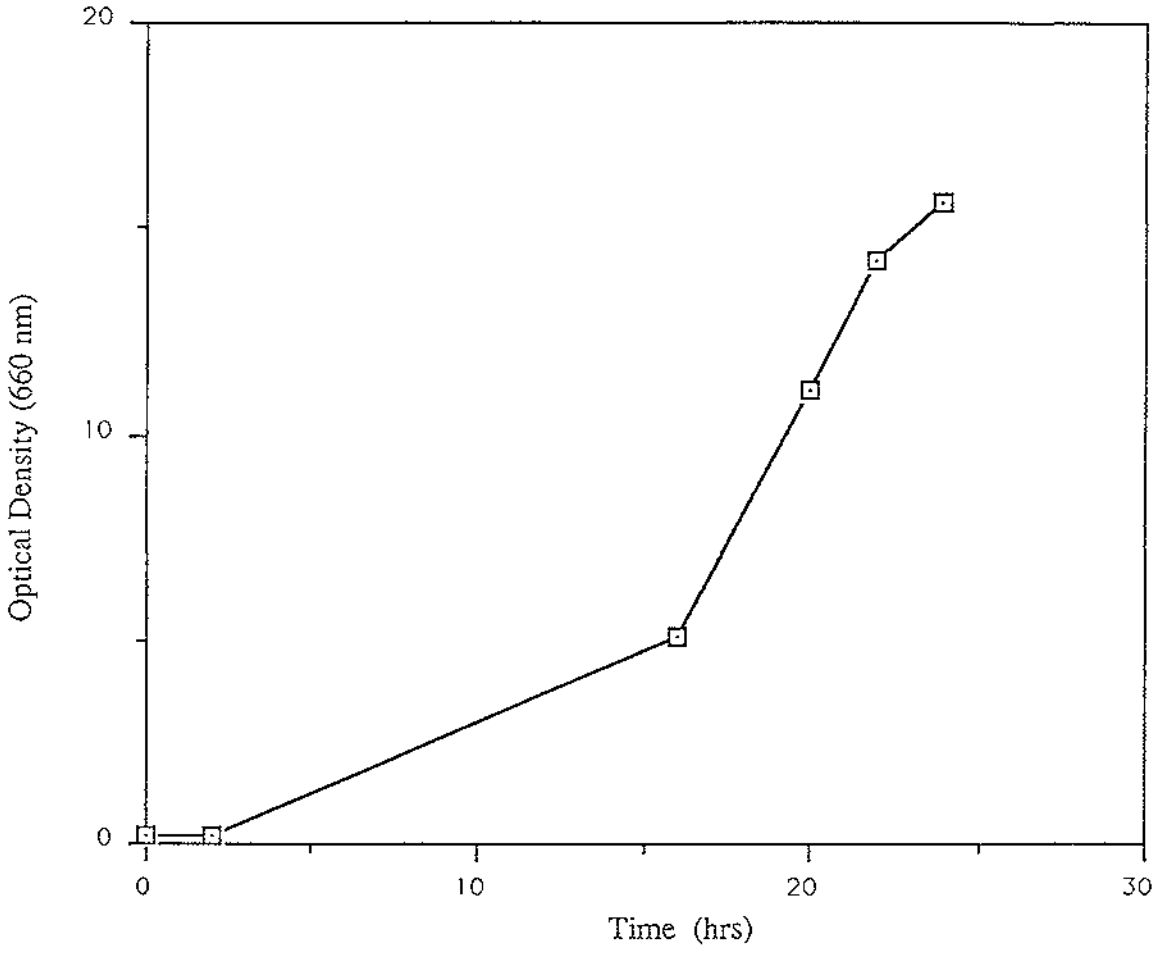

Fig. 2.2 Growth curve of *Zymomonas mobilis*

cells grown for 24 hours at pH 5.0, 30 °C,

21 % glucose, aerobic conditions

(e) The same as in (a), except the pH was titrated to 6.0 after centrifugation, instead of before centrifugation.

(f) The method is the same as in the method section.

(g) This method involved only the French press. The *Z.mobilis* cells were resuspended in pH 6.0 MES buffer and no detergent or DNAse and lysozyme was used.

The results in Table 2.1 showed that GFOR activity was related to total protein concentration: the higher the protein concentration, the higher the enzyme activity. Methods involving the French press gave the highest GFOR activity and specific activity. The method which involved both detergent and French press (f) was used for enzyme extraction instead of the method using French press only (g), because method (f) gave lower protein concentration than method (g). Protein loading is important in dye-ligand chromatography (refer to section 1.2).

2.3.3 Enzyme Stability and Storage

2.3.3.1 Buffer and pH Dialysis

In the original method, $(NH_4)_2SO_4$ precipitation was used for concentrating the GFOR solution eluting from the blue column, and the proteins were resuspended in pH 5.5 acetate buffer with 1M $(NH_4)_2SO_4$. But $(NH_4)_2SO_4$ precipitation caused significant loss of enzyme activity. Therefore, an alternative method of preparing an enzyme solution for the hydrophobic column was required. The enzyme pool eluted from the blue column was ultrafiltered and dialysed overnight against different buffer conditions as shown below:

(A) 20 mM potassium acetate, pH 5.5;

(B) 20 mM potassium acetate, 1M $(NH_4)_2SO_4$, pH 5.0;

(C) 20 mM potassium acetate, 1M $(NH_4)_2SO_4$, pH 5.5;

(D) 20 mM potassium acetate, 1M $(NH_4)_2SO_4$, pH 6.0;

(E) MES, pH 5.5;

(F) MES, pH 6.0;

(G) MES, 1M $(NH_4)_2SO_4$, pH 5.5.

After dialysis, the total activity, conductivity and precipitation of the enzyme solutions were measured. Table 2.2 showed that dialysis induced precipitation of protein, particularly at low pH and in buffers containing $(NH_4)_2SO_4$. To alleviate the precipitation problem, the enzyme sample should be dialysed for a shorter period of time.

2.3.3.2 Storage Stability of GFOR

Both enzyme extract and purified enzyme were tested for stability at different storage temperatures. The enzyme activity of the enzyme extract was measured at hourly intervals at two temperatures: 25°C and 5°C. The purified enzyme was stored at three temperatures: 25°C, 5°C, -18°C (frozen). The enzyme activities were measured over the period of days.

Glucose-fructose oxidoreductase from enzyme extract was not stable over a short period of time. After 24 hours, almost all of the oxidoreductase activity was lost at both

TABLE 2.1 Extraction of glucose-fructose oxidoreductase from *Zymomonas mobilis* under different conditions.

Method	GFOR Activity (μmol min^{-1} ml^{-1})	[Protein] (mg/ml)	Specific Activity (μmol min^{-1} mg^{-1})
(a) usual detergent extraction	6.8	8.0	0.85
(b) acetic acid titration	11.16	8.0	1.40
(c) extraction volume adjusted with cells	10.15	16	0.63
(d) enzyme added before detergent	3.95	8.8	0.42
(e) titate pH after spinning	12.36	11.6	1.07
(f) detergent + French press	26.69	14.8	1.8
(g) French press only	33.7	20	2.7

TABLE 2.2 Stability of glucose-fructose oxidoreductase during dialysis under different conditons. The enzyme was dialysed overnight at 25 °C

Conditions	Total Activity after dialysis (μmol min^{-1} ml^{-1})	Conductivity (m MHO)	Precipitation
(A) no dialysis MES,pH 6.5	9.08	9.0	-
(B) potassium acetate + $(NH_4)_2SO_4$ pH 5.5	4.87	14	heavy
(C) potassium acetate + $(NH_4)_2SO_4$, pH 5.5	5.84	15	heavy
(D) potassium acetate $(NH_4)_2SO_4$, pH 6.0	6.97	13	moderate
(E) potassium acetate, pH 5.5	8.39	1.1	heavy
(F) MES pH 5.5	8.33	2.7	moderate
(G) MES pH 6.0	9.75	2.7	moderate
(H) MES, $(NH_4)_2SO_4$, pH 5.5	6.23	19	modetrate

temperatures: 25°C and 5°C (Fig 2.3). Therefore, the enzyme extract was loaded onto the dye columns immediately after extraction.

Glucose-fructose oxidoreductase was much more stable after purification than in the enzyme extract. The purified oxidoreductase was stable in solution at 5°C and -18°C for several days (Fig 2.4) The enzyme stored at 25°C showed a steady decline in activity, and after 25 days the enzyme activity was completely lost. The best condition to store the purified enzyme was at -18°C. Thawing and refreezing did not affect the oxidoreductase activity.

The rate of loss of activity of unpurified glucose-fructose oxidoreductase was much faster than that of the purified enzyme. This was probably due to the presence of proteases in the enzyme extract, which digested the oxidoreductase as well as other proteins. These proteases were removed during the purification stage.

2.3.4 Enzyme Purification:

2.3.4.1 Dye-ligand Chromatography:

Glucose-fructose oxidoreductase was eluted from the Remazol Brillian Blue column with 30 mM potassium phosphate and 100 mM NaCl (Fig. 2.5). Gluconolactonase was not present in this enzyme preparation because heat treatment was used in the enzyme extraction. The GFOR activity peak shown in Fig 2.5 is broad, compared to that of Zachariou and Scopes (1986). The oxidoreductase activity peak could be reduced to a smaller volume by eluting the enzyme with 0.5 M NaCl as reported by Zachariou and Scopes (1986). Because the enzyme extract contained a higher protein concentration than that of Zachariou and Scopes (1986), 0.5 M NaCl elution may elute more of other proteins as well, thus reducing the purity of the glucose-fructose oxidoreductase.

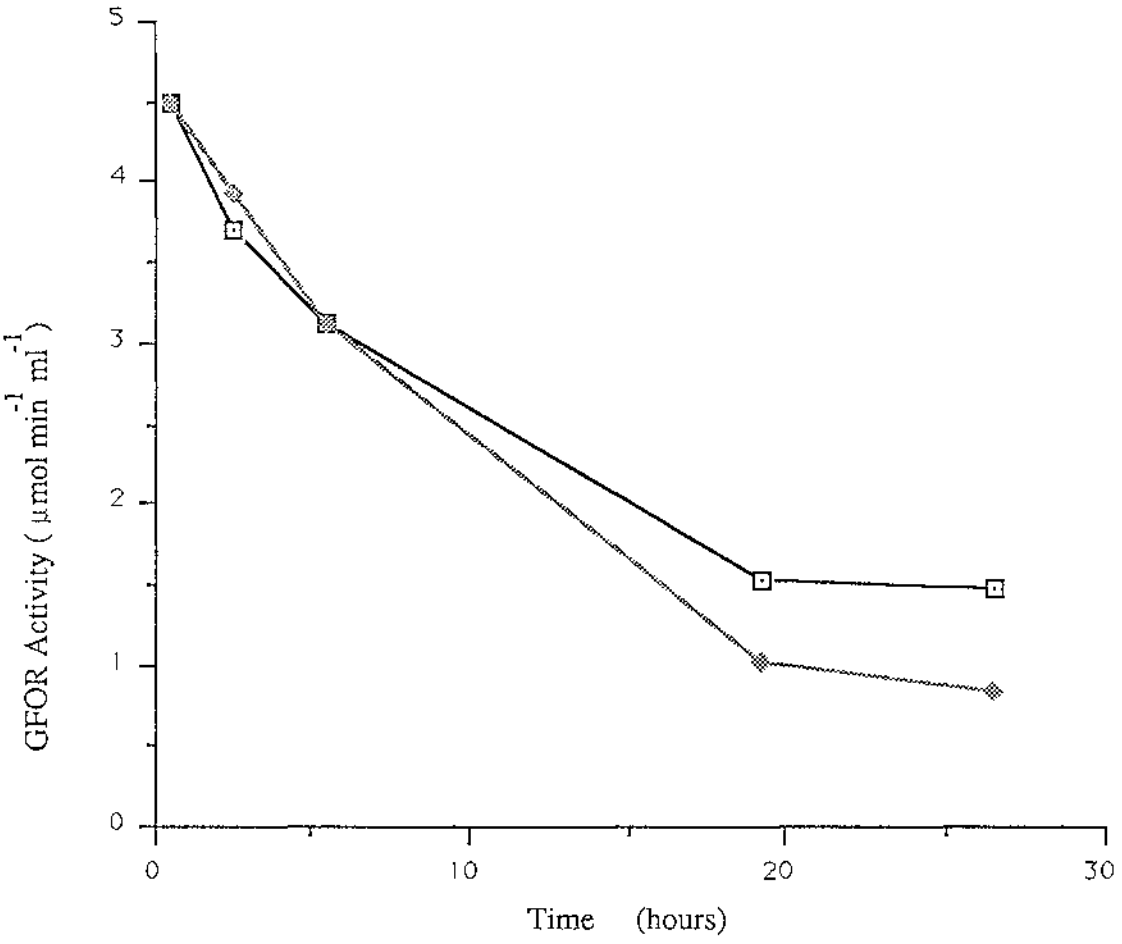

Fig. 2.3 The stability of glucose-fructose oxidoreductase of enzyme extract over a period of time.

25° C, 5° C

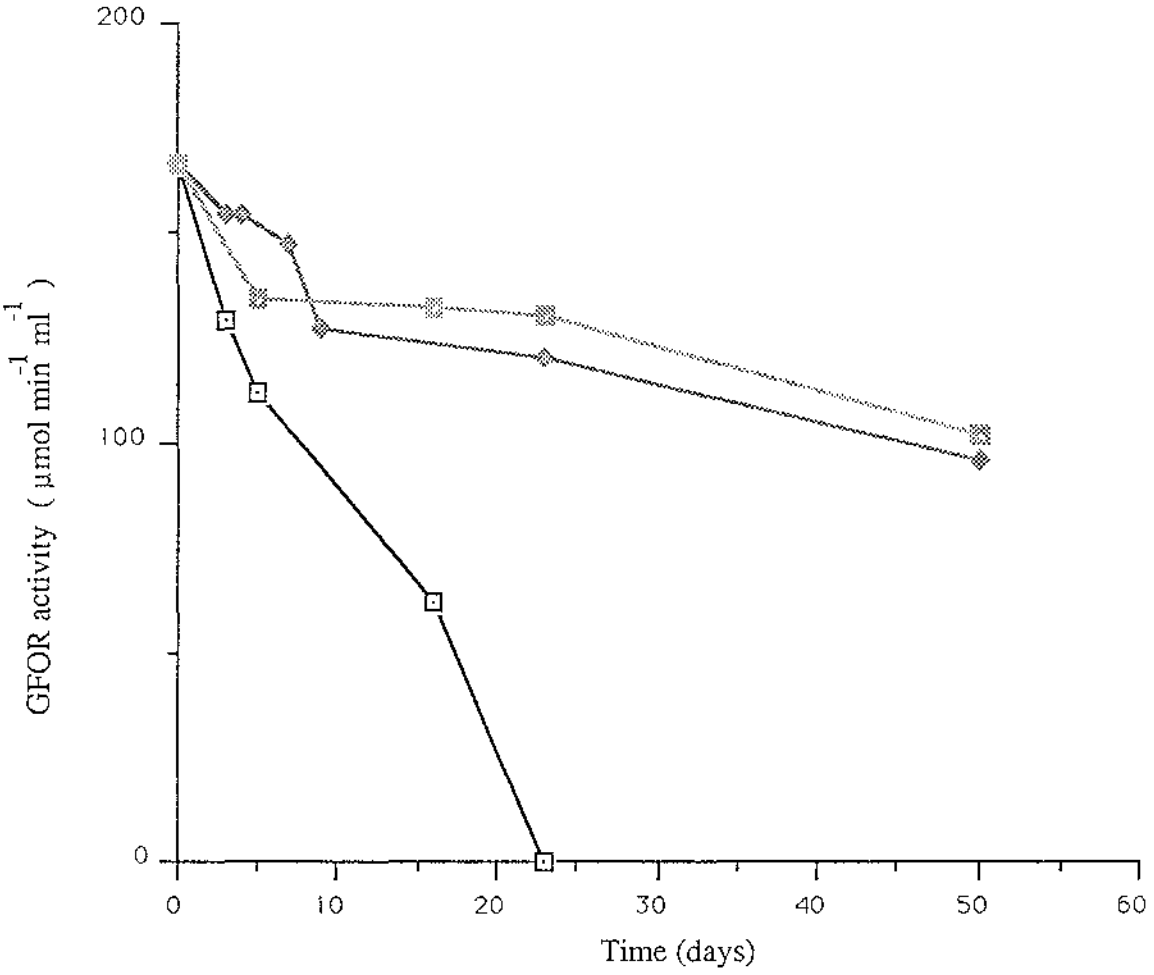

Fig 2.4 The stability of purified glucose-fructoseoxidoreductase over a period of time.

25°C, 5°C, -18°C

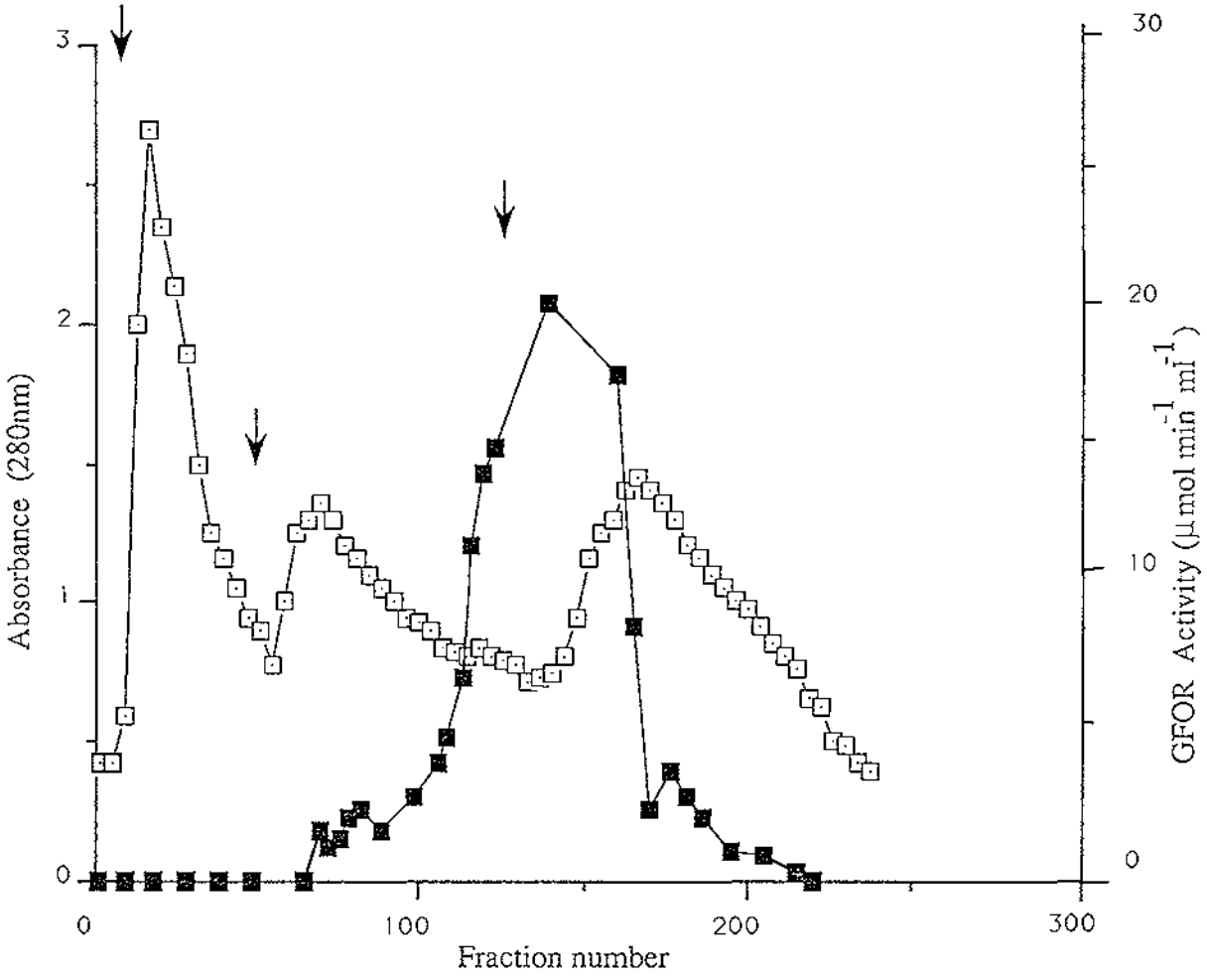

Fig. 2.5 Elution profile of glucose-fructose oxidoreductase (GFOR) from the Remazol Brilliant Blue R8001 column

The protein was eluted with 30mM potassium phosphate (KPi), 30mM KPi + 50 mM NaCl, and 30 mM KPi + 100 mM NaCl consecutively (shown by the arrows).

The column was run at 25° C, pH 6.5, flow rate 3.6 ml/min,

□ absorbance at 280 nm,

■ GFOR activity

2.3.4.2 Hydrophobic Chromatography:

The main principle of this enzyme purification is clearly shown to be selective binding (Fig. 2.6). Most of the protein did not bind to the thio C_4-Sepharose hydrophobic column. Glucose-fructose oxidoreductase was eluted in a small volume, well separated from the main protein peak. The purified enzyme was stored at $5^{\circ}C$ for short term use; for long term use, the oxidoreductase was stored at $-18^{\circ}C$.

2.3.4.3 Summary of Glucose-fructose Oxidoreductase Purification

This modified purification method for glucose-fructose oxidoreductase showed a similar purity of the purified enzyme to that of Zachariou and Scopes (1986) (Table 2.3). The final GFOR specific activity was 218 μmol min^{-1} mg^{-1}, compare to 200 μmol min^{-1} mg^{-1} of Hardman and Scopes (1986). But this modified enzyme extraction method produced a higher yield of glucose-fructose oxidoreductase from a similar amount of *Z.mobilis* cells. Also, the recovery of the final step of enzyme purification was much higher (73%) in the modified method than in the original method of Zachariou and Scopes (1986) (34%)

The purity of glucose-fructose oxidoreductase from the Remazol Blue R column was much lower than that obtained by Zachariou and Scopes (1986). A possible reason for this was that the protein composition of the French press extract was different from that of the detergent extract. Therefore, the types of protein binding to the dye columns were different. This may cause a change the in elution profile of GFOR and other proteins . For instance, GFOR was eluted outside the protein peaks and at different places from that of Zachariou and Scopes (1986). More proteins remained bound to the blue column after 50 mM NaCl elution in the modified system than in the system of Zachariou and Scopes (1986).

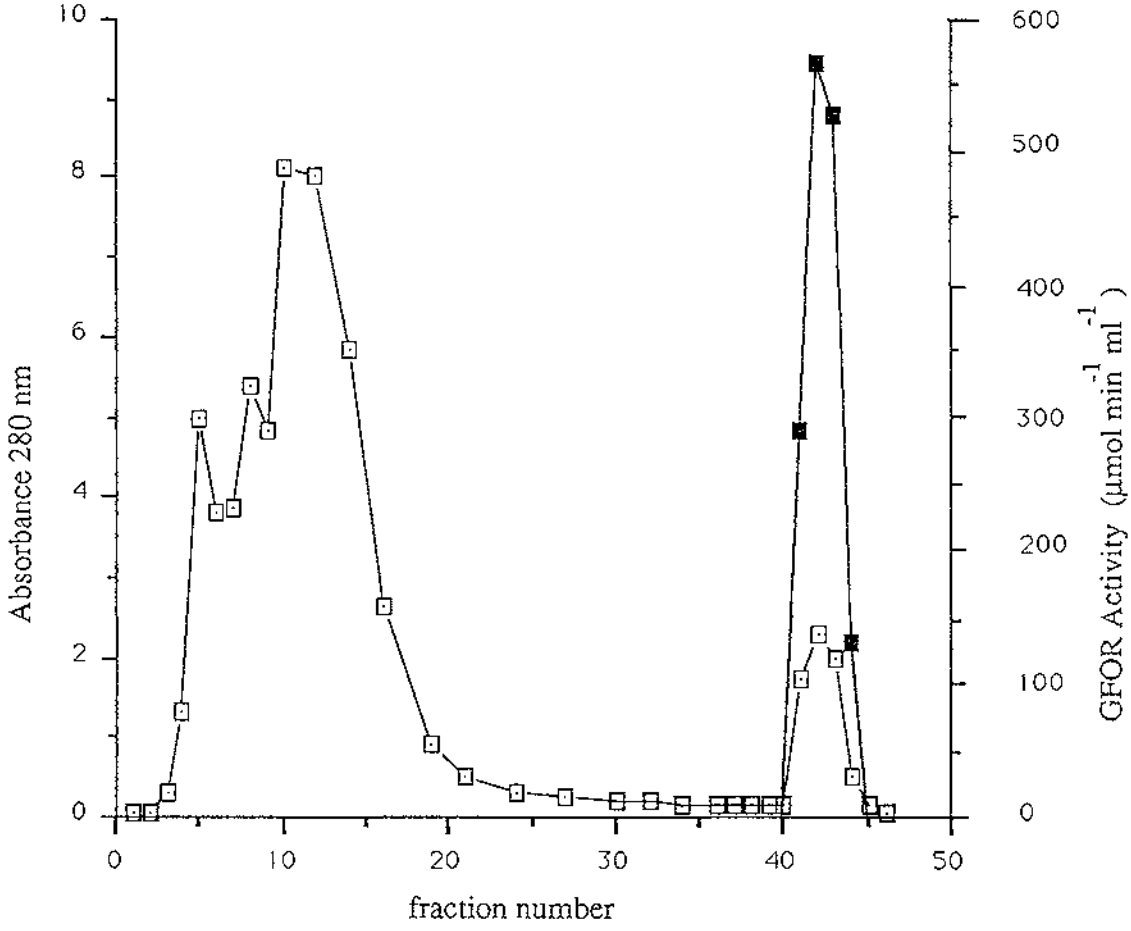

Fig. 2.6 Elution profile of glucose-fructose oxidoreductase (GFOR) from the Thio C_4 - Sepharose column

Glucose-fructose oxidoreductase was stepwise eluted by omitting 1 M $(NH_4)_2SO_4$ from the 20 mM potassium acetate buffer, pH 5.5.

The column was operated at pH 5.5, 25° C and a flow rate of 1.5 ml/min

-□- absorbance at 280 nm

-■- GFOR activity

TABLE 2.3 Summary of glucose-fructose oxidoreductase purification for 67 g of wet *Zymomonas mibilis* cells

Samples	Protein (mg)	Total Activity (μ mol min^{-1} ml^{-1})	Specific Activity	% Recovery
enzyme extract	6097	6935	1.1	100
not bound to dye columns	4420			
30 mM potassium phosphate + 100 mM NaCl	1647	6259	3.8	90
ultrafiltered GFOR	258	6031	23.4	87
dialysis with 1M $(NH_4)_2SO_4$ + 20 mM potassium acetate	120	5422	45.2	78
off C_4 column	23	5021	218	73

2.3.4.4 Ion Exchange Chromatography:

Ion exchange chromatography on CM-Sepharose was used in purifying the oxidoreductase for one sorbitol stopped-flow experiment. Glucose-fructose oxidoreductase was eluted by stepwise elution (Fig. 2.7) instead of a salt gradient, because salt gradient elution gave a broad oxidoreductase elution peak (unpresented results). Ion exchange chromatography gave lower GFOR purity than hydrophobic chromatography.

2.3.5 SDS Gel of Purified Enzyme:

The purity of purified glucose-fructose oxidoreductase from the hydrophobic chromatography was checked by SDS gel electrophoresis. The gel showed a single protein band with Coomassie staining (Fig. 2.8).

2.4 SUMMARY

The results showed that the modified enzyme extraction method produced much higher GFOR activity than the original method of Zacharious and Scopes (1986). However, the modified method gave a different pattern of protein bound to the dye columns as shown by the low specific activity at this stage.

Glucose-fructose oxidoreductase was unstable in the enzyme extract at room temperature, but remained stable for months at low temperature after purification. Ammonium sulphate precipitation was not used to store the oxidoreductase, because when the enzyme solution was redissolved, significant GFOR activity was lost.

Dye-ligand chromatography does have the advantages as claimed. For instance, it was rapid, inexpensive, reliable, simple and stable (Atkinson *et al.*., 1981). However, there

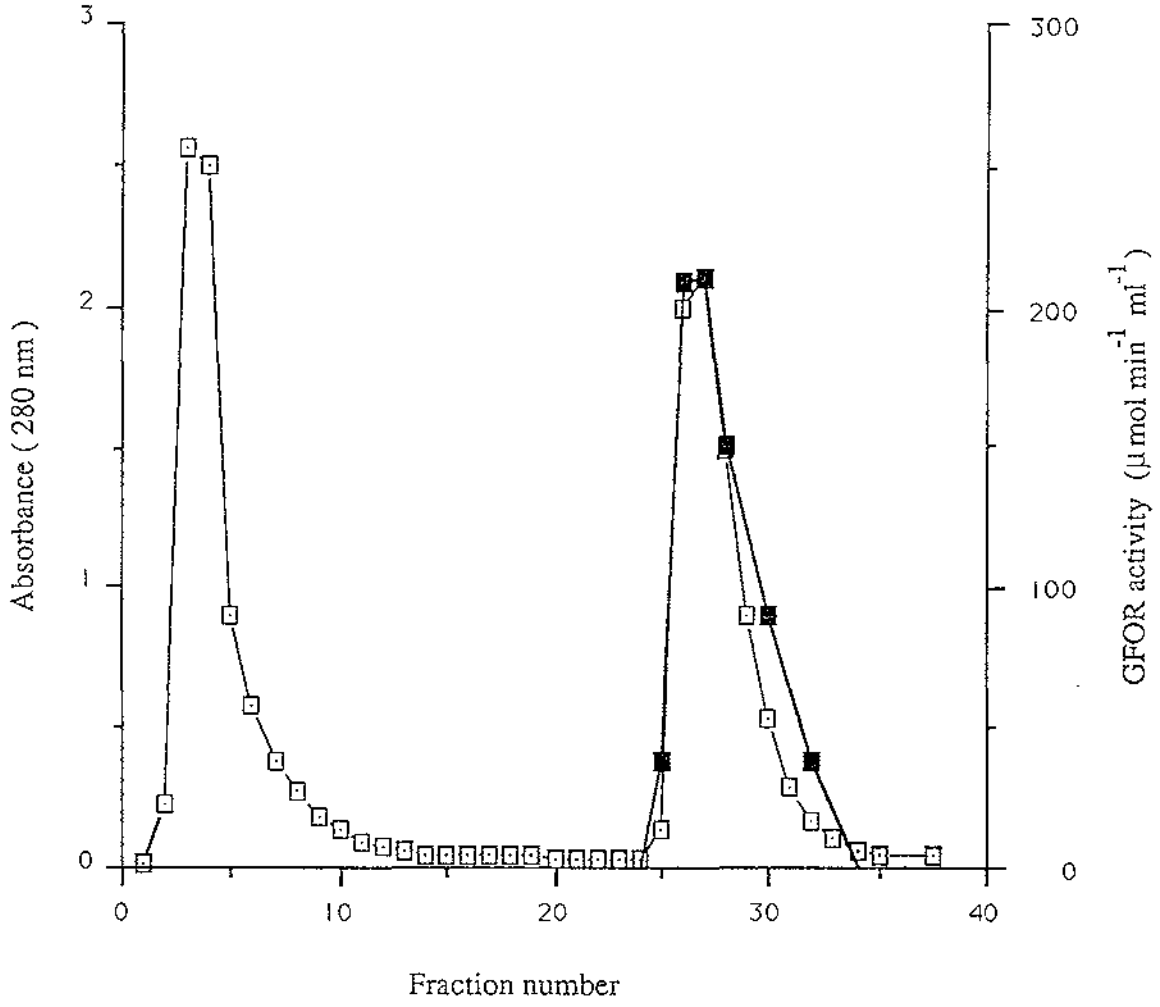

Fig 2.7 Elution profile of glucose-fructose oxidoreductase from CM-Sepharose CL-6B

Glucose-fructose oxidoreductase (GFOR) was eluted stepwisely by 0.2 M NaCl. The buffer was 20 mM potassium acetate, pH 5.5, and the column flow rate was 1.2 ml/min . The enzyme was purified at 25 °C.

-□- absorbance at 280 nm, -■- GFOR activity

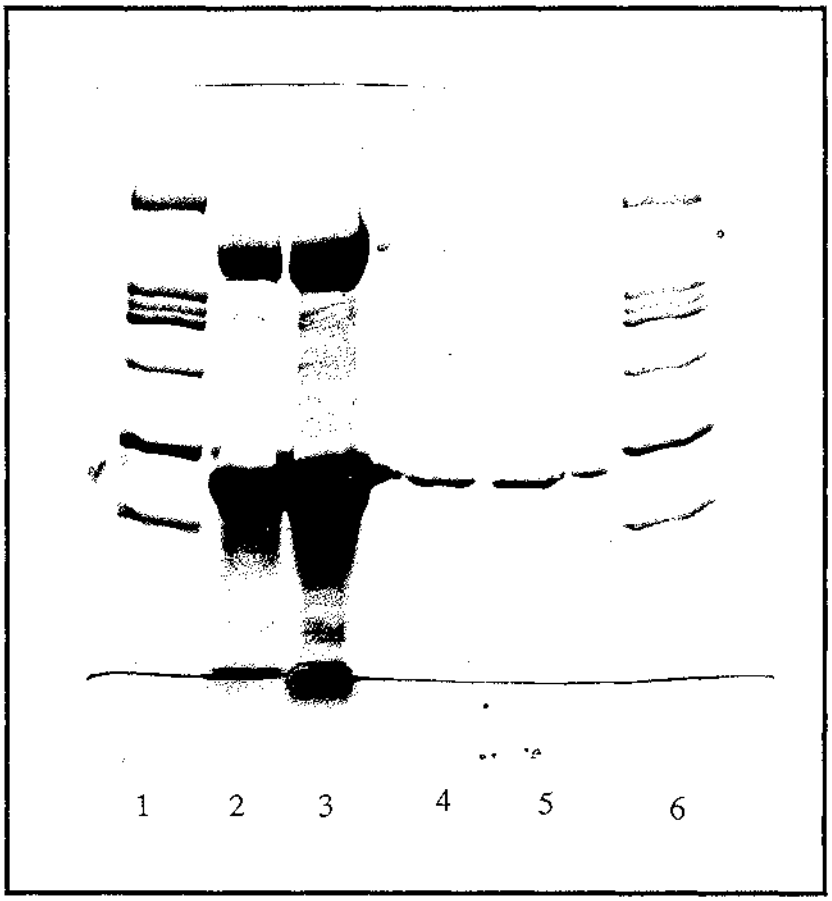

Fig. 2.8 SDS gel of purified glucose-fructose oxidoreductasse

An 8% polyacrylamide gel was run for 4-5 hours and was stained with Coomassie blue overnight.

1 & 6 = molecular weight markers

2 & 3 = purified GFOR from Dr. R.K. Scopes (overloaded)

4 & 5 = My purified GFOR

are pitfalls. Because a high NaCl concentration was not enough to clean the dye columns, a high concentration of urea/NaOH was used to wash the columns. This caused the problem of losing binding sites over a long period. The loss of binding sites led to inconsistency of enzyme elution. Glucose-fructose oxidoreductase was eluted off the Remazol Brillian Blue column earlier as the dye column aged.

Another drawback with dye-ligand chromatography was the stringency for protein loading (Scopes, 1986). When the protein loading was too low, the oxidoreductase bound to the Procion Green (negative column). However, when the protein loading was too high, the oxidoreductase was eluted earlier and the enzyme solution contained more other proteins.

CHAPTER 3

PRESTEADY STATE KINETIC STUDIES ON GLUCOSE-FRUCTOSE OXIDOREDUCTASE

3.1 Introduction

Presteady state kinetics were used here andby Hardman and Scopes (1988) instead of steady state kinetics to study the reaction mechanism catalysed by glucose-fructose oxidoreductase (GFOR). This is because using steady state kinetics we can only obtain the overall rate or rate determining step of the reaction, not the rate constants of individual reactions (Fersht, 1977). The reaction intermediates may be detected and the rates of individual reactions obtained indirectly from steady state kinetics, but may not be detected directly, because the intermediates usually have very fast rates of formation and decay.

Although the presteady state technique is superior in elucidating enzyme reaction mechanisms, steady state kinetics are more important in understanding the overall situation of the cell metabolism and it is the overall catalytic activity of the enzyme which is important in the cell (Fersht, 1977). Because steady state kinetics measure product formation or substrate decay, where these species are at high concentration, only a low enzyme concentration is required. A high enzyme concentration is required for presteady state experiments, since the presteady state method detects enzymatic intermediates and the concentrations of these intermediates are usually very low. They can be detected only if they possess a physical property which can be measured, for example, absorbance or fluorescence.

Absorbance measures the light absorbed by a solution, whereas fluorescence involves detection of light re-emitted at longer wavelength by the absorbing species. Fluorescence is theoretically about 100 times more sensitive than absorbance (Fersht, 1977), but in practice the relative sensitivity is much lower. The greater sensitivity is because fluorescence is measured at right angles to the exciting beam against a dark background, whereas absorbance involves detecting a small change in the transmitted light (Fersht, 1977). The error or fluctuation in the intensity of the incident light will be magnified in the absorbance signal. For fluorescence, the error in the signal is the same as the drift or error in the excitation intensity. Fluorescence experiments generally require much lower enzyme concentrations than absorbance experiments.

Fluorescence quenching is indicative of energy transfer from one group to another in the protein molecule (Fersht, 1977). Two examples of fluorescence quenching are the quenching of NADH fluorescence in aldehyde dehydrogenase (Dickinson, 1985) and in lactate dehydrogenase (Clarke *et al.*., 1989).

For dehydrogenases, absorbance detects only nucleotide ($NADP^+$ /NADPH, NAD^+/NADH) absorbance changes from converting one form to another. At 340 nm NADH and NADPH absorb strongly, while NAD^+ and $NADP^+$ do not. NAD(P)H also fluoresces strongly, but not $NAD(P)^+$. Fluorescence experiments can measure changes in both nucleotide and protein fluorescence and, hence, more changes in the protein can be detected.

This chapter is a continuation of the rapid kinetics work done by Hardman and Scopes (1988). The mechanism proposed for the reaction catalysed by glucose-fructose oxidoreductase on the basis of rapid kinetic studies using absorbance is shown in Fig. 1.3. The whole reaction can be divided into two reversible half reactions, with the reduction of E-$NADP^+$ by glucose or sorbitol and oxidation of E-NADPH by

gluconolactone or fructose. Because all four individual reactions involve changes between E-$NADP^+$ and E-NADPH, they can be studied separately.

Changes in the nucleotide fluorescence of the individual steps of the reaction catalysed by glucose-fructose oxidoreductase were compared with the nucleotide absorbance results obtained by Hardman and Scopes (1988) in order to study the reaction mechanism of GFOR. Protein fluorescence experiments were also carried out to compare with the nucleotide absorbance and fluorescence results.

3.2 Methods

3.2.1 Operation of the Stopped-flow Apparatus

All presteady state kinetic studies were carried out using a Durrum-Gibson D110 stopped-flow spectrophotometer (Durrum Instruments Corporation, Palo Alto, California, USA). This instrument was especially designed to study fast presteady state kinetic reactions (Fig. 3.1). The dead time (the age of the solution at the initial observation) is 2 to 3 ms. Therefore, reaction rate constants up to about 700 s^{-1} can be measured, although only the last quarter of the reaction will be observed for a rate constant of 700 s^{-1} ($t_{1/2}$ = 1 ms).

The samples are loaded into the drive syringes through the reservoir syringes. The top drive syringe is usually used for enzyme and the bottom syringe for substrates. The two solutions in the drive syringes are forced through a mixing jet by a compressed-air driven plunger and the solutions are mixed within 3 ms. When the flow of the solution makes the contact plate hit the rear trigger switch, data collection for the reaction is initiated. The absorbance and fluorescence of the solution are measured at the observation chamber (Fig. 3.2). The input wavelength is selected by a monochromator. A tungsten light source was used for absorbance experiments and a xenon lamp for fluorescence. In fluorescence experiments, the light output is recorded at right angles to the light source,

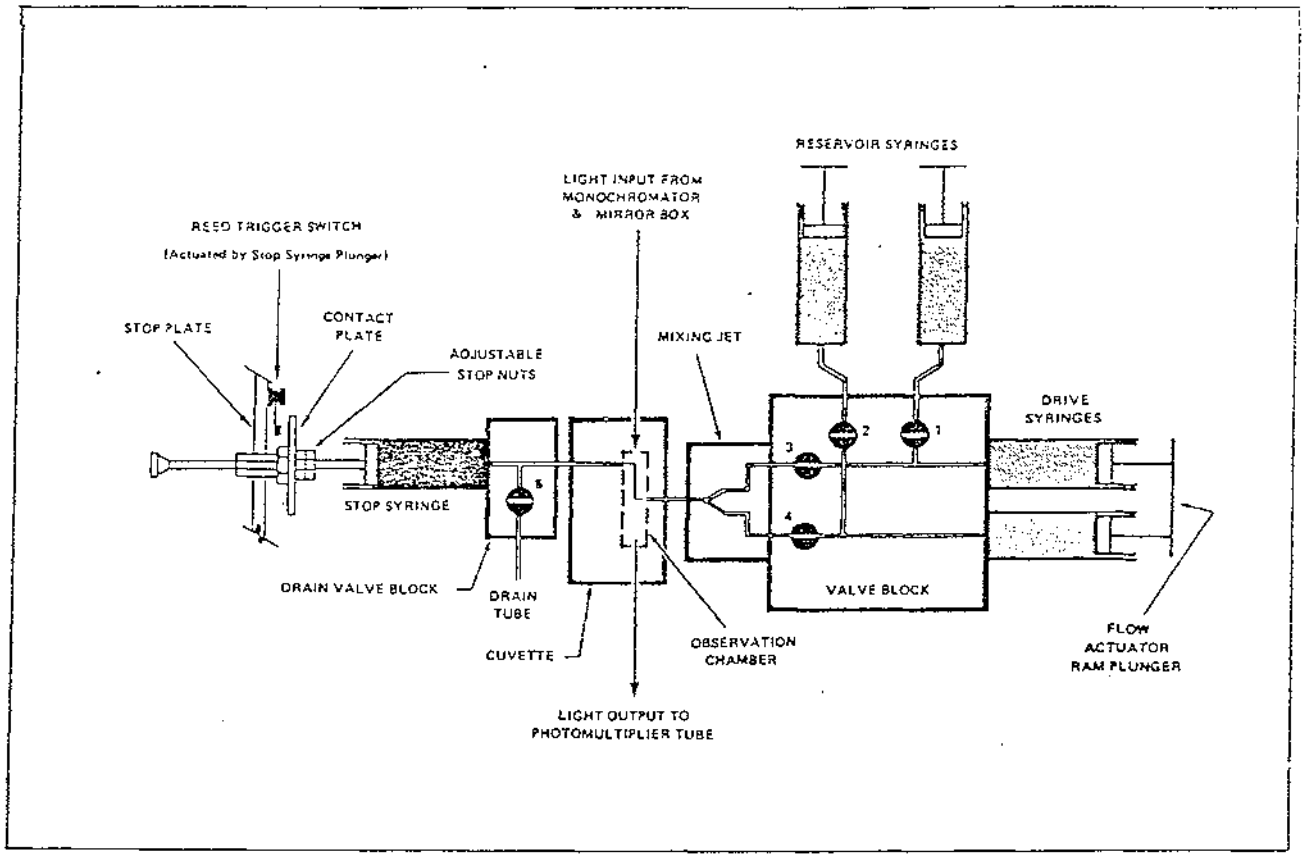

Fig 3.1 Schematic diagram of the stopped-flow apparatus, 1974

Durrum-Gibson D110 spectrophotometer (from Instruction manual)

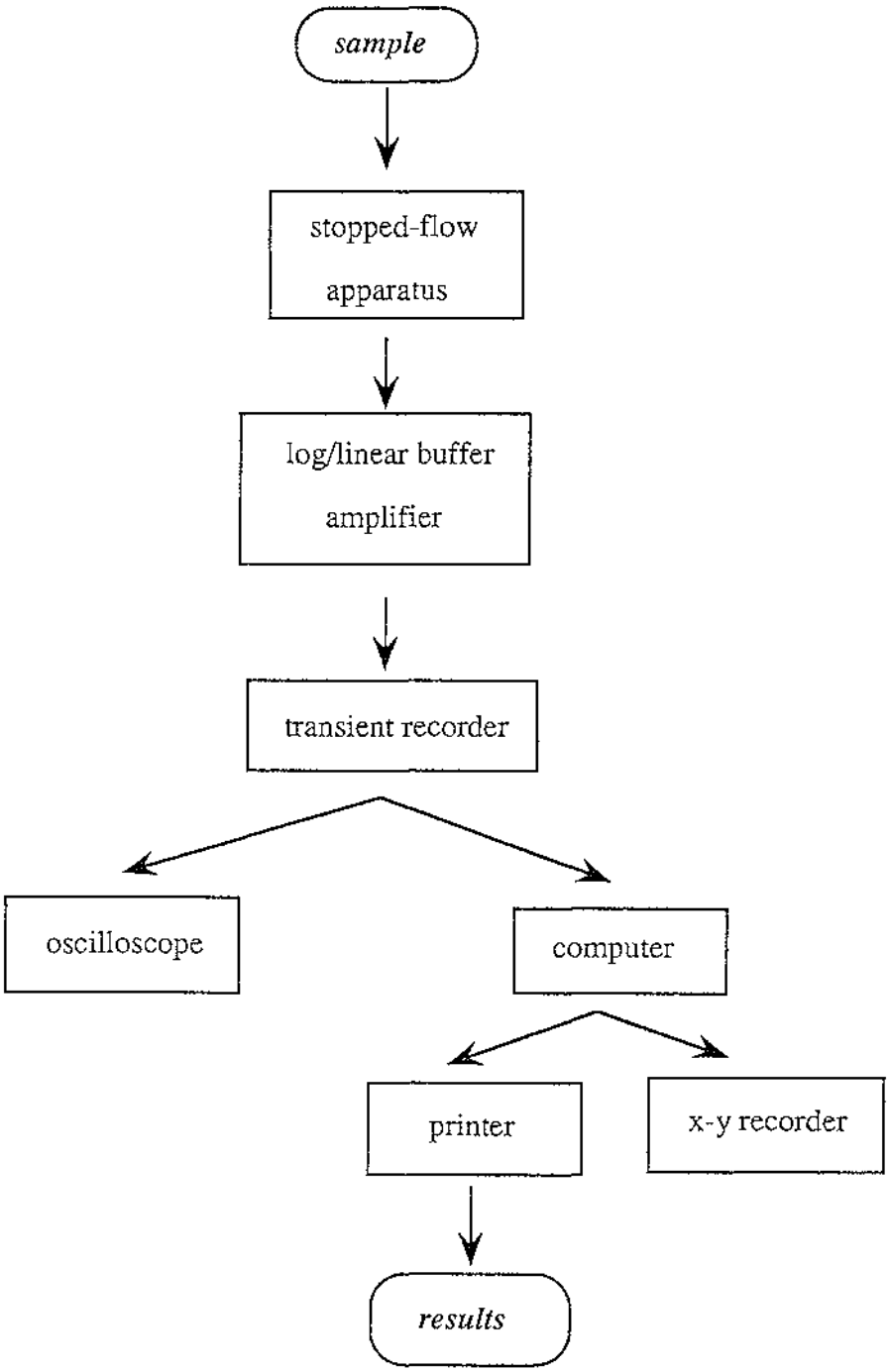

Fig 3.2 A flow chart showing how results from the stopped-flow were observed and processed

but absorbance experiments record the light output straight through from the light source. The output of light signal is monitored by a photomultiplier.

3.2.1.1 Data Collection

The output from the photomultiplier is amplified, converted from transmittance to absorbance if necessary and then detected by a DL 905 transient recorder. The results are visually displaced on an oscilloscope. Data, 1024 points are transferred from the transient recorder to a Cromemco CS-2 microcomputer system, where data can be stored and processed by using a FORTRAN program (STOPFLOW) (Hardman *et al.*., 1985).

3.2.1.2 Data Processing

A FORTRAN program (EXPFIT) (Daniel and Wood, 1971; Hardman, 1983) based on the Marquardt (1963) method was used to carry out non-linear least-squares fitting to calculate the rate constants. The graphs of absorbance or fluorescence against time were plotted using a Bryans 29000 A4 X-Y recorder and the calculated results were printed out on a dot matrix printer. Fig. 3.3 and 3.4 show the printouts of the operations.

Some of the absorbance results were recorded and stored in the transmittance mode. Then the results were converted to absorbance by using a FORTRAN program (modified version of STOPFLOW) before the rate constants were calculated by the non-linear least-squares fitting. The reason for this was that the log converter produced a sharp "spike" at the start of the reaction, which interfered with the processing of the short time scale reactions.

```
                                ITERATION NO.   8

                NON-LINEAR LEAST-SQUARES CURVE-FITTING PROGRAM

EXP(B-BX)+A                 DEP.VAR. MIN.Y=  3.650E+01  MAX.Y=  2.425E+02
RANGE Y=  2.060E+02

                                                                 95%CONFIDENCE LIMITS
IND.VAR(I)  NAME        COEF.B(I)     S.D.COEF.    T-VALUE    LOWER          UPPER
     1      ASYMPT   2.482218E+02     3.75E+00       65.8     2.38E+02     2.58E+02
     2      SLOW AMP 3.506613E+01     1.84E+00       20.7     3.45E+01     4.17E+01
     3      SLOW K   8.903586E-02     2.42E-02        3.7     4.17E-02     1.36E-01
     4      FAST AMP 1.548595E+02     3.30E+00       58.5     1.68E+02     2.01E+02
     5      FAST K   9.600282E-01     3.33E-02       28.9     8.95E-01     1.03E+00
NO. OF OBSERVATIONS                    234
NO. OF COEFFICIENTS                      5
RESIDUAL DEGREES OF FREEDOM            229
RESIDUAL ROOT MEAN SQUARE          3.525021.78
RESIDUAL MEAN SQUARE                12.43577744
RESIDUAL SUM OF SQUARES          2848.602929655
LIST OF RESIDUALS WANTED (ENTER 1) OR NOT (RETURN)?1

OBS. NO.   OBS. Y   FITTED Y   RESIDUAL
     1     36.500     36.937      -.437
     2     52.000     50.440      1.560
     3     64.750     62.916      1.834
     4     74.250     74.448      -.198
     5     83.500     85.100     -1.600
     6     93.750     94.949     -1.199
     7    102.500    104.054     -1.554
     8    112.250    112.472      -.222
     9    120.000    120.257      -.257
    10    126.750    127.457      -.707
    11    134.250    134.116       .133
    12    140.750    140.282       .468
    13    145.500    145.985      -.485
    14    151.000    151.263      -.263
    15    156.000    156.155      -.155
    16    160.500    160.683      -.183
    17    165.000    164.877       .123
    18    168.750    168.765      -.015
    19    172.250    172.369      -.119
    20    176.250    175.710       .540
    21    179.750    178.810       .940
    22    182.500    181.687       .813
    23    185.250    184.558       .692
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Fig 3.3 The printout of the results produced from a sample reaction by the EXPFIT program

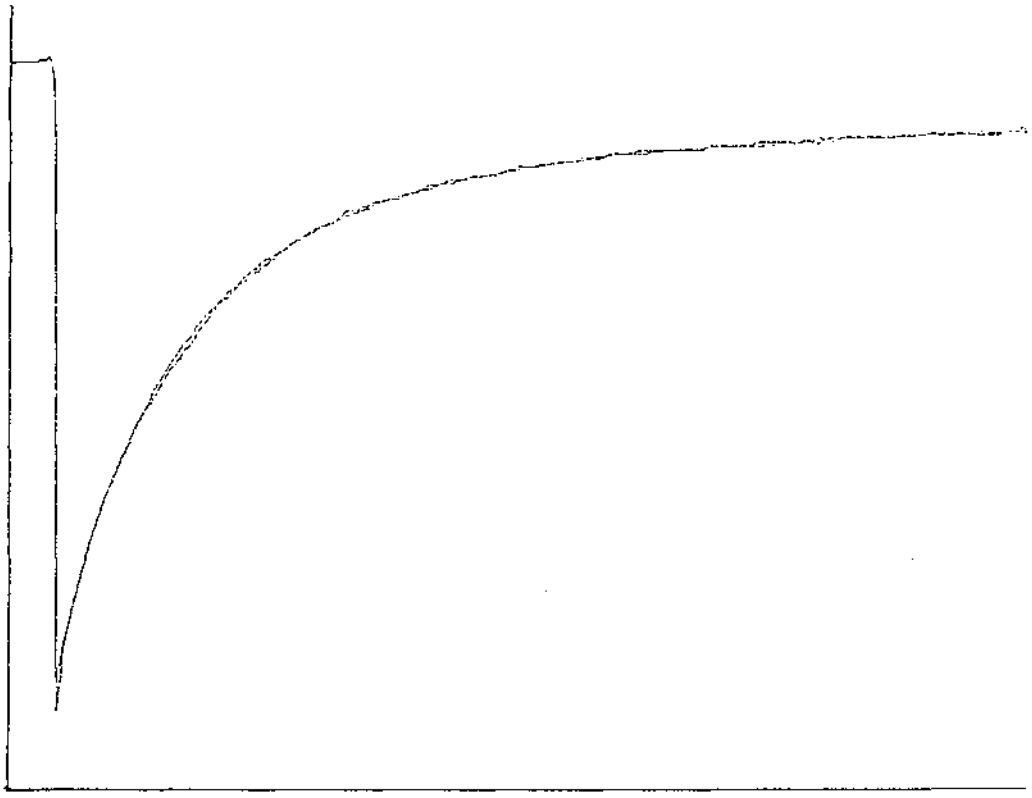


Fig. 3.4 Comparison of the original and the fitted curves for a sample reaction.

The fitted curve was calculated by the program EXPFIT for a single exponential

3.2.2 Preparation of Enzyme Samples for Stopped-flow Experiments

The purified glucose-fructose oxidoreductase from the C_4 column was in the oxidised (E-$NADP^+$) form. Glucose-fructose oxidoreductase from the C_4 column, which was in 0.02 M potassium acetate buffer (pH 5.5), was dialysed against MES buffer (pH 6.5) overnight before use. Reduced enzyme (E-NADPH) was produced by dialysing a GFOR solution against 5 mM glucose in pH 6.5 MES buffer overnight, followed by gel filtration using a Sephadex G-25 (3 cm^2 x 20 cm) column with pH 6.5 Mes buffer. The fractions were collected and the protein concentrations were measured by absorbance at 280 nm. The fractions containing high GFOR activity were pooled.

All solutions were degassed and filtered (through a Sartorius Minisart NML filter) before they were used in the stopped-flow apparatus, in order to reduce interference.

3.2.3 Determination of GFOR Active Sites

The active site concentration of GFOR was calculated by titrating a solution containing 0.5 ml of GFOR solution plus 0.5 ml of pH 6.5 MES buffer with 2 µl aliquots of 1 M glucose. The molar absorption coefficient (ε) for E-NADPH was taken to be 6220, the same as for free NADPH.

3.2.4 Stopped-flow Experiments

3.2.4.1 Nucleotide Absorbance

The instrument was used in absorbance mode, or later in transmittance mode. The following settings were used for nucleotide absorbance experiments: 340 nm and photomultiplier voltage of 325. The GFOR concentration was usually 20 to 30 µM after mixing in the stopped-flow apparatus.

3.2.4.2 Nucleotide Fluorescence

The excitation wavelength was 340 nm. The emitted light passed through Wratten 47B and 2B filters, which have a maximum transmittance at 435 nm. The photomultiplier voltage was 825 volts and the GFOR concentration was usually 5 to 10 μM after mixing.

3.2.4.3 Protein Fluorescence

This method was similar to that for nucleotide fluorescence. The excitation wavelength was 290 nm and emission was observed through a Wratten 18A filter, with has maximum transmittance at 335 nm. The photomultiplier voltage was 550 V. The usual enzyme concentration was 10 to 20 μM after mixing.

3.3 Results and Discussion

3.3.1 The Oxidation of E-NADPH by Gluconolactone

Preliminary experiments on the reduction of E-NADP+ by a low concentration of glucose showed no difference between the absorbance and fluorescence results. Therefore, the oxidation of E-NADPH with gluconolactone was investigated first, using nucleotide fluorescence. The mechanism of oxidation of E-NADPH by gluconolactone has been established by Hardman and Scopes (1988) on the basis of absorbance experiments and is shown in equation 3.1 (also see Fig. 1.3).

$$\text{E-NADPH} + \text{L} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} \text{E-NADPH.L} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{E-NADP}^+\text{.G} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{E-NADP}^+ + \text{G} \qquad (3.1)$$

When the reduced enzyme (E-NADPH) was mixed with water, the level of the fluorescence trace was as shown in Fig. 3.5; this represents the fluorescence of

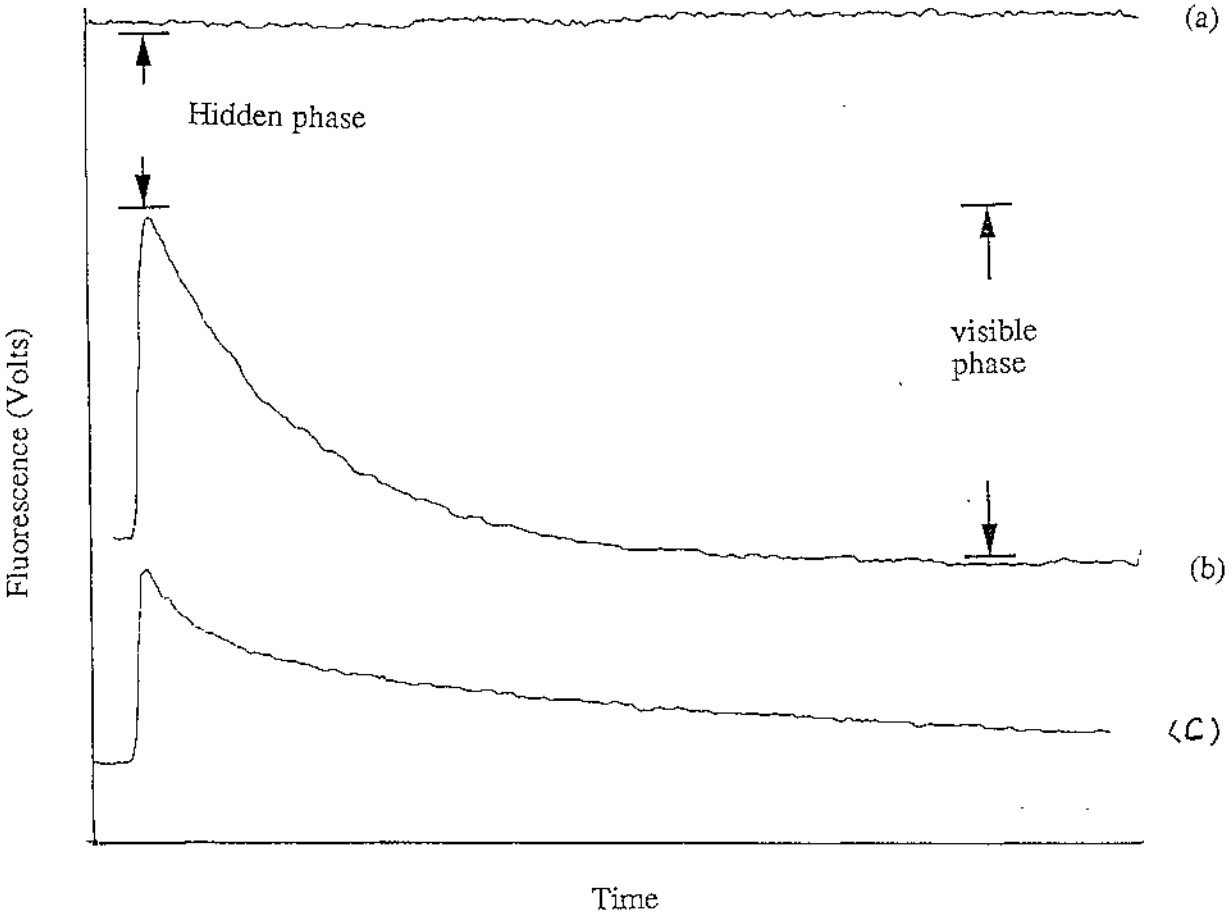


Fig. 3.5 Time course of the fluorescence change on mixing E-NADPH with gluconolactone

The reactions were carried out at pH 6.5, 25 °C and the GFOR concentration was 2.1 μM.

(a) Enzyme alone (mixed with water),

(b) enzyme plus 0.25 mM gluconolactone. The time scale, visible phase rate constant and hidden phase amplitude were: 10 sec, 0.8 s^{-1} and 0.39 V.

(c) enzyme plus 5 mM gluconolactone. The time scale, visible phase rate constant and hidden phase amplitude were: 2 sec, 10 s^{-1} and 1.28V.

E-NADPH at the start of the reactions shown in Fig. 3.5 (the base line). Then when E-NADPH was mixed with gluconolactone at a low concentration (0.25 mM), the fluorescence decreased as E-NADPH was converted to E-NADP$^+$, but the reaction trace started below the base line. The initial decrease in fluorescence was too fast for the stopped-flow instrument to measure and what was observed was the end result of the rapid reaction, followed by a slower phase. The instantaneous drop in fluorescence is referred to as the hidden phase and the slower reaction is called the visible phase. At a higher gluconolactone concentration (5 mM) the amplitude of the hidden phase was greater and that of the visible phase was lower.

When fluorescence experiments were carried out using a wide range of gluconolactone concentrations, the amplitude of the hidden phase was found to increase with increasing gluconolactone concentration (Fig. 3.6). The hidden phase amplitudes could be fitted to a saturation equation (equation 3.2):

$$Amp = \frac{Amp_{max} \times [L]}{K_L + [L]} \qquad (3.2)$$

Where Amp = hidden phase amplitude

Amp_{max} = maximum hidden amplitude at infinite [gluconolactone]

L = gluconolactone

K_L = dissociation constant for gluconolactone

The amplitude of the visible phase decreases with increasing gluconolactone concentration (Fig 3.6). The sum of amplitudes of both visible and hidden phases is almost constant. The amplitudes of the visible phase could be fitted to equation 3.3, which assumes that the visible phase amplitude corresponds to the total fluorescence difference between E-NADPH and E-NADP$^+$ minus the hidden phase amplitude.

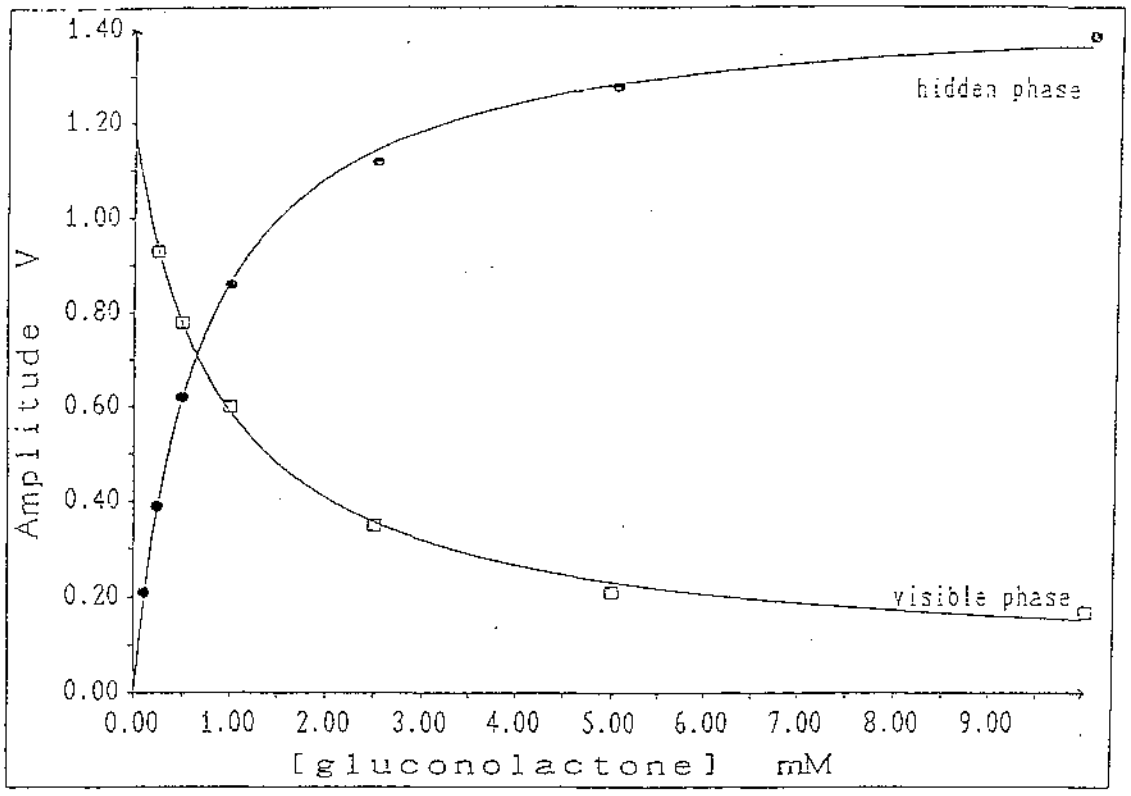


Fig. 3.6 Dependence of the amplitude of fluorescence change on gluconolactone concentration

● hidden phase □ visible phase

The curves represent the computer-calculated best fit lines to equation 3.2 (hidden phase) and equation 3.3 (visible phase).

For hidden phase Amp_{Max} = 1.46 V, K_L = 0.69 ± 0.03 mM

For visible phase Amp_o = 1.17 V, K_L = 0.9 ± 0.1 mM,
Amp_{diff} = 1.11 V

$$Amp = Amp_0 - \frac{Amp_{diff} \times [L]}{K_L + [L]} \qquad (3.3)$$

Where Amp = visible phase amplitude

Amp_0 = visible phase amplitude when [L] = 0

Amp_{diff} = difference in visible phase amplitude between [L] = 0 and infinite [L]

The values of the parameters in both equations 3.2 and 3.3 were calculated by nonlinear fitting. The values of Amp_{max} and Amp_{diff} were 1.46 ± 0.02 volts and 1.12 ± 0.04 volts, respectively. This shows that the visible phase has approximately 4 % of the total fluorescence of the system at infinite gluconolactone concentration. The K_L values for the hidden phase and the visible phase were 0.69 ± 0.03 mM and 0.9 ± 0.1 mM, respectively. These values are similar to each other and to the K_L value obtained in an absorbance experiment by Hardman and Scopes (1988), 0.35 ± 0.01 mM. This suggests that the mechanism proposed by Hardman and Scopes (1988) also applies to the results obtained here.

The biphasic behaviour was not observed in absorbance, as there was no evidence for any hidden phase of reaction (Hardman, pers. comm.). However, the rate constants for the visible phase in fluorescence correspond to the absorbance results obtained by Hardman and Scopes (1988). Also the present work confirmed that the fluorescence and absorbance results are similar. The rate constant measured using fluorescence at 2 mM gluconolactone was 6.4 ± 0.4 s^{-1}, compared to that of 6.8 ± 0.1 s^{-1} of Hardman and Scopes (1988). The limiting value of the rate constant for the oxidation of E-NADPH by gluconolactone for nucleotide fluorescence was 10 s^{-1}, compare to 8 s^{-1} at 21 $^{\circ}C$ in absorbance (Hardman and Scopes, 1988). Therefore, the visible phase in fluorescence corresponds to the only phase seen in absorbance.

The hidden phase involves a decrease in fluorescence, but not in absorbance. Therefore, the hidden phase does not involve the conversion of enzyme-bound NADPH to $NADP^+$. This suggests that the hidden phase represents the gluconolactone binding step, shown by k_{-3} in equation 3.1. The visible phase was the quenching of the rest of fluorescence, and because it corresponds to the only phase seen in absorbance, it probably represents hydrogen transfer from enzyme-bound NADPH to gluconolactone (the step shown as k_{-2}). These conclusions are summarised in equation 3.4.

$$\text{E-NADPH} + \text{L} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} \text{E-NADPH.L} \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} \text{E-NADP}^+.\,\text{G} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{E-NADP}^+ + \text{G} \qquad (3.4)$$

high	high	low	low absorbance
high	low	low	low fluorescence

At high gluconolactone concentrations, almost all of the E-NADPH molecules have been converted to the E-NADPH.L form within the dead time of the stopped-flow instrument, because the binding of gluconolactone is very fast. Therefore, a large hidden phase and a very small visible phase were observed . At low gluconolactone concentrations, not all of the E-NADPH has been converted to E-NADPH.L in the rapid reaction. Therefore, only a partial hidden phase was observed. As E-NADPH.L is converted to $NADP^+$ forms in the visible phase (controlled by k_{-2}), additional E-NADPH binds gluconolactone to maintain the equilibrium. Most of the fluorescence change therefore occurs in the visible phase.

The data in Fig. 3.6 suggest that the fluorescence of E-NADPH.L is not completely quenched. The amplitude of the visible phase at an infinite gluconolactone concentration represents the difference in fluorescence between E-NADPH.L and $NADP^+$. As mentioned earlier, from the curve fitting results we calculated that E-NADPH.L has about 4 % of the fluorescence of E-NADPH.

Similar quenching of nucleotide fluorescence by carbonyl compounds has been observed for lactate dehydrogenase and aldehyde dehydrogenase. When pyruvate (containing a carbonyl group) was bound to the lactate dehydrogenase-NADH complex, the pyrimidine nucleotide fluorescence was quenched (Clarke *et al.*, 1989). Also, propanal bound to aldehyde dehydrogenase led to the eventual nucleotide fluorescence quenching of bound NADH (Dickinson, 1985). The mechanism suggested for the quenching of fluorescence by carbonyl groups was that the strongly polar carbonyl group interacts with the hydrogen of the NADPH. The positvely polarized carbon withdraws electrons from the pyridine ring hydrogen, thus making NAD(P)H more like $NAD(P)^+$, the low fluorescence form (Clarke *et al.*, 1989; Kitson, 1989).(Fig. 3.7).

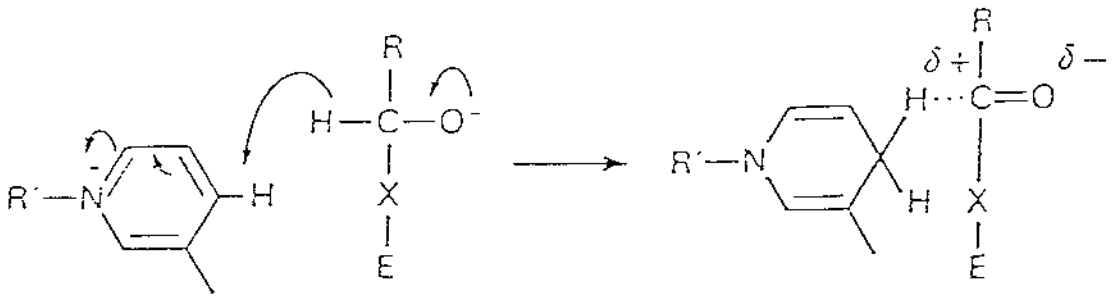
3.3.2 Reduction of the GFOR-$NADP^+$ Complex by Glucose

The experimental results for oxidation of E-NADPH by gluconolactone, in the previous section, suggest that reduction of E-$NADP^+$ by glucose has the mechanism shown in the following equation 3.5 (the reverse of equation 3.4).

$$\text{E-NADP}^+ + \text{G} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-NADP}^+.\text{G} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E-NADPH.L} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{E-NADPH} + \text{L} \qquad (3.5)$$

low	low	high	high absorbance
low	low	low	high fluorescence

Experiments using absorbance suggest that k_2 is greater than k_3 (Hardman and Scopes, 1988). The mechanism of equation 3.5 then implies that the rate of the absorbance change may be faster than the fluorescence change, because absorbance monitors all reduced forms but fluorescence is dominated by the E-NADPH form (E-NADPH.L has about 4 % of the fluorescence of E-NADPH). Therefore, the reduction of E-$NADP^+$ by a wider range of glucose concentrations was studied using nucleotide fluorescence.



E-NADP$^+$ E-NADPH

Fig. 3.7 Mechanism of gluconolactone carbonyl group quenching E-NADPH fluorescence (after Kitson, 1989)

When E-NADP$^+$ was mixed with glucose, an increase in fluorescence was observed (Fig. 3.8). The fluorescence traces could be fitted to a single exponential. The absorbance results reported by Hardman and Scopes (1988) and those obtained here also appeared to fit a single exponential. At low glucose concentration, the rate constants obtained using fluorescence were similar to those for absorbance. However, at higher glucose concentrations, the rate constants for fluorescence experiments were lower than those using absorbance, under the same conditions (Fig. 3.9). The absorbance rate constant was too fast to be measured above 40 mM glucose, while the fluorescence rate constant was still measurable at 100 mM. The fluorescence rate constant reached a limiting value of about 350 s^{-1}. For experiments using absorbance, the limiting value of the rate constant at saturating glucose was calculated to be 2100 ± 130 s^{-1} (Hardman and Scopes, 1988).

The effect of temperature on the rate constant obtained from fluorescence experiments was studied. The rate constant was temperature dependent; the rate constant at 25 $^{\circ}$C was faster than at 20 $^{\circ}$C (Fig. 3.10). The rate constant in fluorescence at 25 $^{\circ}$C was still much lower than the absorbance results at 21 $^{\circ}$C. Therefore, a small difference in temperature between the two sets of experiments was not enough to account for the difference in the rate constants between absorbance and fluorescence (Fig. 3.9).

Firstly, I will use a qualitative explanation for the different rate constants for the two signals. The absorbance change was shown to occur in the k_2 step (Hardman and Scopes, 1988) because that was shown to be the hydrogen transfer step (E-NADP$^+$ to E-NADPH). The gluconolactone experiments indicated that the main nucleotide fluorescence change occurs in the k_3 step (between E-NADPH.L and E-NADPH). The k_2 step has a rate constant of about 2000 s^{-1} and is faster than k_3 (Hardman and Scopes, 1988); therefore the absorbance change is not limited by k_3. Because the fluorescence change occurs in the k_3 step, GFOR must pass through both the

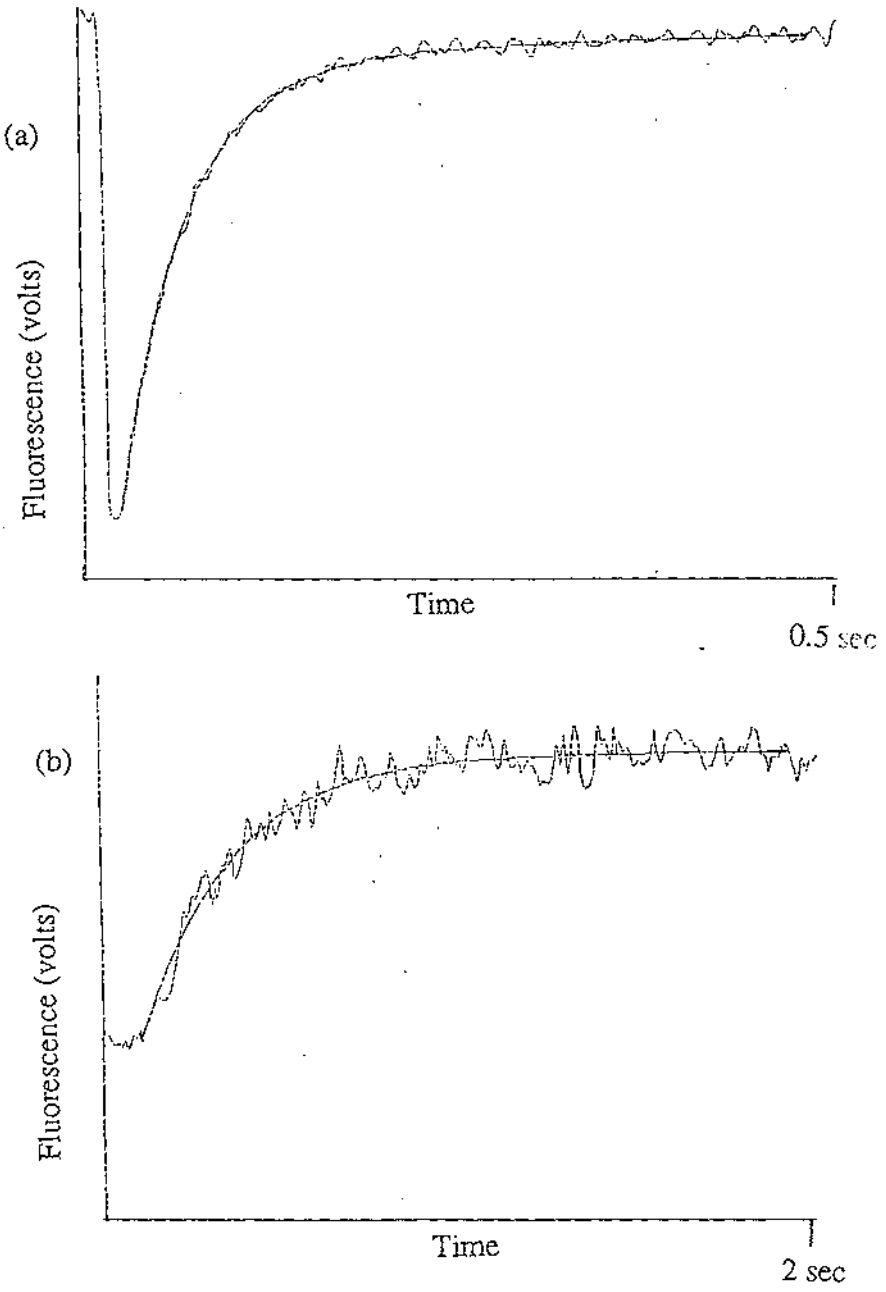


Fig. 3.8 Time course of fluorescence change during reduction of E-NADP$^+$ by glucose

conditions: 21 $^{\circ}$C, pH 6.5 and [enzyme] = 12 μM

(a) 1mM glucose; the rate constant was 21.8 ± 0.3 s^{-1}.

(b) 100 mM glucose; the rate constant was 264 ± 13 s^{-1}

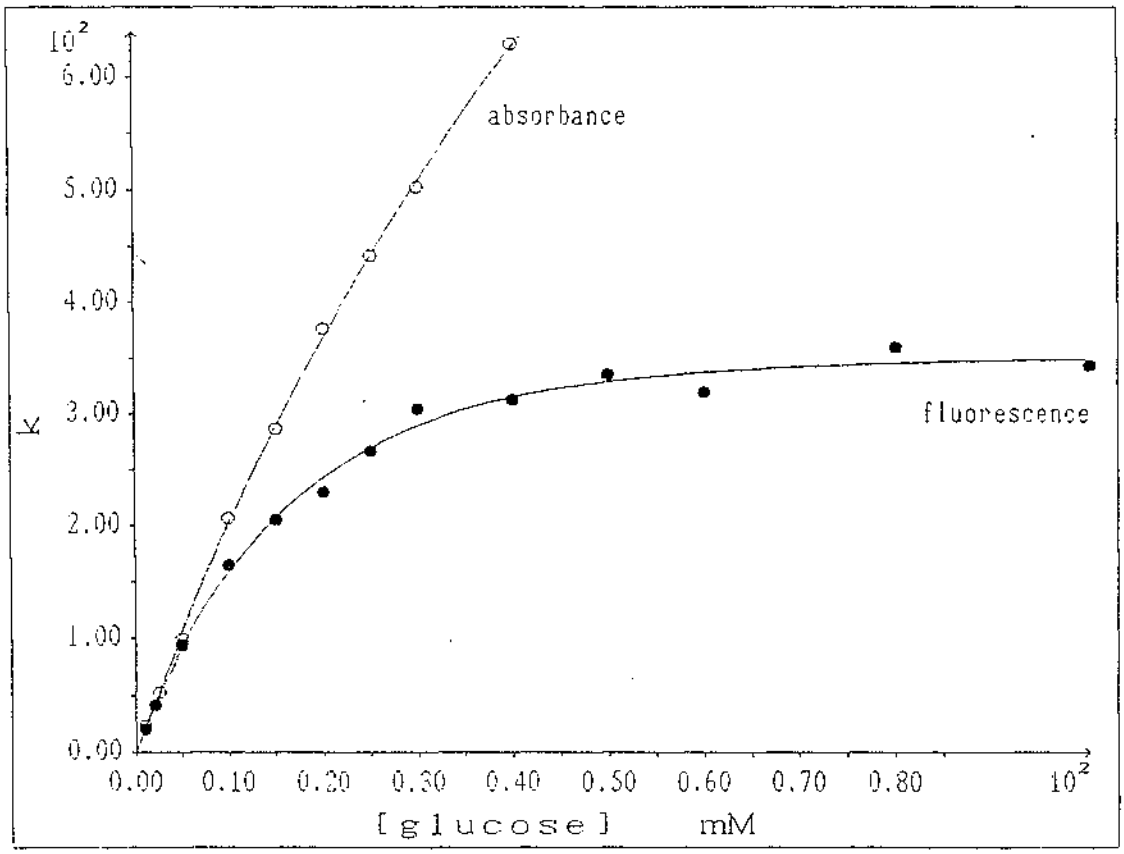


Fig. 3.9 Rate constants for absorbance and fluorescence change at various glucose concentrations under the same conditions, pH 6.5 and 21 °C.

○ absorbance results

● fluorescence results

The lines represent the computer-calculated fits to equations 3.9 (fluorescence) and equation 3.6 (absorbance)

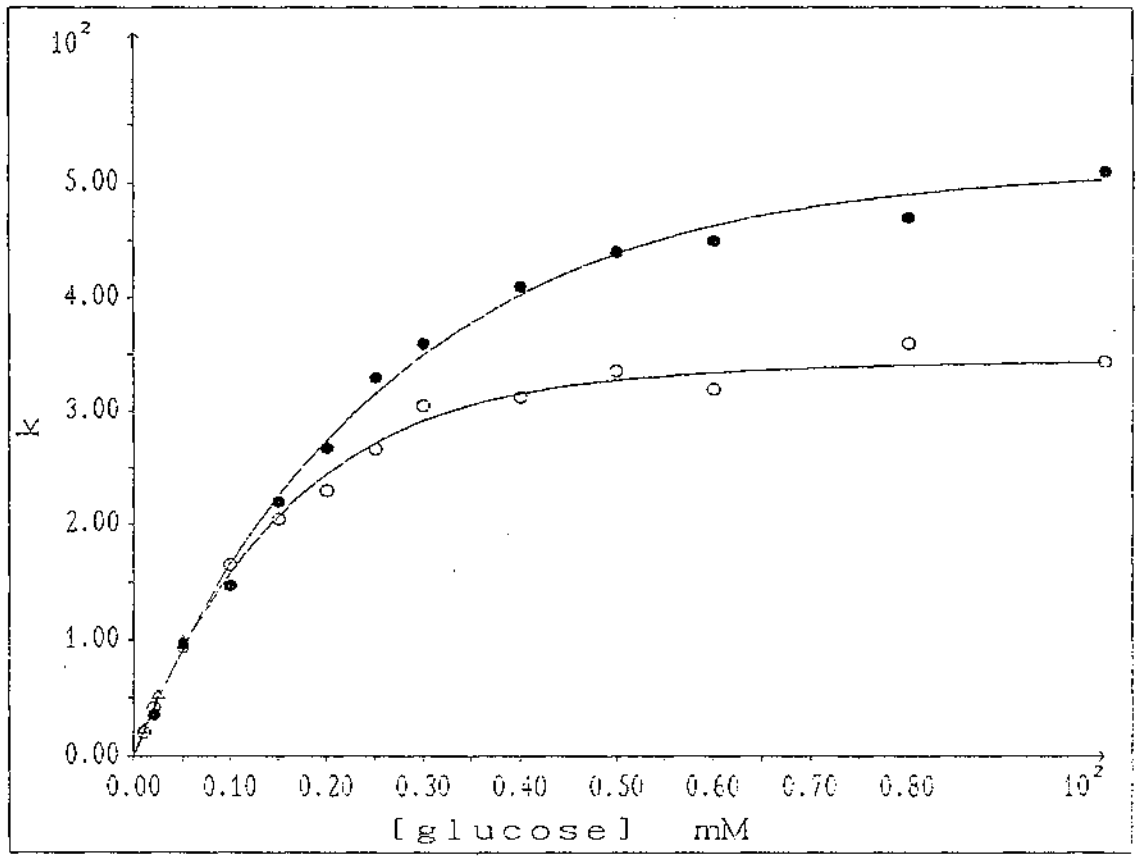


Fig. 3.10 Rate constants for fluorescence change at various glucose concentrations and at different temperatures, at pH 6.5.

○ fluorescence results at 21 °C, [enzyme] was 14.46 μM

● fluorescence results at 25 °C, [enzyme] was 9.6 μM

The lines represent the computer-calculated fits to equation 3.9

k_2 and k_3 steps to give a fluorescence change. The overall rate depends mainly on the rate of the slower step. At high concentrations of glucose, the observed rate constant for fluorescence is limited by k_3; hence the rate constants are slower than for absorbance. But at low concentrations of glucose (lower than the dissociation constant, K_G, 40 mM), the effective rate constant for the conversion of E-$NADP^+$.G to E-NADPH.L ($\frac{k_2\,[G]}{K_G + [G]}$, where $K_G = k_{-1}/k_1$) is low. Since both absorbance and fluorescence require the enzyme to pass through this step, the rate constants for both signals are similar .

The fluorescence and absorbance results can also be analysed quantitatively using equations derived by Bernasconi (1977). For a system of three steps, such as equation 3.5, there will in principle be three relaxations and the reciprocal relaxation times (observed rate constants) will be a function of all six rate constants. However, for most enzyme reactions, the binding of substrate (k_1) is much faster than the other rate constants. The fastest relaxation time will be dependent only on k_1 and k_{-1}, and will be too fast to be observed using a stopped-flow spectrophotometer. The other two reciprocal relaxation times will be functions of k_2, k_{-2}, k_3 and k_{-3}. Bernasconi discusses two cases which may apply to the mechanism in equation 3.3: case 1 and case 4 (Table 4.1 of Bernasconi, 1977).

Case 1 assumes that the second step (k_2) is much faster than the third step (k_3). The expression for the faster observed rate constant is then $\frac{k_2\,[G]}{K_G + [G]} + k_3$. If k_{-2} is zero, this expression can be simplified to a normal Michaelis saturation equation (equation 3.6) and is consistant with the observed absorbance data of Hardman and Scopes (1988).

$$k = \frac{k_2\,[G]}{K_G + [G]} \qquad (3.6)$$

However, there are three problems with case 1. Firstly, k_{-2} is not zero; experiments with E-NADPH plus gluconolactone showed that it is about 8 s^{-1} (Hardman and Scopes, 1988). Therefore, a plot of the observed rate constant for absorbance against glucose

concentration should pass above the origin. It does not. Secondly, the expression for the slowest observed rate constant is $\frac{k_3 K_2 [G]}{K_G + K_2 [G]}$ when $K_2 >> 1$ (Bernasconi, 1977; Hardman, pers. comm.), which is probably true for equation 3.5. This expression also has the Michaelis equation form and the rate constants for fluorescence were fitted to it. But the value of $\frac{K_G}{K_2}$ (the glucose concentration at half saturation) is inconsistent with the values of K_G, k_2 and k_{-2} from the absorbance experiments of Hardman and Scopes (1988). Thirdly, case 1 is a special case of case 4 when the second step is faster than the third. However, study of case 4 (see below) show that this assumption is not valid at low glucose concentration.

Case 4 is more appropriate for the mechanism of equation 3.5, because it assumes that k_2 and k_3 are similar and the observed reaction consists of two processes; the observed rate constants are combinations of k_2, k_{-2}, k_3 and k_{-3}. We have assumed that k_{-3} is effectively zero, since it is a second order reaction and the concentrations of E-NADPH and gluconolactone were low. If the above assumptions are true, then the sum and product of the reciprocal relaxation times are (Bernasconi, 1977):

$$\text{sum} = \frac{k_2 [G]}{K_G + [G]} + k_{-2} + k_3 \qquad (3.7)$$

$$\text{product} = \frac{k_2 [G] k_3}{K_G + [G]} \qquad (3.8)$$

The two reciprocal relaxation times are given by equations 3.9 and 3.10.

$$k_S = \frac{\text{sum} - \sqrt{(\text{sum}^2 - 4 \times \text{product})}}{2} \qquad (3.9)$$

$$k_f = \frac{\text{sum} + \sqrt{(\text{sum}^2 - 4 \times \text{product})}}{2} \qquad (3.10)$$

Since the fluorescence change occurs during the k_3 step, the observed rate constant for this change will be equal to the slower of the two rate constants.

The fluorescence rate constants (Figs 3.9 and 3.10) were fitted to equation 3.9, with the sum and product defined by equations 3.7 and 3.8 and k_{-2} fixed at 8 s^{-1} (Hardman and Scopes, 1988). The lines of best fit are shown in Figs 3.9 and 3.10. This equation gave a better fit than the normal saturation equation. Therefore, case 4 is a good explanation for the fluorescence results. Fitting of the data at 21 $^{\circ}C$ to equation 3.9 gave a k_3 value of 373 ± 14 s^{-1}. This is the rate constant for gluconolactone dissociation, which is the overall rate determining step in the forward direction, because it is the slowest step found by me and by Hardman and Scopes (1988). From fittting to equation 3.9, k_2, and K_G were calculated to be 593 ± 73 s^{-1} and 26 ± 5 mM, respectively. Fitting of the 25 $^{\circ}C$ fluorescence data to equation 3.9 with k_{-2} fixed at 10 s^{-1}, gave a k_3 value of 519 ± 15 s^{-1}, k_2 of 1004 ± 138 s^{-1} and a K_G of 50 ± 10 mM.

The interpretation of the absorbance data is more complex. Computer calculations and simulation (done by Dr. M.J. Hardman) showed that at low glucose concentration, where the effective rate constant for the second step ($\frac{k_2.G}{K_G + G}$) is low, the absorbance change will be dominated by the slower of the two processes. The observed rate constant will be given by equation 3.9 (k_s) and will be the same as that for the fluorescence change. At high concentrations of glucose the absorbance change is dominated by the faster of the two processes (k_f) (equation 3.10), which is itself dominated by k_2. Therefore, the absorbance change will be faster than the fluorescence change, which is dominated by k_3. At intermediate glucose concentrations, the two phases should have comparable amplitudes and reciprocal relaxation times. The two processes would therefore be very difficult to separate. Thus, in practice with the noise level in the stopped-flow apparatus, the reaction traces cannot be fitted to two exponentials. The rate constants obtained from fitting the absorbance results obtained by Hardman and Scopes (1988) and my absorbance results to a single exponential were actually the combination of the two

processes. The computer simulations showed that the rate constant for fitting to a single exponential would be between k_S and k_f and that the values of this rate constant at various glucose concentrations would give a good fit to the saturation equation.

The conclusion of the reaction mechanism drawn from both absorbance and fluorescence results for glucose and gluconolactone is shown below:

$$\text{E-NADP}^+ + \text{G} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E-NADP}^+.\text{G} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E-NADPH.L} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{E-NADPH} + \text{L}$$

high	high	low	low absorbance
high	low	low	low fluorescence

At 25 $^{\circ}$C the fluorescence results of rate constants are $K_G = 26 \pm 5$ mM, $k_2 = 593 \pm 7$ s^{-1}, $k_3 = 372 \pm 14$ s^{-1} and $k_{-2} = 10$ s^{-1}. The absorbance values of K_G, k_2 and k_{-2} from Hardman and Scopes at 21 $^{\circ}$C are: 94 ± 7 mM, 2100 ± 130 s^{-1} and 8 s^{-1} respectively. The absorbance values are significantly greater than the fluorescence values, but the k_{-2} values are similar.

3.3.3 The Oxidation of E-NADPH by Fructose

The mechanism of oxidation of E-NADPH by fructose has been established by Hardman and Scopes (1988) and is shown in equation 3.11.

$$\text{E-NADPH} + \text{F} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{E-NADPH.F} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{E-NADP}^+.\text{S} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \text{E-NADP}^+ + \text{S} \qquad (3.11)$$

The fluorescence results corresponded to the absorbance results obtained by Hardman and Scopes (1988). The reduction of fructose by E-NADPH involves a single phase

fluorescence decrease and the rate constant is linearly dependent on fructose concentration up to 1500 mM (Fig. 3.11).

The highest fructose concentration that could be used in absorbance experiments was 400 mM. The k_{max} of the reaction cannot be obtained from the absorbance experiments, because higher fructose concentrations cannot be used, since the refractive index of the fructose solution caused an additional fast process (Hardman and Scopes, 1988). But for fluorescence experiments, concentrations up to 1500 mM fructose can be used. At 1500 mM fructose the rate constant was about 700 s^{-1} any higher rate constant cannot be measured, because it has reached the limit of the stopped-flow. The linearity up to 1500 mM implies that the dissociation constant for fructose is greater than 5 M. Since Hardman and Scopes (1988) showed that the limiting value for 1 M fructose was suggested to be 430 s^{-1}, the new k_{max} for the reduction of fructose is therefore greater than 2000 s^{-1}. This is much higher than the dissociation constants for the other substrates (Hardman and Scopes, 1988). This is because GFOR only binds the open chain form of fructose and less than 1% of fructose exists in the open chain form over the temperature range of 16°C to 44°C (Goux, 1985).

Although fructose also contains a carbonyl group (Fig. 3.12), fluorescence quenching on binding was not observed. This was because the fructose K_d was much greater than the gluconolactone K_d, over 5 M and 0.35 mM, respectively. The gluconolactone concentration used in the fluorescence experiments was well above the K_d and therefore E-NADPH was saturated with gluconolactone. But the fructose concentrations used were well below the K_d. At the fructose concentrations used in the fluorescence experiments there were not enough bound fructose molecules to influence the fluorescence of E-NADPH.

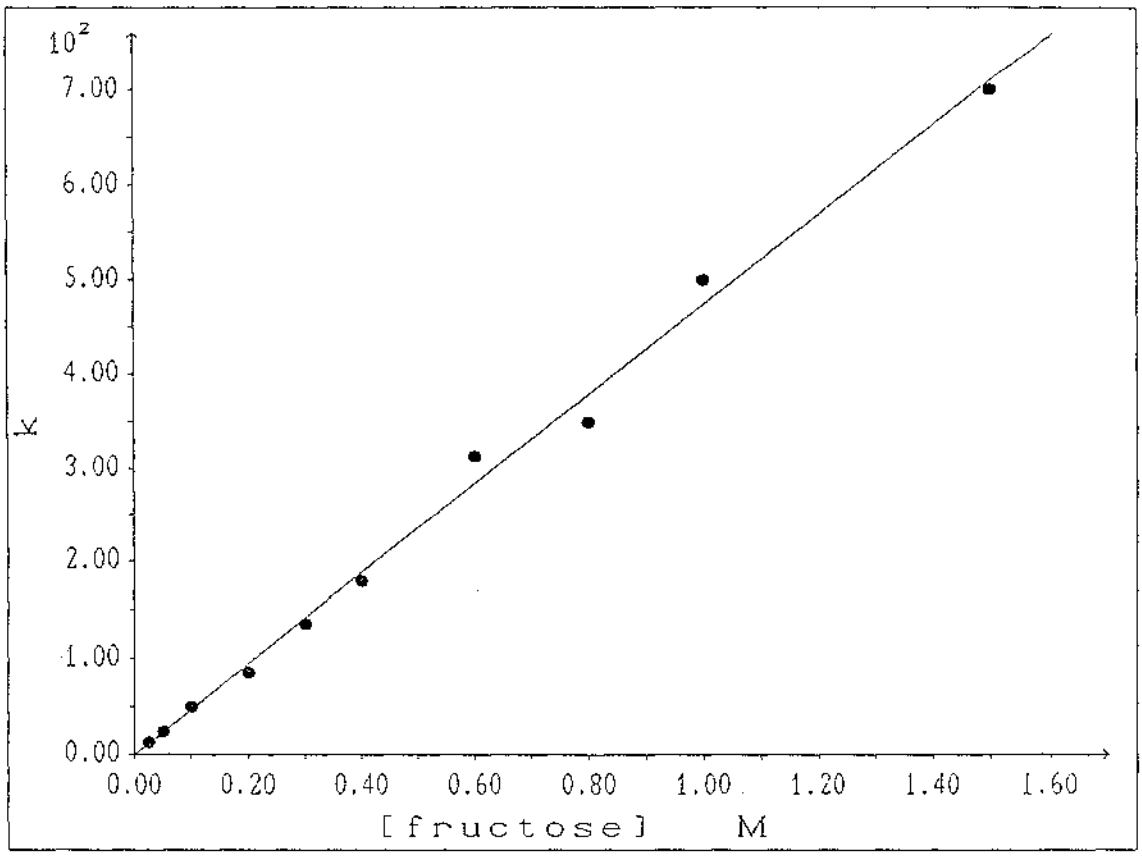
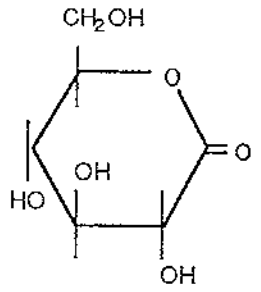
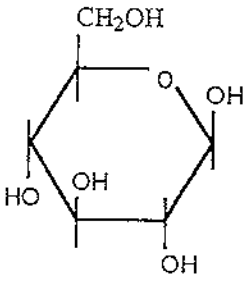


Fig 3.11 Dependence of fluorescence rate constant on fructose concentration

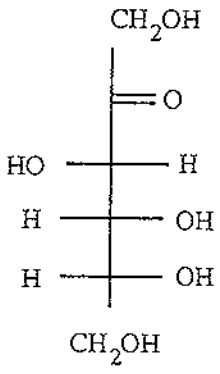
The reaction was carried out at 25 $^{\circ}$C, pH 6.5 and [GFOR] was 7.23 μM. The line represents linear dependence of rate constants on the fructose concentration.



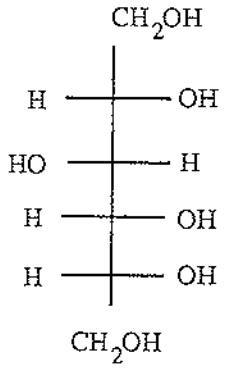
ç-gluconolactone



ß-D-glucose



D-fructose



D-sorbitol

Fig. 3.12 Structures of GFOR substrates

3.3.4 Reduction of E-NADP+ by Sorbitol

The mechanism of reduction of GFOR-NADP+ by sorbitol was shown to be biphasic by Hardman and Scopes (1988). When the data for the later part of the reaction were fitted to one exponential, an initial faster reaction was apparent. The data for the complete reaction fitted well to two exponentials. The reaction mechanism is shown in equation 3.12.

$$\text{E-NADP}^+ + \text{S} \underset{k_6}{\overset{k_{-6}}{\rightleftharpoons}} \text{E-NADP}^+.\text{S} \underset{k_5}{\overset{k_{-5}}{\rightleftharpoons}} \text{E-NADPH.F} \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} \text{E-NADPH} + \text{F} \qquad (3.12)$$

The fluorescence experiments also gave a biphasic curve for the reduction of E-NADP+ by sorbitol. But the fluorescence results at 25°C did not correspond to the absorbance results at 21°C published by Hardman and Scopes (1988). The fluorescence rate constants were faster and the fast phase was a higher proportional of the total amplitude (Table 3.1). Many experiments were carried out to find the reason for the differences. The factors tested were: difference between fluorescence and absorbance, temperature difference, pH difference, enzyme concentration, difference in enzyme purification, difference in processing the results, the time for recording the reaction.

3.3.4.1 Difference Between Fluorescence and Absorbance

Absorbance and fluorescence experiments were carried out on the same day, using the same enzyme concentration and at the same temperature and pH. The two sets of results showed good agreement (Table 3.2).

TABLE 3.1 Initial results from this work and those of Hardman and Scopes (1988) for rate constants plus percentage amplitude in the faster of the two phases for the reduction of E-NADP+ by sorbitol at pH 6.5

[Sorbitol] (mM)	Fluorescence Experiment (25°C)			Absorbance experiments (Hardman and Scopes, 1988; Hardman unpublished results) (21 °C)		
	% fast phase	fast rate constant (s^{-1})	slow rate constant (s^{-1})	% fast phase	fast rate constant (s^{-1})	slow rate constant (s^{-1})
10	58	0.26	0.02	9	0.13	0.052
25	74	0.43	0.03	17	0.237	0.0865
50	86	0.85	0.038	21	0.385	0.144
100	81	1.5	0.027	16	0.79	0.237
500	86	3.5	1.05	55	0.74	0.303

TABLE 3.2: Rate constants plus percentage of total amplitude in the faster phase for the reduction of E-NADP$^+$ by sorbitol at 25 $^{\circ}$C, pH 6.5 and 11.25 μM enzyme concentration.

	Absorbance Results			Fluorescence Results		
[Sorbitol] (mM)	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
10	38 ± 1	0.40 ± 0.06	0.047 ± 0.008	40 ± 5	0.40 ± 0.09	0.042 ± 0.003
100	87 ± 3	1.5 ± 0.1	0.07 ± 0.02	83 ± 4	1.4 ± 0.2	0.2 ± 0.2
500	99 ± 1	3.0 ± 0.7		85 ± 7	3.3 ± 0.2	1.2 ± 0.3

3.3.4.2 Effect of Temperature

The fluorescence experiments at three temperatures (21°C, 25°C, 30°C) were carried out on the same day at the same pH (6.5) and enzyme concentration (11 μM). The results did not show any significant difference (Table 3.3).

3.3.4.3 Effect of pH

The fluorescence experiments of sorbitol reduction reaction were carried out at different pH (6.0, 6.5, 7.0), at 25°C and similar GFOR concentrations to test whether a small difference in pH may have contributed to the difference between the results obtained by me and those of Hardman and Scopes (1988). These experiments were conducted on different days. These results showed more variation than in other experiments, but the differences in Table 3.4 were not as large as those in Table 3.1. Also the original results in Table 3.1 were supposed to be at the same pH.

3.3.4.4 The Effect of Enzyme Concentration

The difference in enzyme concentration was tested, because a lower enzyme concentration for fluorescence is usually used . The more diluted enzyme may show some difference from the enzyme at higher concentration. Different enzyme concentrations were used in the absorbance experiments of the reduction of E-$NADP^+$ by 100 mM sorbitol at 23°C. The results showed that different enzyme concentrations had very little effect on the percentage of fast phase and the rate constants (Table 3.5).

3.3.4.5 Effect of a Difference in the Method of Purification of GFOR

I used a C_4 hydrophobic column as the final purification step, instead of the CM-Trisacryl column used by Hardman and Scopes (1988). It was thought that one of these columns

TABLE 3.3 Fluorescence results for rate constants and percentage of total amplitude in the faster phase for reduction of E-NADP$^+$ by sorbitol at different temperatures. The pH was 6.5 and the enzyme concentration was 11 μM.

Temperature ($^{\circ}$C)	[Sorbitol] (mM)	% Fast Phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
21	10	48 ± 9	0.29 ± 0.07	0.03 ± 0.007
	50	75 ± 4	0.87 ± 0.02	0.064 ± 0.009
	500	84 ± 7	2.9 ± 0.3	0.7 ± 0.2
25	10	51 ± 7	0.27 ± 0.02	0.032 ± 0.001
	50	77 ± 2	0.81 ± 0.09	0.04 ± 0.005
	500	89 ± 2	3.3 ± 0.1	0.5 ± 0.1
30	10	58 ± 6	0.20 ± 0.07	0.03 ± 0.01
	50	80.5 ± 0.6	0.85 ± 0.05	0.058 ± 0.006
	500	91± 1	3.5 ± 0.3	0.4 ± 0.1

TABLE 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 pHs. The experiment was carried out at 25°C and the enzyme concentration was 9.24 μM.

pH	[Sorbitol] (mM)	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
6.0	10	58 ± 4	0.26 ± 0.03	0.029 ± 0.005
	25	74 ± 3	0.43 ± 0.02	0.03 ± 0.003
	50	86 ± 5	0.85 ± 0.03	0.038 ± 0.02
	100	81 ± 10	1.5 ± 0.1	0.27 ± 0.03
	500	86 ± 4	3.5 ± 0.1	1.05 ± 0.05
6.5	25	95 ± 0.7	0.41 ± 0.04	0.03 ± 0.02
	50	89 ± 11	0.65 ± 0.09	0.03 ± 0.02
	100	84 ± 1	1.18 ± 0.05	0.08 ± 0.02
	500	100	1.6 ± 0.2	
7.0	10	62 ± 4	0.279 ± 0.07	0.034 ± 0.04
	25	78 ± 2	0.46 ± 0.09	0.039 ± 0.007
	50	86 ± 2	0.4 ± 0.03	0.022 ± 0.009
	100	88 ± 5	1.4 ± 0.06	0.26 ± 0.4

TABLE 3.5 The absorbance results for the reduction of E-NADP$^+$ at different concentrations by sorbitol (100 mM) at 25°C and pH 6.5.

[enzyme] (mM)	% Fast amplitude	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
2.4	100	1.0 ± 0.3	
4.8	80 ± 13	1.5 ± 0.3	0.3 ± 0.2
11.25	82 ± 6	1.4 ± 0.2	0.2 ± 0.1
22.3	77 ± 3	1.2 ± 0.2	0.1 ± 0.02

might have removed some type of small molecules from GFOR, which the other did not, and this may have caused the differences in the results. Alternatively, the ammonium sulphate precipitation used in the present method may have affected the kinetics of the purified enzyme. Glucose-fructose oxidoreductase samples from two different steps during purification were used in absorbance experiments of the sorbitol oxidation reaction. The sources of enzyme were: CM-Sepharose purified enzyme (ion exchange chromatography), and the enzyme from the Remazol Brilliant Blue R column and prior to the C_4 or CM column step. The absorbance results from different enzyme sources (Tables 3.6 and 3.7) showed no differences from the fluorescence results for C_4 purified enzyme in Table 3.1.

3.3.4.6 Differences in Data Processing

The FORTRAN program "Expfit" written by Dr. M.J. Hardman was used to calculate the rate constants and the amplitudes of a recorded curve. The program can fit between any chosen points of the recorded curve, and depending on the points fitted, the processed results can be different. For a reaction trace there are a total of 1024 points, with 50 points for pretrigger. Different points were chosen to fit the recorded data from an absorbance experiment with 50 mM sorbitol. The results showed that there were no differences in rate constants and % amplitude for different ranges of points fitted (Table 3.8).

3.3.4.7 Time Scale for Recording the Sorbitol Reaction

The total time used for recording the reaction may affect the fitted rate constants and amplitudes of a particular reaction. If the results were recorded too early in the reaction, the slow phase would be missed out; if the results were recorded too late in the reaction , the main part of the reaction would be reduced to a small part of the graph. Both situations led to inaccurate calculation of the rate constants and amplitudes.

TABLE 3.6 Absorbance results of reduction of sorbitol after GFOR was purified by CM-Sepharose column. The experiment was carried out at 25°C, pH 6.5 and the enzyme concentration was 9.6 μM.

[Sorbitol] (mM)	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
10	47 ± 7	0.3 ± 0.2	0.03 ± 0.01
20	60 ± 1	0.44 ± 0.03	0.04 ± 0.004
50	67 ± 10	1.1 ± 0.3	0.3 ± 0.1
100	100	1.2 ± 0.1	
500	100	3.0 ± 0.4	

TABLE 3.7 Absorbance results of reduction of sorbitol after GFOR was purified from the Remazol Brilliant Blue R column at pH 6.5, 25°C and the enzyme concentration was 16.07 μM.

[Sorbitol] (mM)	% Fast phase	Fast rate constants (s^{-1})	Slow rate constants (s^{-1})
10	40 ± 20	0.14 ± 0.05	0.03 ± 0.1
15	42 ± 2	0.34 ± 0.07	0.055 ± 0.003
50	75 ± 3	0.77 ± 0.03	0.09 ± 0.03
100	86 ± 1	1.2 ± 0.1	0.1 ± 0.01
500	100	2.7 ± 0.2	

TABLE 3.8 The rate constants and % fast phase for the reduction of E-NADP$^+$ by 50 mM sorbitol when different points were fitted. The experiment was carried out at 25°C, pH 6.5 and the enzyme concentration was 9.6 μM.

Points fitted	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
70 - 990	66 ± 1	1.22 ± 0.02	0.37 ± 0.08
80 - 990	68 ± 1	1.19 ± 0.02	0.36± 0.01
90 - 990	68 ± 1	1.17 ± 0.02	0.35 ± 0.01
100 - 990	69 ± 1	1.17 ± 0.02	0.35 ± 0.01

An absorbance experiment was carried out at 25°C using 50 mM sorbitol and 9.6 μM GFOR (Fig. 3.13). The results showed that the short time scale gave higher rate constants, but a smaller fast phase amplitude (Table 3.9). This was predicted since the reaction has not completed. The amplitude of the fast phase was underestimated and the rate constants were overestimated since the change in absorbance was faster than normal. For a longer time scale the fast amplitude was similar to that shown in Table 3.8, since the reaction was completed. But the rate constant was slower because the actual reaction was reduced to a small area, which was difficult to measure. Although the results were significant, the values did not correspond to those of Hardman and Scopes (1988) for the same time scale and same sorbitol concentration (shown in bold in Table 3.9).

There were no differences between absorbance and fluorescence results for the reduction of sorbitol. But they were different from those absorbance results obtained by Hardman and Scopes (1988). The reasons for the differences have not been elucidated. The results showed that a slight change in conditions, such as temperature, pH and enzyme concentration did not cause any significant difference in the results. Also, methods of enzyme purification and the difference in data processing showed no difference in the results. However there may be two explanations. The difference in the time scale used for recording the reaction could be one reason. The results in Table 3.9 showed that the results were variable depending on the time for recording the reaction.

The second possible reason could be that there was a machine drift at the end of the reaction. This drift is significant if the reaction was very slow. The reduction of E-$NADP^+$ by sorbitol was very slow, compared to the other three reactions. This drift can alter the calculated phase amplitude and rate constants. Therefore, reduction of E-$NADP^+$ by sorbitol may be only one phase.

The most likely reason for the differences in the reduction of E-$NADP^+$ by sorbitol results obtained by me and those obtained by Hardman and Scopes (1988) is because the

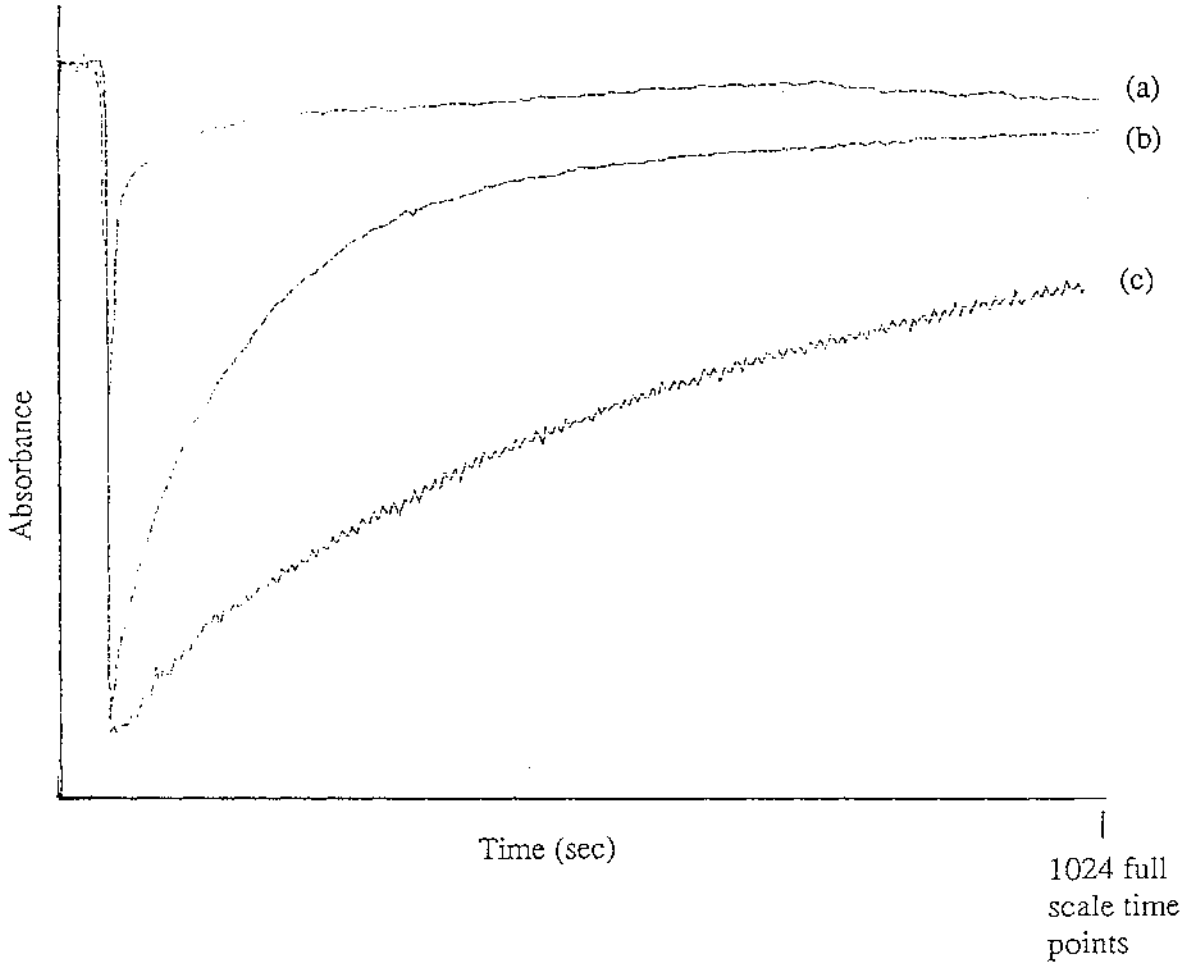


Fig. 3.13 Change in absorbance for the reaction of E-$NADP^+$ with 50 mM sorbitol on different time scales at pH 6.5, 25 °C and enzyme concentration of 9.6 μM.

(a) time scale was 276 seconds, (b) time scale was 10 seconds,
(c) time scale was 2 seconds

TABLE 3.9 The absorbance results for the reduction of E-NADP+ by 50 mM sorbitol on different time scales at 25°C, pH 6.5 and enzyme concentration of 9.6 μM.

Time (sec)	% Fast phase	Fast rate constant (s^{-1})	Slow rate constant (s^{-1})
2	13 ± 2	10 ± 2	0.88 ± 0.02
5	19 ± 5	3 ± 0.8	0.71 ± 0.05
10	62 ± 9	1.1 ± 0.1	0.38 ± 0.06
21	80 ± 5	0.9 ± 0.1	0.12 ± 0.05
50 (Hardman and Scopes, 1988)	**21**	**0.4**	**0.14**
50	81 ± 2	0.8 ± 0.02	0.047 ± 0.002
276	75 ± 3	0.6 ± 0.3	0.04 ± 0.02

rate constants for the two phases are similar in magnitude. Therefore, the rate constants are difficult to distinguish. The amplitudes of the two phases are dependent on the their rate constants (equation 42 of Frost and Pearson, 1961).

3.3.5 Protein Fluorescence

All of the reactions were carried out at 25 $^{\circ}$C and pH 6.5. Typical traces of protein fluorescence on mixing E-NADP$^+$ or E-NADPH (as appropriate) with four substrates, glucose, gluconolactone, fructose and sorbitol are shown in Fig. 3.14. The protein fluorescence decreased when E-NADP$^+$ was mixed with glucose or sorbitol and protein fluorescence increased when E-NADPH was mixed with gluconolactone or fructose. The change in protein fluorescence was small, about 2 to 4%. This small change in signal was difficult to work with and therefore only concentrations of substrates which gave larger amplitudes were measured.

On mixing 1 mM of glucose with E-NADP$^+$ a decrease in protein fluorescence was observed, and a rate constant of 13.5 $\pm$ 0.7 s $^{-1}$ was obtained (Fig 3.14 (a)). This rate constant was similar to the nucleotide fluorescence and nucleotide absorbance rate constants for the same glucose concentration which were 22 $\pm$ 2 s^{-1} and 21 $\pm$ 0.5 s^{-1}, respectively. The protein fluorescence signal was measurable at only one glucose concentration; rate constants was not obtainable at higher glucose concentration, because the protein fluorescence signal was low. The fluorescence quenching was only 2%.

The protein fluorescence increased on mixing 5 mM gluconolactone with E-NADPH and a rate constant of 12 $\pm$ 1 s^{-1} was obtained (Fig. 3.14 (b)). The values obtained using nucleotide fluorescence and nucleotide absorbance at the same concentration of gluconolactone and under the same conditions were similar, 10 $\pm$ 1 s^{-1} and 9.3 $\pm$ 0.4 s^{-1}. The protein fluorescence quenching was also low, 7%.

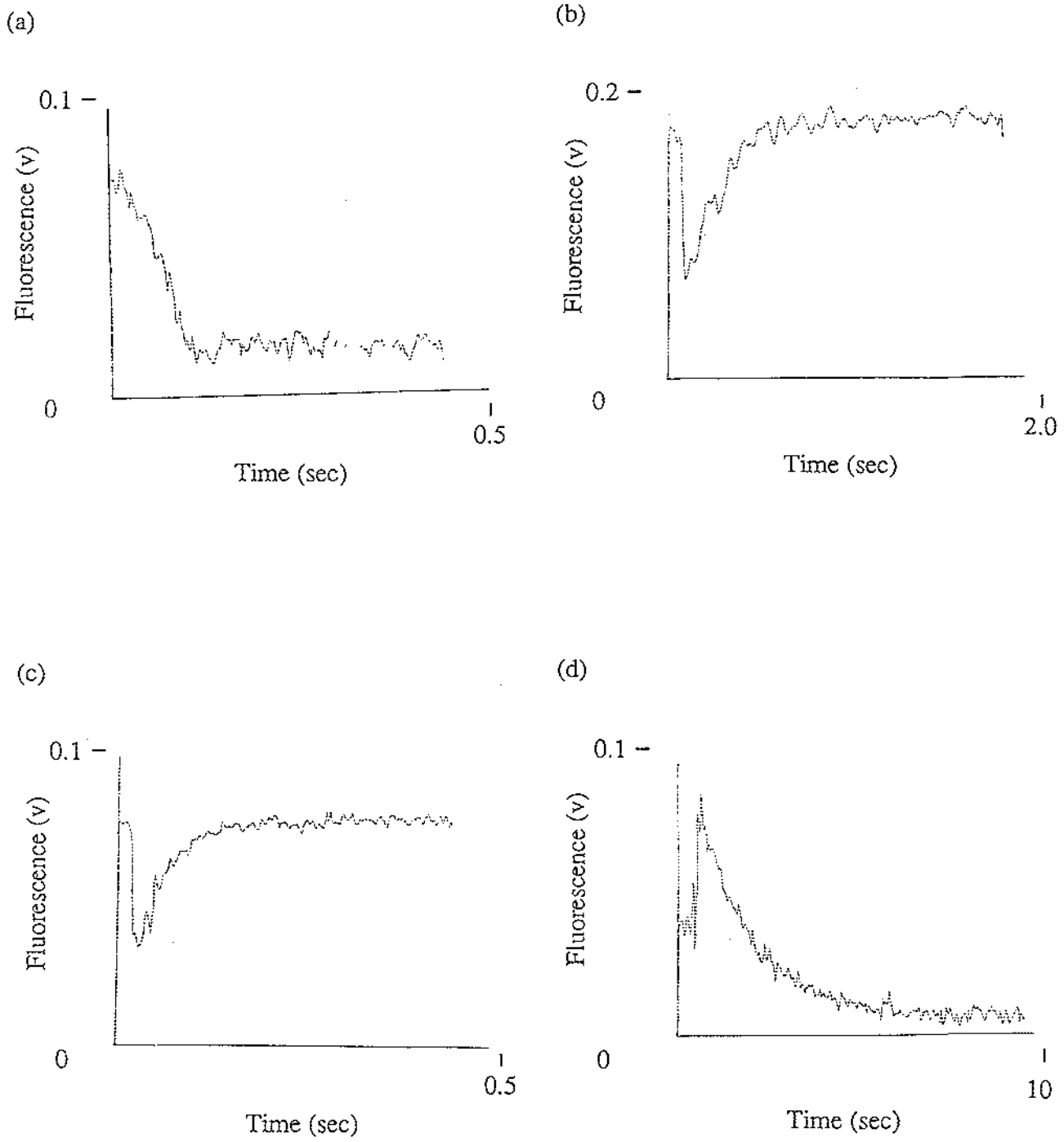


Fig. 3.14 Protein fluorescence traces of oxidation and reduction of GFOR by substrates

(a) 1 mM glucose, 50.6 μM [E-$NADP^+$], 25°C
(b) 50 mM fructose, 16.25 μM [E-NADPH], 25°C
(c) 5 mM gluconolactone, 11.7 μM [E-NADPH], 25°C
(d) 50 mM sorbitol, 2.5 μM [E-$NADP^+$], 25°C

On mixing 50 mM sorbitol with E-NADP$^+$ the protein fluorescence decreased, as shown in Fig. 3.14 (d) and there was 2 % fluorescence quenching. The rate constant was 0.8 ± 0.3 s^{-1}, which is similar to the fast rate constants for nucleotide fluorescence and nucleotide absorbance, 1.1 ± 0.3 s^{-1} and 0.77 ± 0.03 s^{-1}, respectively for the same sorbitol concentration and conditions.

An increase in protein fluorescence was observed on mixing fructose with E-NADPH (Fig. 3.14 (b)). The fluorescence quenching was also small, 4 %. Several fructose concentrations were used (Table 3.10). The rate constants did not increase with the increase in fructose concentration, but at low fructose concentration, such as 50 mM, the rate constant is similar to that of nucleotide fluorescence, 24.3 ± 0.4 s^{-1} at the same concentration and condition.

TABLE 3.10 The protein fluorescence rate constants of oxidation of E-NADPH by fructose at 25°C, pH 6.5 and the enzyme concentration was 17.84 μM.

[Fructose] (mM)	Rate Constants (s^{-1})
10	17 ± 3
25	14 ± 3
50	19 ± 2
100	22 ± 3

The rate constants for the reduction of fructose using protein fluorescence were independent of enzyme concentration (Table 3.11).

TABLE 3.11 Protein fluorescence rate constants of oxidation of E-NADPH by 50 mM fructose at different enzyme concentrations. The experiment was conducted at 25°C and pH 6.5.

[enzyme] (μM)	Rate constants (s^{-1})
5.4	18 ± 1
6.0	15 ± 1
16.25	18 ± 1

Rate constants were obtained at only low substrate concentration, because the fluorescence change was small and was difficult to measure at higher substrate concentrations. However, rate constants were measurable at higher fructose concentrations and this showed that the rate of the protein fluorescence reaction was independent of concentrations of substrates and enzyme (Table 3.10 and 3.11). The rate constants obtained here may not be caused by an artifact, because all four individual reactions were different, and the rate constants corresponded to the nucleotide fluorescence results at the same concentrations.

For all four reactions, the change in protein fluorescence was in the opposite direction to the changes in nucleotide fluorescence and absorbance, which reflects the change between enzyme bound $NADP^+$ and bound NADPH. Protein fluorescence quenching usually results from transferring energy from tryptophan to NADPH, because the excitation wavelength for protein fluorescence here was 290 nm, which is in the range of tryptophan excitation wavelength, 270 to 305 nm (Timasheff, 1970). The GFOR amino acid sequence sent from Dr. R.K. Scopes showed that it contains 3 tryptophan residues per subunit.

The rate constants from protein fluorescence are similar to those obtained using nucleotide fluorescence and absorbance at the same concentrations. This suggests that the protein fluorescence of reduced forms of the enzyme is quenched by the bound NADPH. Another conclusion which can be drawn from these protein fluorescence results is that the protein fluorescence change was not the result of protein conformational change.

Glucose-fructose oxidoreductase has very low protein fluorescence quenching compared with other dehydrogenase systems which have protein fluorescence quenching. For example, in lactate dehydrogenase, it has 87% protein fluorescence quenching (Holbrook *et al..*, 1975) and 50% quenching of alcohol dehydrogenase protein fluorescence (Shore, 1975). This indicated that the tryptophan is far away from the NADPH, because the magnitude of the protein fluorescence quenching is related to the distance apart between the two fluorescent chromophores. Protein fluorescence quenching is proportional to $\frac{1}{r^6}$ where r is the distance between the two fluorescent chromophores; the closer the two groups together the stronger the protein fluorescence signal (Timascheff, 1970).

3.4 Summary

The nucleotide fluorescence results agreed well with the mechanism for glucose-fructose oxidoreductase proposed by Hardman and Scopes (1988) on the basis of absorbance results. Extra information was obtained by nucleotide fluorescence. This information was the quenching of the fluorescence of E-NADPH by gluconolactone when it was bound to the oxidoreductase. This quenching led to the difference between the nucleotide absorbance and fluorescence signals in the glucose/gluconolactone half reaction. The hidden phase in the oxidation of E-NADPH by gluconolactone showed that the binding of gluconolactone to GFOR was fast, but the results for reduction of E-$NADP^+$ by glucose showed that the release of gluconolactone was slow.

The fructose reduction was shown to be linearly dependent on fructose concentration. Higher fructose concentrations could be used in nucleotide fluorescence, but saturation was still not reached. However, the new K_D value for reduction of fructose was estimated to be greater than 5 M.

There were no differences between absorbance and fluorescence results for the oxidation of sorbitol. But the current results are different from those obtained by Hardman and Scopes (1988). Both absorbance and fluorescence showed that the reaction is biphasic. The reason for the difference may be due to the two phases in the sorbitol oxidation reaction having similar rate constants and therefore, being difficult to separate under the conditions used to measure the rate constants. This may be a task for future experiments.

The protein fluorescence rate constants were similar to those for nucleotide fluorescence and absorbance at certain low concentrations of substrates. The direction of protein fluorescence change corresponded to the reverse of the nucleotide fluorescence change. This suggests that protein fluorescence was measuring the quenching of tryptophan fluorescence by NADPH, but not measuring other protein conformational changes. Higher substrate concentrations were also attempted, but their fluorescence signals were small, therefore, they were difficult to measure and it was not pursued any further.

CHAPTER 4
DYE-PROTEIN BINDING STUDIES

4.1 Introduction

Since the discovery of reactive clothing dyes which have strong affinity for proteins, the nature of dye-protein binding has been studied. It still remains a mystery because no satisfactory answers have been found. However, a few general suggestions have been proposed.

Dyes contain chemical groups similar to those of proteins. For example, dyes contain fused aromatic rings which show structural resemblance to aromatic amino acids (Scopes, 1986). Therefore, aromatic amino acids are sometimes used in eluting proteins from the dye columns. The polyanionic nature and the sulphonic acid groups of the dyes also resemble the phosphodiester backbone of nucleic acids (Atkinson, *et al.*., 1981). In this project phosphate ion and salt were used in the elution of glucose-fructose oxidoreductase from the Remazol Brilliant Blue column.

Dyes also contain a variety of side groups which could form noncovalent bonding interactions, such as electrostatic interaction, hydrophobic interactions and hydrogen bonding (Scopes, 1986). These bonding forces are also found in proteins. Therefore, the dye-protein interactions may be similar to the protein-protein interactions.

The dye which has been studied in most detail is Cibacron Blue F-3GA. This dye binds to proteins containing a nucleotide fold in particular (Thompson and Stellwagen, 1976; Biellman *et al.*., 1979). It has been clearly shown that the dye binds to the nucleotide-binding site of alcohol dehydrogenase. But this was not indicative of how Cibacron Blue

F-3GA binds to proteins in general, since it also binds to proteins without a nucleotide-binding site (Hey and Dean, 1983). Thompson and Stellwagen (1976) showed a spectral shift upon the titration of phosphoglycerate kinase with Cibacron Blue F-3GA dye. This indicated that there was some form of interaction between enzyme and dye. In this chapter, the nature of dye-protein binding was briefly investigated.

4.2 Methods

4.2.1 Measurement of Spectral Shift of Dye-Protein Binding

Twenty µl aliquots of 22 µM glucose-fructose oxidoreductase was gradually added to 0.7 ml of dye, which was of similar concentration to the final GFOR concentration. The mixture solution was scanned from 300 nm - 820 nm by a HP8452A Diode Array Spectrophotometer interfaced to IBM compatible computer.

4.3 Results and Discussion

4.3.1 Spectral Shift of Dye-protein Binding:

All three dyes have a maximum absorbance wavelength in the visible range, 300 nm - 800 nm (Figures 4.1, 4.2 and 4.3):

695 nm for Procion Green H4G,

421 nm for Procion Yellow H-E4R, and

610 nm for Remazol Brilliant Blue R8001

No shift in absorbance maximum upon the binding of the dye to the enzyme was observed. Though the yellow dye showed a small increase in its maximum absorbance, this may be due to the influence of the protein absorbance at 280 nm, because the maximum absorbance was near this region. Naturally the absorbance should increase in

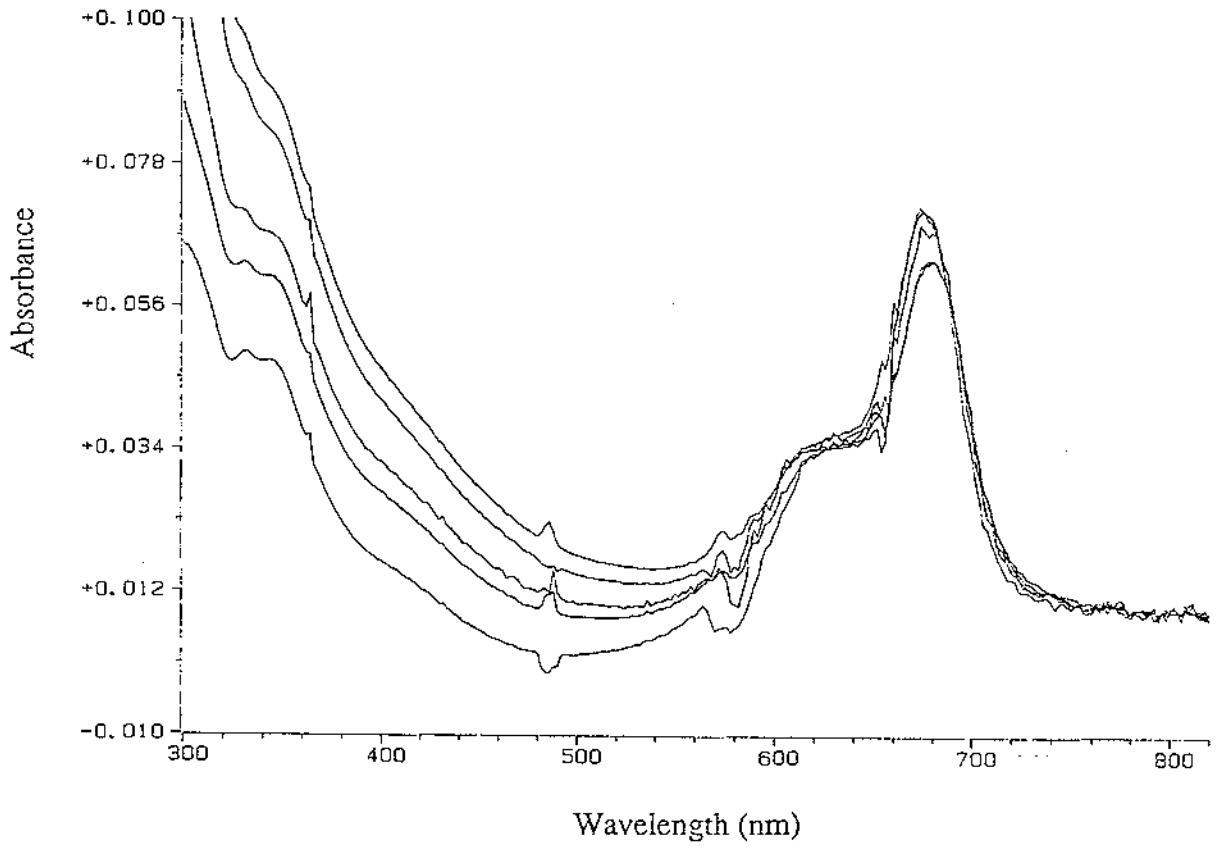


Fig. 4.1 Spectrum of Procion Green H-4G dye plus GFOR

The experiment was carried out at 25°C, pH 6.5, and [dye] was 1.46 μg/ml. The solution absorbance spectrum was scanned as 20 μl aliquots of 22 μM of GFOR were added 1 ml of solution

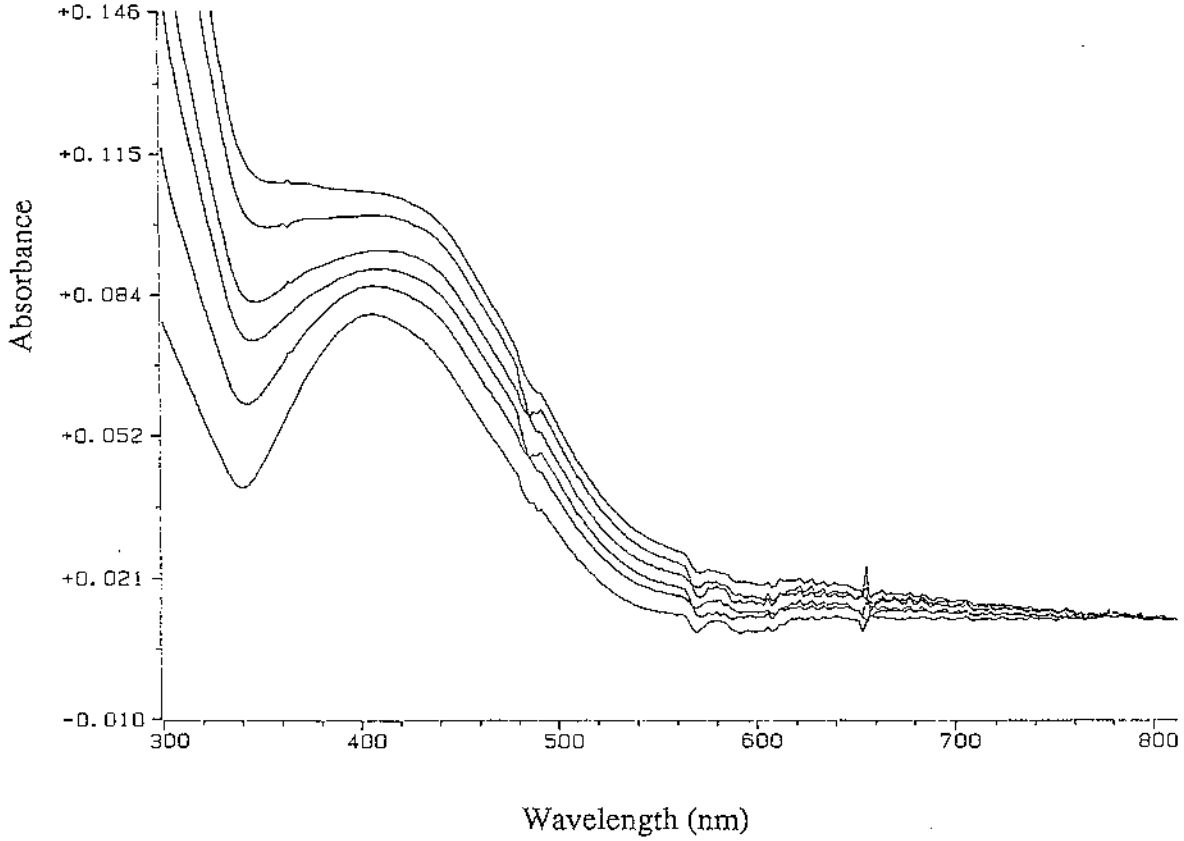


Fig. 4.2 Spectrum of Procion Yellow H-E4R dye plus GFOR

The experiment was carried out at 25 °C, pH 6.5, and [dye] was 4.2 μg/ml. The solution absorbance spectrum was scanned as 20 μl aliquots of 22 μM of GFOR was added.

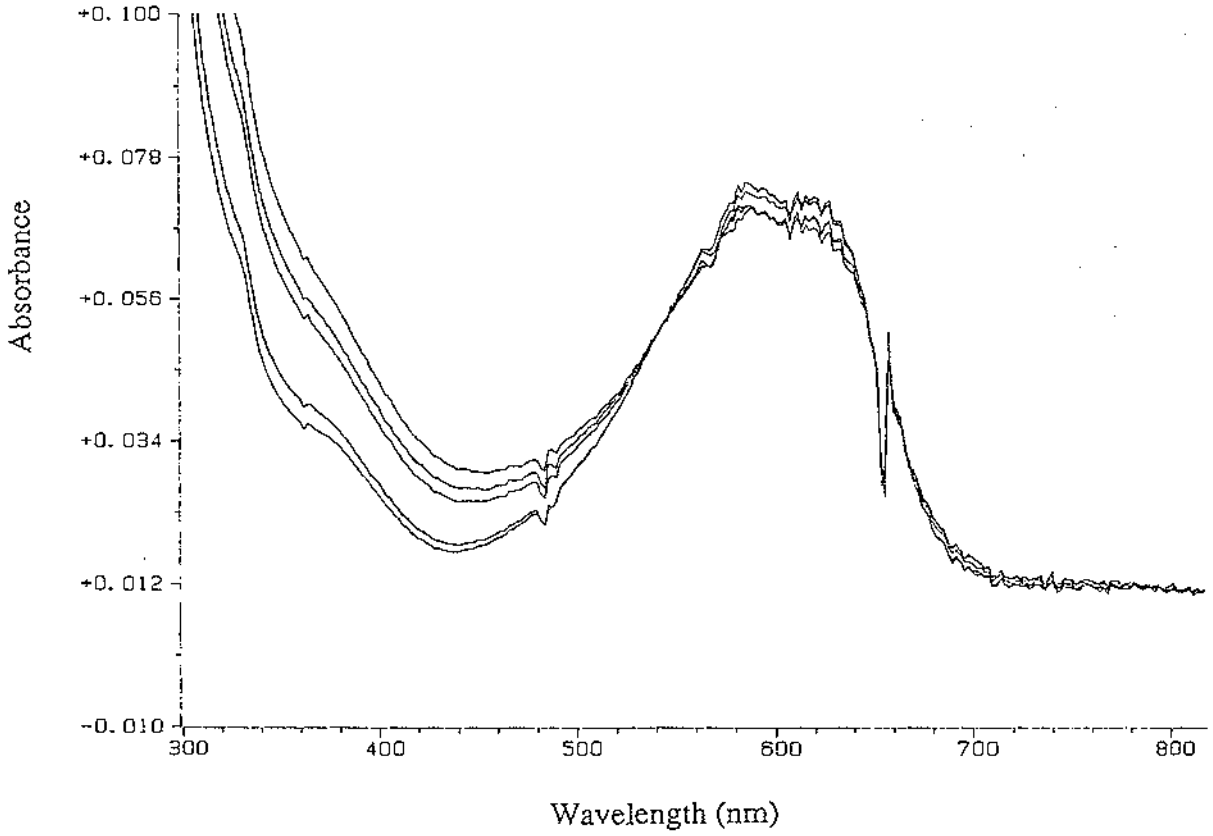


Fig. 4.3 Spectrum of Remazol Brilliant Blue R8001 dye plus GFOR

The experiment was carried out at 25 °C, pH 6.5, and [dye] was 5.2 µg/ml. The solution absorbance spectrum was scanned as 20 µl aliquots of 22 µM of GFOR was added.

this region, because protein (GFOR) was added. Because the blue and green dye absorbance maxima were further away from 280 nm, their absorbance increases were smaller.

4.3.2 Active Site of Glucose-fructose Oxidoreductase:

The activity of GFOR was measured when the dye bound to the enzyme. The binding of the dye to Glucose-fructose oxidoreductase did not affect the oxidoreductase activity. Table 4.1 shows that the enzyme activity remained essentially unchanged and therefore the dyes did not bind to the enzyme's active site.

TABLE 4.1 Effect of dye binding on glucose-fructose oxidoreductase activity. The values are duplicate assays.

Sample	GFOR + MES buffer	GFOR + Green Dye	GFOR + Blue Dye	GFOR + Yellow Dye
GFOR Activity (μmol min $^{-1}$ ml $^{-1}$)	43.72, 41.30	42.00 44.55	45.94 41.60	45.50 41.30

4.3.3 Reversibility of Dye-Protein Binding

The dye-GFOR complex solution after the spectral shift scanning experiment was dialysed overnight against pH 6.5 MES buffer to test out whether the dye-protein binding was reversible over a long period of time. The absorbance was measured before and after dialysis. There were no significant decreases in absorbance of all three dye-GFOR

complex solution after overnight dialysis, compared to the solution with GFOR in MES buffer only (Table 4.2). This suggests that the dye-protein bond may not be broken and this bond is strong. If the dye-enzyme complex was weak, the bond should be broken upon dialysis over a long period of time and the dyes should leave the dialysis bag. The size exclusion of the dialysis bag was to exclude molecules with molecular weight less than 10,000 to 15,000. The molecular weights of the dyes were less than 2,000, for example, Remazol Brilliant Blue has a molecular weight of 627.

TABLE 4.2 Absorbance maxima of dye-glucose-fructose oxidoreductase after overnight dialysis.

Dye	Absorbance maxima wavelength (nm)	GFOR + MES buffer	Dye + GFOR (before dialysis)	Dye + GFOR (after dialysis)
Yellow	421	0.046	0.118	0.127
Green	695	0.012	0.0813	0.052
Blue	610	0.017	0.083	0.04

4.4 Conclusion

The binding of dye to glucose-fructose oxidoreductase does not alter the conformation of either the protein or the dyes, because no significant absorbance change or spectral shift was detected. This suggests that the bonding forces between the dye and the protein may only be surface forces, for example, hydrogen bonds, electrostatic interaction, and hydrophobic interactions. These surface forces do not cause any significant conformation changes to the protein.

Dye-protein binding that causes minimum enzyme conformation change is ideal in enzyme purification during dye-ligand column chromatography, because the purified enzyme in its correct conformation could be used for studies involving the active form of the enzyme.

The activity of glucose-fructose oxidoreductase was not affected upon the formation of the dye-protein complex. This shows that the dye-binding site on the oxidoreductase was not at the active site, nor did the binding cause any conformational changes which could affect the enzyme activity.

Also the dialysis result showed that the dye-enzyme binding was strong, since the dyes could not be diffused out of the dialysis bags. However, this bond could be disrupted by salts and phosphate ions, because the dye-protein bonding is believed to involve ionic interactions. This property is useful in enzyme purification when eluting enzyme from the column. Further work is required to study the dialysis of dye-enzyme complex against high salt concentration. The dyes would be expected to diffuse out from the glucose-fructose oxidoreductase.

CHAPTER 5

CHEMICAL MODIFICATION OF GLUCOSE-FRUCTOSE OXIDOREDUCTASE

5.1 Introduction

Glucose-fructose oxidoreductase (GFOR) has been shown to contain a tightly bound cofactor, $NADP^+$. It does not contain any metal ions, since treatment with o-phenanthroline or EDTA did not affect GFOR activity (Zacharious and Scopes, 1986). Furthermore, no external sulphhydryl groups were found, since GFOR was not modified by dithiobis (dinitrobenzoate) after the subunits were exposed with the treatment of sodium dodecyl sulphate (Zacharious and Scopes, 1986).

In this chapter the effect of diethyl pyrocarbonate (DEPC) on GFOR was briefly investigated. Diethyl pyrocarbonate (ethoxyformic anhydride) specifically modifies histidine residues (Melchior and Fahrey, 1970).

5.2 Methods

5.2.1 Measurement of DEPC

Diethyl pyrocarbonate concentration was measured based on the methods of Motion (1986) and Melchior and Fahrey (1970). Diethyl pyrocarbonate was first dissolved in absolute ethanol. Then the DEPC concentration was determined by the change in absorbance at 233 nm when an aliquot of DEPC solution was added to 10 μM of histidine solution. The ε (absorbance coefficient) was 3200 l mol^{-1} cm^{-1}.

5.2.2 Modification of Glucose-fructose Oxidoreductase with Diethyl Pyrocarbonate

Two ways of observing the effect of DEPC on GFOR were used, as follows:

(a) Diethyl pyrocarbonate at different concentrations was added to GFOR assay mixtures without prior incubation of GFOR with DEPC. Glucose-fructose oxidoreductase acivity was measured immediately. The control experiment contained the same volume of ethanol, but no DEPC. The ethanol concentration was below 4% in the final enzyme assay mixture.

(b) Glucose-fructose oxidoreductase (2 μM) was incubated with DEPC at different concentrations in pH 6.0 phosphate buffer at 25°C. Then samples were taken for the GFOR activity determination at various time intervals (Morris and McKinley-McKee, 1972).

5.2.3 UV Absorbance Spectrum of Modified GFOR

The UV absorbance spectra of 1 ml solutions of 2 μM glucose-fructose oxidoreductase, and 20 μM or 281.2 μM DEPC were scanned from 190 nm to 500 nm. The scanning was carried out using a HP8452A Diode Array Spectrophotometer interfaced to an IBM compatible computer. The reaction was scanned for 60 minutes at various time intervals.

5.3 Results and Discussion

5.3.1 The Enzymic Activity of Modified GFOR

When 2 μl aliquots of 3.75 mM DEPC were added to the GFOR assay mixture (without prior incubatof of GFOR with DEPC), an activation of GFOR activity was shown (Fig. 5.1). The GFOR activity increased with increasing addition of DEPC (Fig. 5.1).

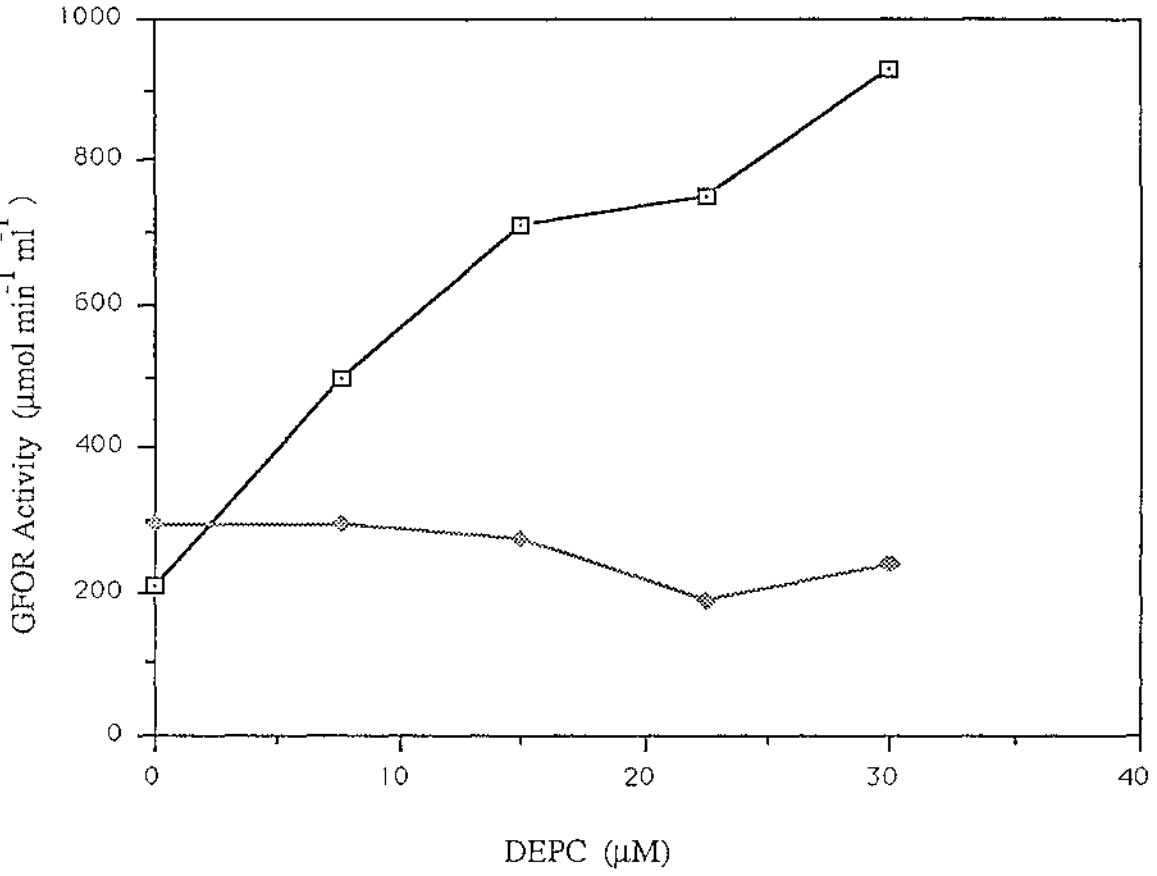


Fig 5.1 Activation of glucose-fructose oxidoreductase with diethyl pyrocarbonate

Glucose-fructose oxidoreductase was not incubated with DEPC before activity measurement, but DEPC was added at the start of the enzyme assay. The reaction was carried out at 25°C, pH 6.5 , and the enzyme concentration was 0.5mM. The enzyme activity assay took 0.5 min.

control experiment with no DEPC, only absolute ethanol

GFOR activity with DEPC

The activity of GFOR after prior incubation with DEPC was also measured. Glucose-fructose oxidoreductase was incubated with different DEPC concentrations for various time intervals. Initial activation of GFOR activity was observed at low DEPC concentrations (23 μM and 28.2 μM) (Fig. 5.2). This activation of GFOR activity occurred within the first 5 minutes, followed by inactivation of GFOR activity at later times. The enzyme activity was not completely inhibited at low DEPC concentrations. However, at high DEPC concentrations, initial activation of glucose-fructose oxidoreductase activity was not observed, and the oxidoreductase activity was completely inhibited within 10 to 25 minutes.

When GFOR was incubated with various concentrations of DEPC were incubated for 60 minutes, GFOR activity decreased with increasing DEPC concentration (Fig. 5.3). The results showed that the effect of DEPC on glucose-fructose oxidoreductase was both time and concentration dependent. This suggests that modifying a group in GFOR by DEPC may activate the enzyme, but complete modification of this group could deactivate GFOR.

5.3 2 UV Absorbance Spectrum of GFOR Modified by DEPC

The UV absorbance spectra of the GFOR modified by two concentrations of DEPC concentrations (20 μM and 281.2 μM) were scanned. The peak absorbance for the rmodification of GFOR by DEPC started at 242 nm, then shifted towards lower wavelength (blue shift), from 242 nm to 234 nm after 60 minutes incubation with DEPC (Fig 5.4 and Fig 5.5). This blue shift was characteristic of carbethoxylation of histidine (Morris and McKinley-McKee, 1972). However, the slope of absorbance increase with time at 242 nm in Fig 5.6, but in other histidine modified systems, the slope of absorbance change at 242 nm decrease with time (Pradel and Kassab, 1968; Huc *et al.*, 1971; Morris and McKinley-McKee, 1972). The loss of GFOR activity did not correspond to the increase in absorption at 242 nm (Fig 5.7). Therefore, the number of

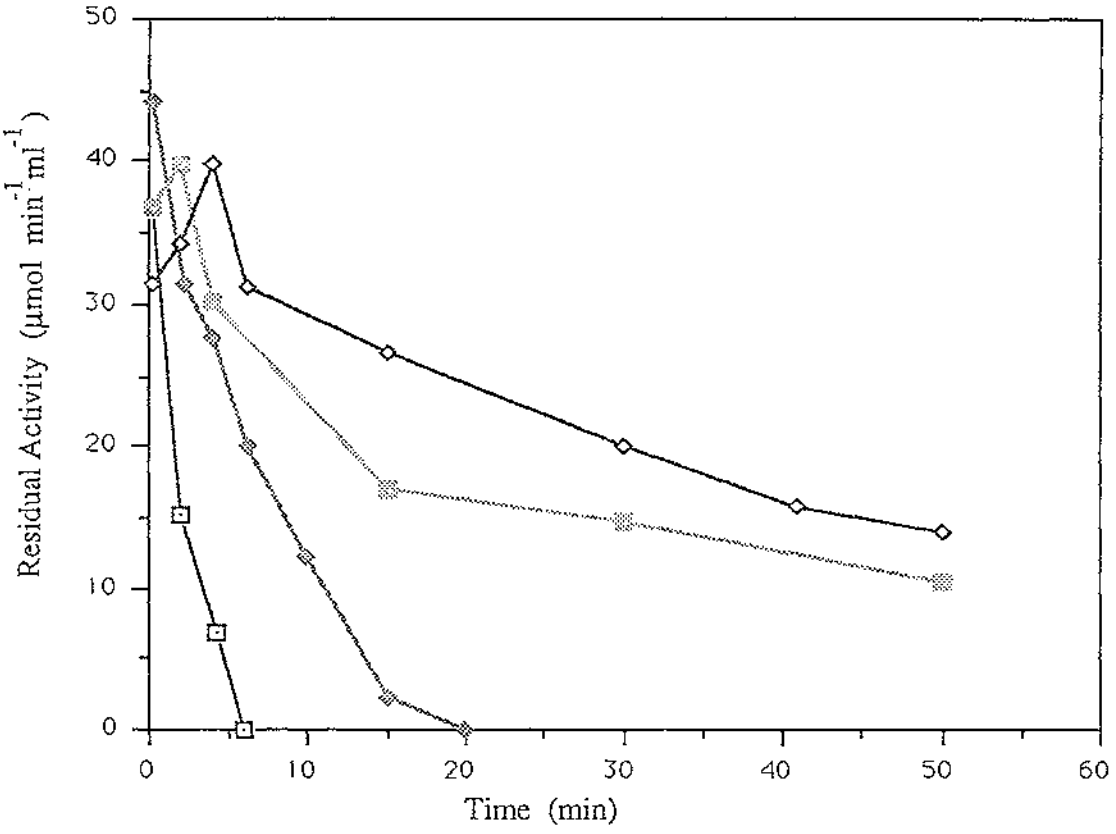


Fig 5.2 The effect of diethyl pyrocarbonate on glucose-fructose oxidoreductase during 60 minute incubation with various DEPC concentrations.

Glucose-fructose oxidoreductase was incubated with various DEPC concentrations for various time interval in pH 6.0 phosphate buffer at 25°C, and the enzyme concentration was 2 μM. Samples were taken and GFOR activity was assayed for 0.5 minutes.

- 281.2 μM of DEPC,
- 56.24 μM of DEPC,
- 23.43 μM of DEPC,
- 20.09 μM of DEPC

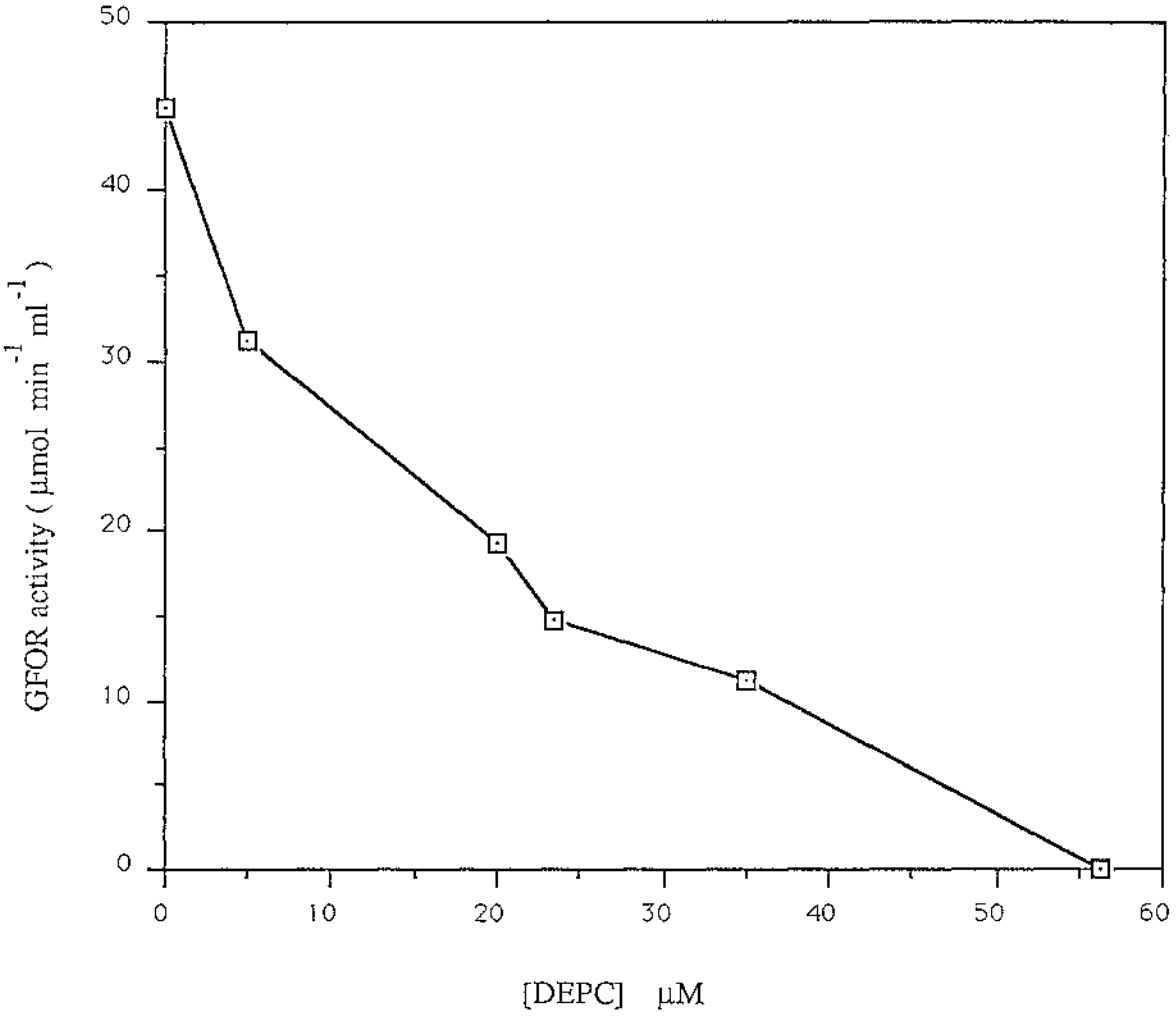


Fig 5.3 The effect of different DEPC on glucose-fructose oxidoreductase at after 60 minutes incubation

The incubation mixture contain 2 µM of GFOR and different concentrations of DEPC in pH 6.0 phosphate buffer. The enzyme activity was measured at 25 °C.

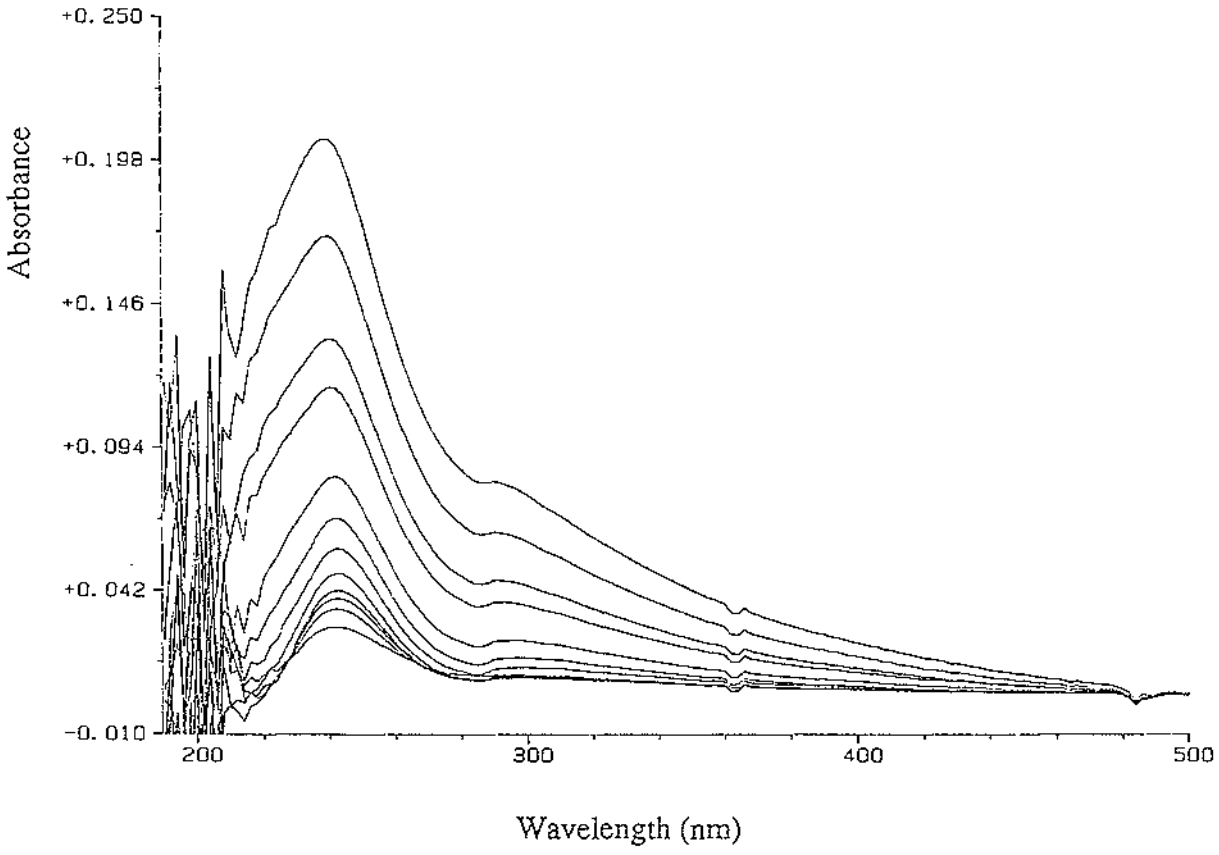


Fig. 5.4 UV spectrum of the effect of 20.9 μM of DEPC on GFOR at various time intervals.

The reaction mixture with 2 μM GFOR and 20.9 μM DEPC was incubated in pH 6.0 phosphate buffer at 25°C for 60 minutes. The UV spectrum of the above reaction mixture was scanned at various time intervals.

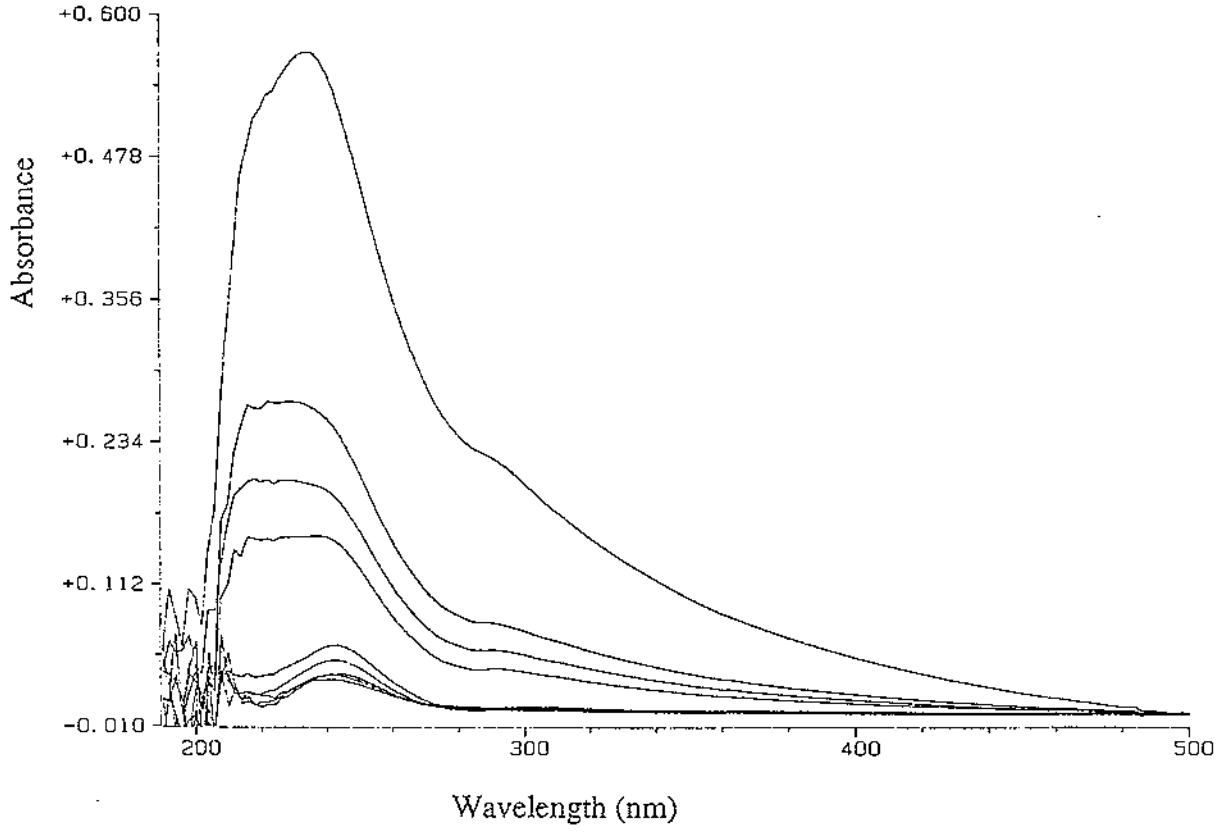


Fig. 5.5 UV spectrum of the effect of 281 μM DEPC on GFOR at various time intervals.

The reaction mixture with 2 μM GFOR and 281 μM DEPC was incubated in pH 6.0 phosphate buffer at 25 °C for 60 minutes. The UV spectrum of the reaction mixture was scanned at various time intervals.

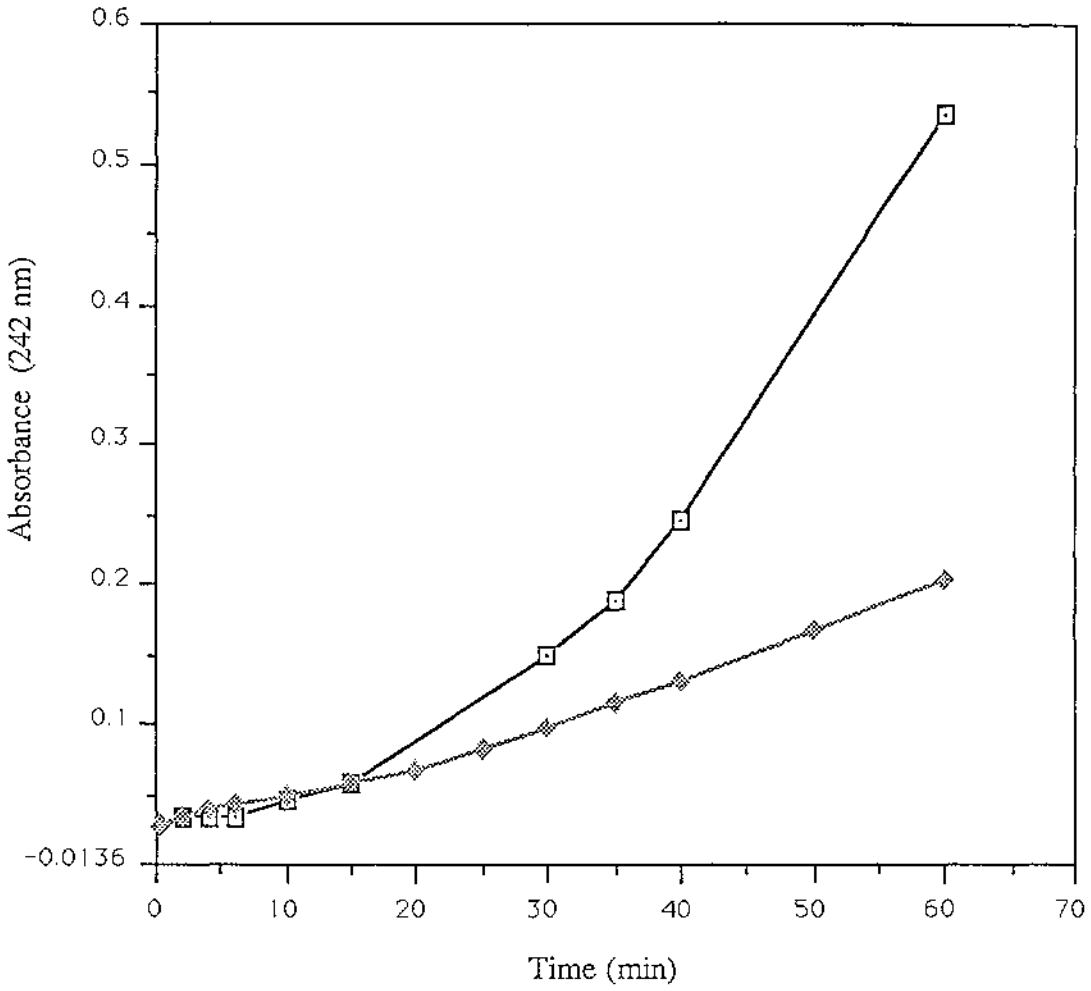


Fig 5.6 The absorbance change at 242 nm over 60 minutes

The absorbance change at 242 nm from fig 5.4 and 5.5.

281.2 μM DEPC, 20.9 μM DEPC

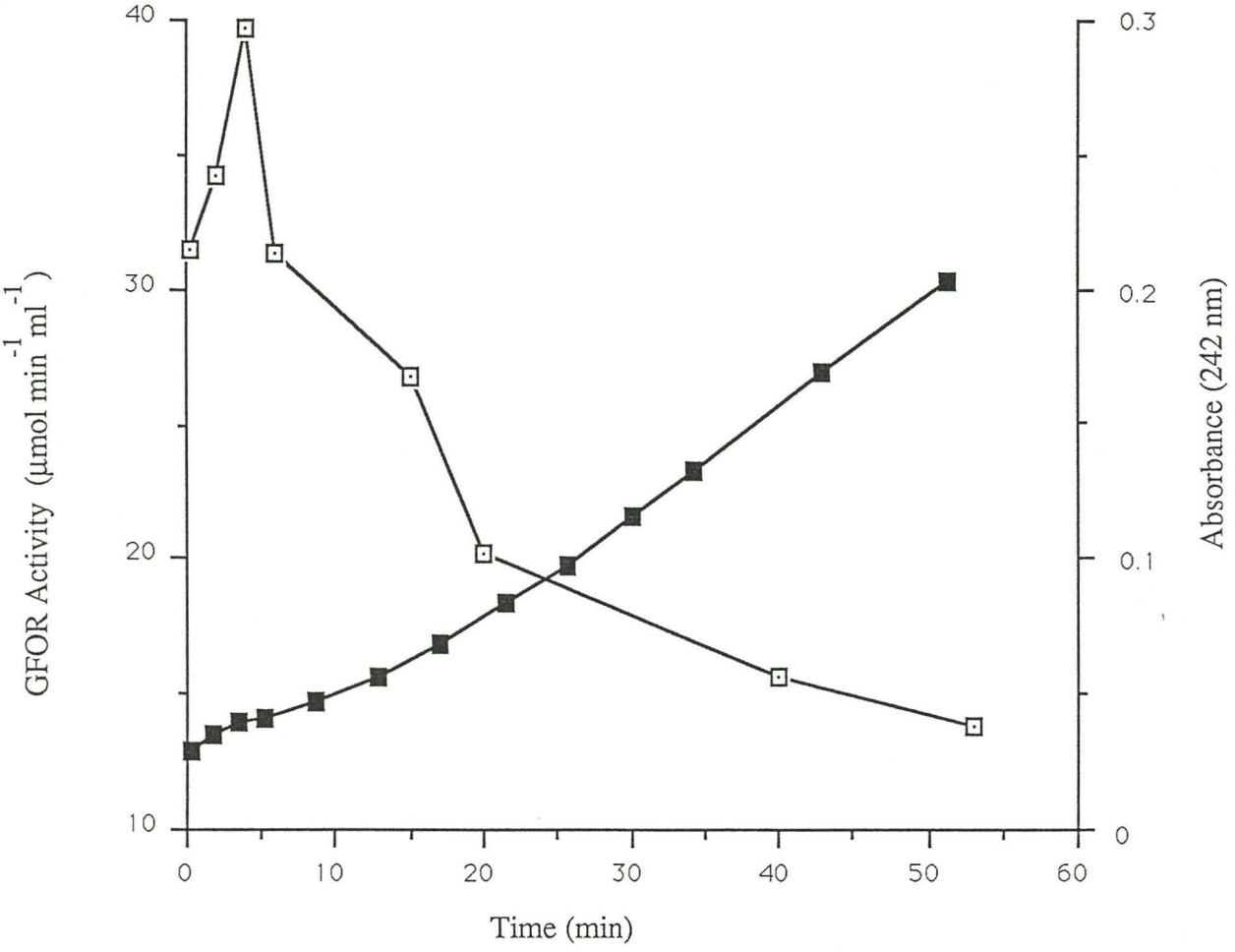


Fig. 5.7 The graphs of GFOR activity and absorbance at 242 nm of 20.09 μM DEPC against time

⊡ GFOR activity

■ absorbance at 242 nm

histidine residues modified per mole of glucose-fructose oxidoreductase could not be calculated.

There may be three reasons for the lack of correspondence between the loss of oxidoreductase activity and the absorbance increase at 242 nm. One reason could be that the protein was denatured. Therefore, the rate of loss of glucose-fructose oxidoreductase activity was faster than the absorbance increase at 242 nm. At the end of 60 minutes incubation with DEPC, the enzyme solution became cloudy, indicating that the protein had precipitated. Protein precipitation would also lead to an increase in absorbance. Another reason may be that DEPC did not specifically modify histidine.residues.

The specificity of diethyl pyrocarbonate can be checked with various methods, for example, analysis of the effect of DEPC on glucose-fructose oxidoreductase activity and absorbance at 242 nm before and after denaturaion of GFOR with 6 M urea or 1% SDS, and excess DEPC (Pradel and Kassab, 1968).

There are other chemical reagents which specifically modify histidine residue such as diazo-1-H-tetrazole (Apitz-Castro and Suarez, 1970). They can be used to test the specificity of histidine modification.

The best test for the specificty of DEPC modification of histidine groups on glucose-fructose oxidoreductase is perhaps to carry out reverse inhibition studies by using hydroxylamine (Huc *et al.*, 1971; Morris and McKinley-McKee, 1972). Hydroxylamine can specifically remove carbethoxylation on histidine residues and the enzyme activity is restored.

5.3.3 Protection of GFOR by Substrates Against DEPC Inactivation

Glucose-fructose oxidoreductase (oxidized or reduced) was incubated with substrates: glucose, fructose and sorbitol, and DEPC before the enzyme activity was measured.

The oxidised form of the oxidoreductase (4.4 μM) was incubated with 281.2 μM of DEPC and various sugar concentrations (1M glucose, 0.5 M glucose, 1M sorbitol, 0.5 M sorbitol, and 1M fructose in pH 6.0 phosphate buffer). Glucose-fructose oxidoreductase activity was measured at various time intervals.

The enzyme (E-$NADP^+$) was not protected against DEPC inhibition by the subtrates, not even glucose, which was known to bind to the oxidised form of the enzyme (Hardman and Scopes, 1988).

The reduced glucose-fructose oxidoreductase (E-NADPH) (4.4 μM) was also incubated with the same concentration of DEPC under same conditions, but only with fructose.

Inhibition of the reduced glucose-fructose oxidoreductase (E-NADPH) by DEPC was not protected by fructose, which was also known to bind to this form of the enzyme (Hardman and Scopes, 1988)

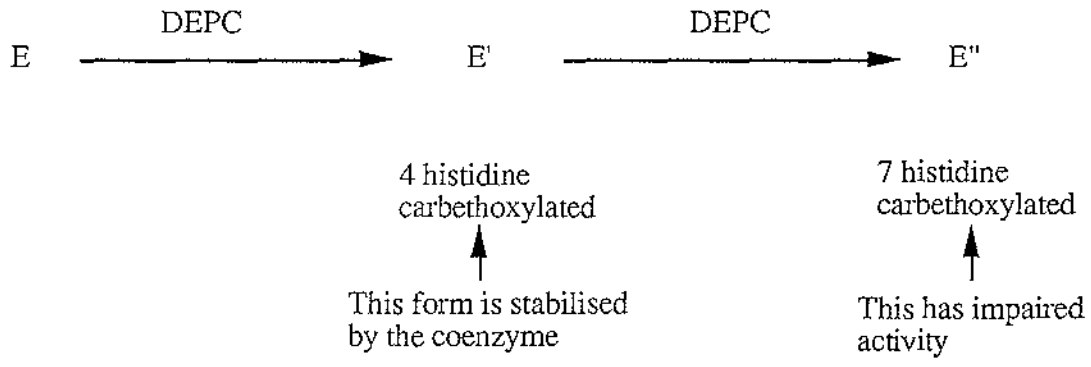
.

This lack of substrate protection of GFOR against DEPC inactivation suggests that the group which was modified by DEPC is not involved in the enzymatic reaction. But this modified group may be important in maintaining the active conformation of the oxidoreductase.

5.4 Conclusion

The results from the modification of glucose-fructose oxidoreductase by diethyl pyrocarbonate suggest that DEPC modifies catalytically important GFOR histidine residues. The results in Section 5.3.1 showed patterns of enzyme activation and inhibition by DEPC similar to those of liver alcohol dehydrogenase (Morris and McKinley-McKee, 1972). The evidence for the DEPC modification of histidine residue was demonstrated, by a blue shift from 242 nm to 234 nm (Section 5.3.2). This is a positive test for carbethoxylation of histidine.

The role histidine plays in glucose-fructose oxidoreductase could be of conformational and/or catalytic importance. If more than one histidine residue were involved, the initial activation seen in Fig 5.2 may be due to the modification of one of the histidine residues which stabilized the binding of substrates or increased the k_{cat}. But complete modification of histidine residues caused denaturation of the GFOR. Morris and McKinley-McKee (1972) found that alcohol dehydrogenase underwent three subtle conformational changes (refer to the diagram below). At low DEPC concentrations, the first conformation formed as a result of DEPC modification was stabilised by the coenzyme. Carbethoxylation of the rest of the histidine residues denatured the enzyme. At high concentrations of DEPC, excess DEPC was available and all histidine residues were probably modified simultaneously in alcohol dehydrogenase. Therefore, the activation phase was not observed.



The substrates did not protect glucose-fructose oxidoreductase against DEPC deactivation, suggesting that the modified histidine residues were not at the active site. Nevertheless, they could be near the active site, as seen in octopine dehydrogenase (Huc *et al.*, 1971).

CHAPTER 6

REMOVAL OF COENZYME FROM GLUCOSE-FRUCTOSE OXIDOREDUCTASE

6.1 Introduction

Glucose-fructose oxidoreductase contains a tightly bound cofactor, $NADP^+$ (Zachariou and Scopes, 1986), which remains tightly bound throughout the catalytic cycle (Hardman and Scopes, 1988). This feature is rare among enzymes which use pyridine nucleotide as cofactor. The pyridine nucleotide usually leaves the enzyme during some stage of the catalytic cycle. For example, aldehyde dehydrogenase has NAD^+ which is bound as a substrate and then leaves the enzyme as NADH (Bennett *et al.*., 1982). There is one enzyme which shares the above property of GFOR, lactate-malate oxidoreductase.

In order to understand the nature of the $NADP^+$-enzyme complex, removal of $NADP^+$ is required. Zachariou and Scopes (1986) separated $NADP^+$ from GFOR by precipitation with perchloric acid. But since the apoprotein was denatured, no study was done on the apoprotein. Allen (1966) also found that $NADP^+$ from lactate-malate oxidoreductase was removed only by methods which gave inactive apoenzyme.

The information obtained from apoenzyme studies could be useful in understanding the nature of the enzyme-$NADP^+$ complex. This has been illustrated by the studies done on flavin proteins. The type of binding (noncovalent or covalent) involved in FAD-enzyme complexes was established by the treatment used to remove the FAD. Dialysis or acidic denaturation was employed to remove noncovalently bound flavin and a covalently or peptide-bound flavin was removed by proteases (Friesel and McKenzie, 1970; Singer *et al.*., 1971).

The properties of the apoenzyme and reconstituted holoenzyme can be studied and compared with those of the native enzyme (Choong *et al.*, 1975).

The third way by which the cofactor binding to the enzyme can be studied is the use of probes to detect intermediates of the catalysed reaction (Gihisla *et al.*, 1977; Entsch *et al.*, 1980). These probes are FAD containing a substituted reactive group. Depending on the reactive group, these probes can be used in different ways. Massey *et al.* (1984) used probes to reveal the solvent accessibility of FAD in flavoproteins. Strittmatter (1961) found a sulphhydryl group of cytochrome b_5 reductase was involve in FAD-apoenzyme binding.

Finally, the function of the prosthetic group can be studied. FAD in glucose oxidase is involved in oxidation and also stabilizes the tertiary structure of the protein (Swoboda, 1969).

6.2 Methods

6.2.1 Acidic Ammonium Sulphate Precipitation of GFOR

This method was based on that of Choong *et al.*. (1975), with slight modification. Saturated $(NH_4)_2SO_4$ solution at pH 1.5 was added to GFOR solution (3 to 10 mg/ml) in 0.1 M sodium acetate buffer (pH 5.7), to give 90 % saturation with $(NH_4)_2SO_4$. This solution was centrifuged for 10 minutes. The precipitate was resuspended in the same buffer, and the acid was neutralised by the addition of 2.5 M sodium acetate. Glucose-fructose oxidoreductase was again reprecipitated with pH 1.5 $(NH_4)_2SO_4$ solution and this was repeated for two cycles. The final GFOR precipitate was then resuspended in pH 6.5 $(NH_4)_2SO_4$ solution to give 80 % saturation. The enzyme solution was again centrifuged and was resuspended in pH 6.5 MES. This solution was scanned from 200

nm to 600 nm for any remaining $NADP^+$ that were present using a Unicam SP800 UV Spectrophotomer, before it was incubated with 0.1 mM $NADP^+$ for reconstitution.

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

(a) This method was based on that of Ghisla *et al.*. (1977). Glucose-fructose oxidoreductase solution (25 to 30 μM) was dialysed against 50 volumes of a buffer which contained the following: 1 M KBr solution, 1 M urea, 0.5 mM EDTA in pH 6.5 MES buffer at 2 to 4 °C. The dialysis buffer was changed every 12 hours until 2 % or less of the total GFOR activity was left. The apoprotein was then dialysed against 0.5 mM EDTA in 10 mM MES (pH 6.5). The apoenzyme was incubated with $NADP^+$ for reconstitution.

(b) This method was based on the procedure of Hill (1988). A solution containing 1 mg/ml of GFOR was dialysed against 30 mM NAD^+ in pH 6.5 buffer over night, followed by dialysing the GFOR solution against 1 litre of pH 6.5 MES buffer without NAD^+. The second step was repeated twice until little or no residual GFOR activity was left. The apoenzyme was then incubated with $NADP^+$ for reconstitution.

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

A solution of GFOR solution (1 mg/ml) was passed through an activated charcoal column (3 cm^2 x 5 ml). Then the column was washed with pH 6.5 MES buffer. The fraction containing protein was collected.

6.3 Results and Discussion

6.3.1 Acidic Ammonium Sulphate Precipitation of GFOR

The UV spectrum showed no absorbance at 259 nm or near this region. This indicated that $NADP^+$ was removed. The addition of $NADP^+$ to the apoenzyme did not reconstitute GFOR activity, even after 2 hours of monitoring the enzyme activity. A possible reason may be that the protein was denatured and this denaturation was irreversible.

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

After eight to nine days of dialysis against 1 M KBr and 1 M urea, over 10 % of the original enzyme activity was still present, much higher than the expected value (2 % or less). This method was too mild to remove $NADP^+$ from GFOR.

Almost 90 % of the GFOR activity was lost after several days of dialysis against KBr/urea, compared to about 10 % in the usual dialysis in enzyme purification. This may be due to the breakage of salt bridges and other ionic bonds in GFOR by high salt concentration, but not all of the bonds which bound $NADP^+$ to the enzyme. Therefore, GFOR activity was lost.

The dialysis of GFOR against NAD^+ retained 72 % of the original enzyme activity. Also, the dialysate showed some precipitation. One reason the NAD^+ dialysis method retained higher original GFOR activity than KBr/urea dialysis was that the NAD^+ dialysis was milder than the KBr/urea dialysis. Also, the duration of NAD^+ dialysis was shorter than KBr/urea; GFOR is unstable after prolonged dialysis, especially at low pH (refer to Section 2.3.3.1).

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

The absorbance of the GFOR solution before and after the charcoal column was measured at 280 nm. It showed that the protein was not eluted off the column, but remained bound by the charcoal. This was expected, since charcoal is designed to remove organic compounds, and proteins are one example of organic molecules. The charcoal may bind the hydrophobic regions of GFOR as in hydrophobic chromatography. Glucose-fructose oxidoreductase binds to a thio C_4-Sepharose hydrophobic column in protein purification (refer to Section 2.3.4.2).

6.4 Conclusion

The results indicated that $NADP^+$ bound tightly to the GFOR apoenzyme. The evidence for this was that $NADP^+$ could not be removed by mild ionic disruption, such as dialysis against KBr/urea, but only by "harsh" methods which resulted in irreversible protein denaturation. These methods included perchloric acid (Zachariou and Scopes, 1986) and acidic $(NH_4)_2SO_4$ precipitation (Allen, 1966). Dialysis against potassium bromide and urea should have removed $NADP^+$ from glucose-fructose oxidoreductase if the $NADP^+$ was bound to the enzyme by weak ionic bonds. The nature of the $NADP^+$ - enzyme interaction probably involved strong non-covalent bonds, such as hydrophobic bond and hydrogen bonds. The complex bond cannot be covalent because $NADP^+$ was removed completely by acidic $(NH_4)_2SO_4$ precipitation. The cofactor is linked to the protein by noncovalent bonds if the cofactor is released on denaturation of the protein, without any protease present (Singer *et al* ., 1971; Friesel and McKenzie, 1970)

The third conclusion that could be drawn from the results is that the cofactor is important in stabilizing GFOR. When the pyrimidine nucleotide is removed, the structure collapses or changes, resulting in different bonds forming and burying the cofactor binding site.

This property was found in glucose oxidase (Swoboda, 1969). This could be the reason why $(NH_4)_2SO_4$ treated GFOR apoenzyme did not reconstitute to fully functional holoenzyme when $NADP^+$ was added. The $NADP^+$ binding site on the apoenzyme may be buried and unavailable for the catalytic reaction.

CHAPTER 7
SUMMARY

7.1 Summary of Results

The properties of glucose-fructose oxidoreductase (GFOR) are mainly contributed by the cofactor, $NADP^+$. Not only was $NADP^+$ responsible for the oxidative and reductive function of GFOR, but also for the stability of GFOR. The cofactor was shown to be bound tightly to the enzyme even during the oxidation and reduction reactions. Also, preliminary experiments using an indicator (chlorophenol red), were conducted to detect any free proton released into the enzyme solution. The absorbance changes over a period of time were recorded on a HP8452A Diode Array Spectrophotometer. The results showed that the proton was not released during the redox reaction.

The reaction mechanism was studied by presteady state kinetics using the stopped-flow apparatus. Both nucleotide fluorescence and protein fluorescence results complemented the absorbance results. The nucleotide fluorescence experiments also revealed fluorescence quenching by gluconolactone when bound to E-NADPH. The oxidation of E-NADPH by gluconolactone was biphasic in nucleotide fluorescence and the slow phase in fluorescence corresponded to the only phase found in absorbance.

For the reduction of E-$NADP^+$ by glucose was also shown to involve two steps, the absorbance rate constants were much faster than the fluorescence rate constants, because they were measuring different steps. The absorbance step preceded the fluorescence step.

The fluorescence changes in both glucose and gluconolactone reactions occurred during the gluconolactone association/dissociation step. The differences between the absorbance and fluorescence results were believed to be due to the quenching of E-NADPH fluorescence

by gluconolactone when gluconolactone was bound to the enzyme. This is because gluconolactone has a strongly polar carbonyl group which withdraws electrons from the NADPH nicotinamide ring, resulting in the NADPH becoming more like $NADP^+$ (a low fluorescence species).

Glucose-fructose oxidoreductase exhibits unusual stability. When GFOR was purified, it was stable at room temperature for weeks, and could withstand freezing and thawing for months. Furthermore, the dye-enzyme binding studies showed that the binding of the dye to GFOR did not affect the enzyme activity. The types of dye-protein binding may involve many kinds of strong forces, for example, hydrophobic interactions, electrostatic interactions, hydrogen bonding and other forces which could cause disturbances to protein structure. But the scanning spectrum results showed no conformational change.

However, the stability of GFOR was dramatically affected when $NADP^+$ was removed from the enzyme by acidic ammonium sulphate precipitation. The removal of the nucleotide from glucose-fructose oxidoreductase denatured the protein and this denaturation was irreversible even upon the addition of $NADP^+$.

The unusual stability of GFOR may be an advantage for *Zymomonas mobilis*, since it grows in a "harsh" fermenting environment: pH as low as 3.7, 11.2 % ethanol (v/v) (Doelle and Doelle, 1989).

Structural studies have been carried out on GFOR. These studies include modification with diethyl pyrocarbonate and comparison of the GFOR subunit sequence with the conserved nucleotide binding site sequence from other dehydrogenases. Preliminary studies with diethyl pyrocarbonate suggested the involvement of histidine residues in the catalytic reaction, and/ or their importance in maintaining the correct enzyme conformation.

The GFOR sequence is known, but its tertiary structure has not yet been elucidated. Therefore, the $NADP^+$ binding domain of GFOR was located by comparing the sequence of GFOR with the most conserved pyrimidine nucleotide binding sequence from other dehydrogenases.

The conserved nucleotide binding domain included hexapeptide with the following sequence:

gly - x - gly - x x - gly

There is a basic residue (lysine) five or six residues before the hexapeptide and a hydrophobic residue (valine) two residues before the first glycine. Also an aspartate residue about twenty-two residues following the hexapeptide, which is believed to have an important functional role in the binding of coenzyme, is also commonly found in the nucleotide binding areas of other dehydrogenases (Nagata *et. al*, 1988; Hempel and Jornvall, 1989).

With the aid of a computer program (University of Wisconscin (GCG) sequence analysis software package on a Vax computer), the cofactor binding sequence was located near the sequence from residue 206 to residue 241 of GFOR.

*	206	VGSLMDIGIYGLNGTRYLLGEEPSEVRAYTYSDPNP	241
#	2	VVAVLGVGNVGMGVAMSLLMMGSPMAAKVILVDINE	37

* GFOR amino acid sequence

\# general conserved (NAD^+/$NADP^+$) binding sequence

The underlined letters a the conserved sequence mentioned in the text

The GFOR cofactor sequence showed homology to the conserved sequence of other dehydrogenases. But there are slight variations. For example, instead of having the first glycine as in other dehydrogenases, GFOR sequence has an aspartate (D) . Also, GFOR has a leucine residue instead of valine two residues before the first glycine of the hexapeptide. However, the structures of leucine and valine are very similar.

Now the tertiary structure of the $NADP^+$ binding domain can be studied by comparing with the known tertiary structures of other dehydrogenases. This will enable the importance of the residues in that domain to be understood and help to understand the reaction mechanism catalysed by GFOR and the nature of the binding between the $NADP^+$ and GFOR. It has been suggested in chapter 6 that $NADP^+$ is bound to the enzyme by noncovalent bonds.

7.2 Future Work

From the above summary, there remain many unanswered questions about this enzyme and the reaction it catalyses. Further work is required to better understand this enzyme, as described below:

(1) the use of gluconolactone analogues (with a carbonyl group), such as oxamate, to test whether they will quench the nucleotide fluorescence of bound NADPH.

(2) the confirmation of the involvement of histidine in GFOR by using hydroxylamine to carry out reverse carboxymethylation of histidine residue/residues. If this test is positive, then each of the eight histidines in the GFOR subunit can be tested by using a chemical group to block other histidine molecules.

(3) the reaction mechanism can be studied further by temperature jump experiments, because the reaction is reversible. Temperature jump experiments involve incubating an

equilibrated mixture in an absorbance or fluorescence cell at one temperature, then raising the temperature by 5 to 10°C in 0.1 to 1 μsec (Fersht, 1977). The rate of relaxation to a new equilibrium can be measured. The rate of reaction can be measured in such a fast time, because the mixing of the solution is cut out and therefore there is no dead time limit.

(4) compariing the tertiary structure of the nucleotide binding domain of GFOR with that of other dehydrogenases with known tertiary structure. This will enable site-directed mutagenesis to be carried out on the residues at nucleotide binding site or near this site, so that the importance and function of the residues can be assessed.

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