Kinetic Mechanism from Steady-State Kinetics of the Reaction Catalysed by Baker's-Yeast Glucose 6-Phosphate Dehydrogenase in Solution and Covalently Attached to Sepharose

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1. The reaction catalysed by glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) from baker's yeast was studied in 42 mM-glycylglycine buffer, pH 7.4 at 25°C, by initial-velocity studies and by the use of NADPH as a product inhibitor. 2. The reactions catalysed by both the soluble enzyme and a stable enzyme covalently attached to CNBr-activated Sepharose 4B probably follow an ordered reaction mechanism with NADP⁺ and NADPH as the leading reactants. 3. The kinetic constants obtained for the soluble enzyme were: $K_m^{NADP^+}$, 19 μ M; $K_m^{Glc-6-P}$, 51 μ M; $K_s^{NADP^+}$, 23 μ M; K_s^{NADPH} , 15 μ M. Similar values were obtained for the immobilized enzyme. 4. The assay of the immobilized enzyme was done by using a micro packed-bed recirculation reactor, and the advantages of this technique are discussed.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) was discovered by Warburg & Christian (1931). Since then the enzyme has been isolated from various sources (Bonsignore & De Flora, 1972). The steady-state kinetics of several NADP⁺-specific glucose 6-phosphate dehydrogenases isolated from micro-organisms have been investigated. Where the reaction mechanism has been determined by kinetic methods, it has usually been proposed that the reaction catalysed by the enzyme follows an ordered reaction pathway (Sanwal, 1970; Olive *et al.*, 1971; Afolayan, 1972) with NADP⁺ and NADPH as the leading reactants.

This proposed reaction mechanism is based on steady-state initial-velocity studies and product-inhibition studies with NADPH. For this reaction mechanism, NADPH should act as a competitive inhibitor with NADP⁺, and as a non-competitive inhibitor against glucose 6-phosphate (Cleland, 1970).

In Goheer *et al.* (1976), we reported the properties of baker's-yeast glucose 6-phosphate dehydrogenase covalently attached to CNBr-activated Sepharose 4B. The reaction mechanism of soluble enzyme from this source has not been reported. Also, before the completion of the work reported in the present paper, only Preuveneers *et al.* (1973) had compared the reaction mechanism of a soluble enzyme, D-3-

hydroxybutyrate dehydrogenase, with that for the same enzyme after covalent immobilization. Most studies on the kinetics of immobilized enzymes have been done by using a packed-bed reactor or continuous stirred-tank reactor systems (Gould, 1975), but with both of these reactors there are diffusion problems. These problems can be decreased, but not eliminated, by the use of an increased stirrer speed or flow rate, but because of this problem only apparent kinetic constants can be obtained. Ford *et al.* (1972) described a micro packed-bed recirculation reactor for kinetic studies on immobilized enzymes. They proved that this technique, under appropriate conditions, gave real kinetics for trypsin, which is a pseudo-one-substrate enzyme.

We have shown that a reactor similar to that used by Ford *et al.* (1972) can be used for more complex kinetic studies on a two-substrate enzyme (Gould & Goheer, 1973). In the present paper, we report further studies on the steady-state kinetics of immobilized baker's-yeast glucose 6-phosphate dehydrogenase. The kinetic mechanism of the soluble enzyme has also been investigated and compared with that of the immobilized enzyme.

Materials and Methods

Materials

Glucose 6-phosphate dehydrogenase (type XV) from baker's yeast and D-glucose 6-phosphate (disodium salt) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2

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7BH, U.K. NADP⁺ and NADPH were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. CNBr was bought from Ralph N. Emmanuel, Wembley, Middx. HA0 1PY, U.K. Sepharose 4B was obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. All other reagents were analytical grade and obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Methods

Preparation of immobilized glucose 6-phosphate dehydrogenase. CNBr-activated Sepharose 4B was prepared by the method of Axen & Ernback (1971) as described in Goheer *et al.* (1976), but the coupling of the enzyme to this carrier was modified to prepare a more active preparation. To do this, 100 units (where 1 unit is 1 μ mol of substrate transformed/min) of glucose 6-phosphate dehydrogenase was allowed to react with only 100mg of CNBr-activated Sepharose 4B. The reaction was done in the presence of glucose 6-phosphate and NADP⁺ in a reaction volume of 2ml. After 24h at 4°C the gel was washed with solutions of 0.1M-NaHCO₃ and 1M-NaCl.

Assay of immobilized enzyme. The kinetics of the immobilized glucose 6-phosphate dehydrogenase were investigated by using a recirculation reactor, which was modified from that of Ford *et al.* (1972). The immobilized enzyme (approx. 10mg) was retained between two nylon filters, with mean pore diameters of 14 μ m, supported on a stainless-steel screen in a 13mm ultrafiltration cell obtained from Millipore (U.K.) Ltd., London NW10 7SP, U.K. The assay solution was passed through this micro packed-bed reactor into an open reservoir, where it was continuously stirred. The solution was then recirculated by a DCL-micro-pump through a 1cm flow-through cuvette and the linear change in extinction was recorded at 340nm with a Cecil u.v. spectrophotometer. Upstream of the reactor, a piece of tubing about 15cm (6in) long passed through a constant-temperature water jacket maintained at 25°C. Before the reaction was started, the assay solution was recirculated through the system with a by-pass filter in the position of the reactor for a few minutes. During this time the solution reached 25°C and the filter removed any small particles that would otherwise have entered the pores of the filters in the reactor. The reaction was then started by switching the reactor into the recirculating stream.

The amount of immobilized enzyme in the recirculation reactor was selected to ensure that the fractional conversion per pass was less than 2% at the flow rates used. This condition gives an approximately gradientless reactor and makes it possible to obtain initial rate measurements with immobilized enzymes (Ford *et al.*, 1972). The frac-

tional conversion per pass, $\Delta S/S$, can be determined from eqn. (1),

$$\frac{\Delta S}{S} = \frac{V_r \left(-\frac{dS}{dt} \right)}{QS} \quad (1)$$

where S is the substrate concentration, ΔS is the substrate concentration change across the enzyme reactor, V_r is the fluid volume in the system, $-\frac{dS}{dt}$ is the decrease in substrate concentration in unit time and Q is the recirculation flow rate.

The low substrate concentration change per pass is amplified by using high recirculation flow rates. As the flow rate was increased from 1 to 5ml/min the reaction rate increased to a constant value. This value was maintained until 30ml/min, but then the reaction rate decreased when a flow rate of 40ml/min was used (Gould & Goheer, 1973). After these preliminary studies a flow rate of 10ml/min was used to study the kinetics of the immobilized enzyme.

After each kinetic run the volume of the assay solution was measured and then the whole recirculation reactor was washed out with water. The same sample of enzyme was used for all the kinetic runs required for each graphical analysis by the method of Lineweaver & Burk (1934). The immobilized enzyme was shown to be stable by frequent use of a standard assay.

Initial-velocity studies for soluble and immobilized enzyme. Similar reaction conditions were used for the soluble enzyme as those used for the immobilized enzyme, i.e. 42mm-glycylglycine/NaOH buffer at pH7.4, at 25°C and in the presence of 10mm-MgCl₂. The concentrations of the two substrates, NADP⁺ and glucose 6-phosphate, were varied over a 25-fold range. The initial velocity for each kinetic run was determined at 340nm and at known concentrations of both substrates. Further initial velocities were determined for several concentrations of glucose 6-phosphate at different fixed concentrations of NADP⁺.

Product-inhibition studies were done with NADPH. Initial velocities were determined as above, except that different fixed concentrations of inhibitor (including zero) were present in the reaction mixtures.

Results

Initial-velocity patterns for soluble and immobilized glucose 6-phosphate dehydrogenase

The initial-velocity pattern (double-reciprocal plot) for the reaction catalysed by glucose 6-phosphate dehydrogenase in the forward direction is shown in Fig. 1 for the soluble enzyme. The immobilized enzyme also gave an intersecting pattern (Gould & Goheer, 1973). The secondary plots of

Fig. 1. Initial-velocity pattern for soluble glucose 6-phosphate dehydrogenase

[NADP⁺] μM : Δ , 250; $\square$, 35; $\blacktriangle$, 19; $\blacksquare$, 13. [Glucose 6-phosphate] as indicated. Other conditions were as given in the Materials and Methods section.

the slopes and intercepts against the reciprocal of NADP⁺ concentration for both enzyme forms were straight lines. These were used to determine the kinetic constants of both enzyme forms and the values are given in Table 1.

Product-inhibition patterns for soluble and immobilized glucose 6-phosphate dehydrogenase

The competitive-inhibition pattern caused by NADPH, with NADP⁺ as the variable substrate, is shown in Fig. 2 for the immobilized enzyme. The soluble enzyme was also inhibited competitively. The plots of slopes against the concentration of inhibitor were linear for both enzyme forms and the inhibitor constants, K_{is} , were determined from the horizontal intercepts of the replots and were used to calculate the value of K_s^{NADPH} (see Table 1) by the relevant equation given by Cleland (1963).

Non-competitive-inhibition patterns were obtained with NADPH when glucose 6-phosphate was the variable substrate for the soluble enzyme and the immobilized enzyme (Fig. 3). The linear secondary plots of the slopes and intercepts against the concentration of NADPH were used to determine the inhibitor constants from the horizontal intercepts of the slopes (K_{is}) and intercepts (K_{ii}) replots. Values of K_s^{NADPH} were then calculated (see Table 1) by the relevant equation as given by Cleland (1963).

Discussion

Probable kinetic mechanism of glucose 6-phosphate dehydrogenase from baker's yeast

The initial-velocity patterns (double-reciprocal plots) for the soluble and immobilized enzyme were

Fig. 2. Inhibition of the immobilized enzyme by NADPH with NADP⁺ as the variable substrate

Fixed [glucose 6-phosphate], 2 mM, and [NADP⁺] as indicated. [NADPH] μM : Δ , 0.0; $\square$, 10; $\blacktriangle$, 20; $\blacksquare$, 40. Other conditions were as given in the Materials and Methods section.

Fig. 3. Inhibition of immobilized enzyme by NADPH with glucose 6-phosphate as the variable substrate

Fixed [NADP⁺], 0.08 mM, and [glucose 6-phosphate] as indicated. [NADPH] μM : Δ , 0.0; $\square$, 10; $\blacktriangle$, 20; $\blacksquare$, 40. Other conditions were as given in the Materials and Methods section.

of the intersecting type with linear slope and intercept replots. These intersecting patterns are consistent with an ordered, random or Theorell-Chance reaction mechanism, but not with a Ping Pong

Table 1. Kinetic parameters for baker's-yeast glucose 6-phosphate dehydrogenase obtained from the initial-velocity and product-inhibition analysis

Analysis	Kinetic constant	Enzyme concentration (μM)	
		Soluble	Immobilized
Initial velocity	$K_m^{\text{NADP}^+}$	19	56
	$K_m^{\text{Glc-6-P}}$	51	140
	$K_s^{\text{NADP}^+}$	23	10
Product inhibition (NADP ⁺ varied)	K_s^{NADPH} (from K_{1s})	12	20
	K_s^{NADPH} (from K_{1s})	15	3
Product inhibition (glucose 6-phosphate varied)	K_s^{NADPH} (from K_{1s})	17	13
	K_s^{NADPH} (from K_{11})		

mechanism, which gives parallel lines (Cleland, 1970).

Product-inhibition studies with NADPH against NADP⁺ gave a linear competitive pattern. A linear non-competitive pattern was obtained with NADPH against glucose 6-phosphate. These results are consistent with an ordered reaction mechanism, with the coenzymes NADP⁺ and NADPH being the leading reactants. From similar initial-velocity patterns and NADPH-inhibition patterns, Soldin & Balinsky (1968) in their studies on the erythrocyte enzyme, Sanwal (1970), on the *Escherichia coli* enzyme, and Afolayan (1972), on the enzyme from *Candida utilis*, all proposed that glucose 6-phosphate dehydrogenase followed an ordered reaction mechanism.

However, for brewer's-yeast glucose 6-phosphate dehydrogenase, Kuby *et al.* (1974) obtained competitive inhibition by NADPH against glucose 6-phosphate. From their kinetic studies and the results of ligand-binding studies, Kuby *et al.* (1974) concluded that the enzyme followed a rapid-equilibrium reaction mechanism. Malcolm & Shepherd (1972) studied the steady-state kinetics of NADP⁺-specific glucose 6-phosphate dehydrogenase from *Penicillium duponti* and *Penicillium notatum*. The enzyme from both sources gave product-inhibition data that were consistent with those of Kuby *et al.* (1974).

In all the studies on the kinetic mechanism of glucose 6-phosphate dehydrogenases it would have been useful to study the inhibitory effect of the other reactant, D-glucono- δ -lactone 6-phosphate. However, this lactone reacts with water with a half-life of 1.5 min at pH 7.4 (Glaser & Brown, 1955) and is therefore unsuitable for steady-state kinetic studies. The lactone forms 6-phospho-D-gluconate, which did not have any inhibitory effect on glucose 6-phosphate dehydrogenase from *C. utilis* (Afolayan, 1972).

From our kinetic studies there is no evidence that the reaction mechanism of immobilized glucose 6-phosphate dehydrogenase has been altered by covalent attachment to CNBr-activated Sepharose 4B. To our knowledge the reaction mechanism of only one other two-substrate immobilized enzyme has been published (Preuveneers *et al.*, 1973). They found that the reaction pathway for D-3-hydroxybutyrate dehydrogenase from *Rhodospseudomonas spheroides* was not altered by covalent attachment to DEAE-cellulose.

Kinetic constants of soluble and immobilized glucose 6-phosphate dehydrogenase from baker's yeast

The kinetic constants of the soluble and immobilized baker's-yeast glucose 6-phosphate dehydrogenase are given in Table 1. The values obtained for the soluble and immobilized enzyme are similar. The apparent Michaelis constants for the immobilized enzyme are about three times greater than those for the soluble enzyme, whereas the dissociation constant $K_s^{\text{NADP}^+}$ for the immobilized enzyme is about a half of that for the soluble enzyme. The three independent values for the other dissociation constant K_s^{NADPH} are in good agreement for the soluble enzyme. Two of the values for K_s^{NADPH} for the immobilized enzyme are very similar to those obtained from the soluble enzyme.

The Michaelis constants obtained for the soluble baker's-yeast glucose 6-phosphate dehydrogenase were $K_m^{\text{NADP}^+} = 19 \mu\text{M}$ and $K_m^{\text{Glc-6-P}} = 51 \mu\text{M}$. Several workers have given values for these constants for the enzyme from brewer's yeast, including Glaser & Brown (1955), Lowry *et al.* (1961), Rose (1961) and Horne *et al.* (1970). Their values range from 2 to $32 \mu\text{M}$ for $K_m^{\text{NADP}^+}$ and from 20 to $58 \mu\text{M}$ for $K_m^{\text{Glc-6-P}}$. Other micro-organisms with NADP⁺-specific glucose 6-phosphate dehydrogenases that have similar Michaelis constants are *E. coli* (Sanwal,

1970) and *Neurospora crassa* (Scott & Tatum, 1971).

The recirculation reactor

The recirculation reactor was invaluable for our studies on immobilized glucose 6-phosphate dehydrogenase reported here. However, care must be taken to use the correct operating conditions. Preliminary experiments showed that as the flow rate was increased from 30 to 40 ml/min the measured initial rate decreased. This was probably due to the Sepharose-gel particles being compressed at the high flow rates, which might decrease the accessibility of enzyme in some of the pores. An increased pressure within the reactor was observed at the higher flow rate (40 ml/min). The use of non-compressible carriers should overcome this problem. However, the recirculation reactor can eliminate many of the difficulties associated with other reactors used to study the kinetics of immobilized enzyme. Unlike a stirred reactor, no fines are produced in the recirculation reactor used in our experiments. Also the same immobilized enzyme sample could be used for many runs, so long as the enzyme was stable. As the enzyme activity is rate-limiting, this is an important advantage over stirred tank reactors as used by Preuveneers *et al.* (1973).

The use of small quantities of immobilized enzyme decreases the effects of temperature and axial gradients in the reactor. Substrate and product concentration gradients external to the immobilized enzyme matrix can be minimized by the low (<2%) pass conversion rate and eliminated by the use of sufficiently high flow rates (Ford *et al.*, 1972). However, the elimination of the effects of internal diffusion within the Sepharose 4B gel particles on the kinetics of immobilized enzymes is more difficult. These effects need not be apparent except at high substrate concentrations when curved double-reciprocal plots are obtained (Lee & Tsao, 1974). One way of testing for internal diffusion effects is to use immobilized enzyme particles of several different sizes. This was not done with the immobilized enzyme used in this study. It is possible that the small differences observed in the kinetic

constants of the soluble and immobilized enzyme (Table 1) could be due to the effects of internal diffusion.

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References

- Afolayan, A. (1972) *Biochemistry* **11**, 4172–4178
Axen, R. & Ernback, S. (1971) *Eur. J. Biochem.* **18**, 351–360
Bonsignore, A. & De Flora, A. (1972) *Curr. Top. Cell. Regul.* **4**, 21–62
Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 173–187
Cleland, W. W. (1970) *Enzymes*, 3rd edn., **2**, 1–65
Ford, J. R., Lambert, A. H., Cohen, W. & Chambers, R. P. (1972) *Biotechnol. Bioeng. Symp.* **3**, 267–284
Glaser, L. & Brown, D. H. (1955) *J. Biol. Chem.* **216**, 67–79
Goheer, M. A., Gould, B. J. & Parke, D. V. (1976) *Biochem. J.* **157**, 289–294.
Gould, B. J. (1975) in *Handbook of Enzyme Biotechnology* (Wiseman, A., ed.), pp. 5–26, Ellis Horwood, Chichester
Gould, B. J. & Goheer, M. A. (1973) *Biochem. Soc. Trans.* **1**, 1284–1286
Horne, R. N., Anderson, W. B. & Nordlie, R. C. (1970) *Biochemistry* **9**, 610–616
Kuby, S. A., Wu, J. T. & Roy, R. N. (1974) *Arch. Biochem. Biophys.* **165**, 153–178
Lee, Y. Y. & Tsao, G. T. (1974) *J. Food Sci.* **39**, 667–672
Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666
Lowry, O. H., Passonneau, J. V., Schulz, D. W. & Rock, M. K. (1961) *J. Biol. Chem.* **236**, 2746–2755
Malcolm, A. A. & Shepherd, M. G. (1972) *Biochem. J.* **128**, 817–831
Olive, C., Geroch, M. E. & Levy, H. R. (1971) *J. Biol. Chem.* **246**, 2047–2057
Preuveneers, M. J., Peacock, D., Crook, E. M., Clark, J. B. & Brocklehurst, K. (1973) *Biochem. J.* **133**, 133–157
Rose, I. A. (1961) *J. Biol. Chem.* **236**, 603–609
Sanwal, B. D. (1970) *J. Biol. Chem.* **245**, 1626–1631
Scott, W. A. & Tatum, E. L. (1971) *J. Biol. Chem.* **246**, 6347–6352
Soldin, S. J. & Balinsky, D. (1968) *Biochemistry* **7**, 1077–1082
Warburg, O. & Christian, W. (1931) *Biochem. Z.* **242**, 206–227