Kinetic Studies on Dogfish Liver Glutamate Dehydrogenase

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(Received 3 July 1978)

Initial-rate studies were made of the oxidation of L-glutamate by NAD^+ and NADP^+ catalysed by highly purified preparations of dogfish liver glutamate dehydrogenase. With NAD^+ as coenzyme the kinetics show the same features of coenzyme activation as seen with the bovine liver enzyme [Engel & Dalziel (1969) *Biochem. J.* **115**, 621–631]. With NADP^+ as coenzyme, initial rates are much slower than with NAD^+ , and Lineweaver–Burk plots are linear over extended ranges of substrate and coenzyme concentration. Stopped-flow studies with NADP^+ as coenzyme give no evidence for the accumulation of significant concentrations of NADPH-containing complexes with the enzyme in the steady state. Protection studies against inactivation by pyridoxal 5'-phosphate indicate that NAD^+ and NADP^+ give the same degree of protection in the presence of sodium glutarate. The results are used to deduce information about the mechanism of glutamate oxidation by the enzyme. Initial-rate studies of the reductive amination of 2-oxoglutarate by NADH and NADPH catalysed by dogfish liver glutamate dehydrogenase showed that the kinetic features of the reaction are very similar with both coenzymes, but reactions with NADH are much faster. The data show that a number of possible mechanisms for the reaction may be discarded, including the compulsory mechanism (previously proposed for the enzyme) in which the sequence of binding is NAD(P)H , NH_4^+ and 2-oxoglutarate. The kinetic data suggest either a rapid-equilibrium random mechanism or the compulsory mechanism with the binding sequence NH_4^+ , NAD(P)H , 2-oxoglutarate. However, binding studies and protection studies indicate that coenzyme and 2-oxoglutarate do bind to the free enzyme.

Previous limited kinetic studies of the reductive amination of 2-oxoglutarate by NADPH catalysed by dogfish liver glutamate dehydrogenase (EC 1.4.1.3) were interpreted on the basis of a compulsory mechanism with NADPH binding first, followed by NH_4^+ and then 2-oxoglutarate (Corman *et al.*, 1967). For bovine glutamate dehydrogenase the choice of a compulsory mechanism on the basis of limited studies (Frieden, 1959) has been shown to be incorrect (Engel & Dalziel, 1970). It seemed worthwhile to re-investigate this problem with a systematic study. A further point of interest concerned the use of NADH as an alternative coenzyme. Previous work indicated that NADH was a much better coenzyme than NADPH, but kinetic studies with NADH were not undertaken. For the bovine enzyme NADH and NADPH are about equally effective (Engel & Dalziel, 1970).

The allosteric properties of glutamate dehydrogenase have been much investigated in recent years. Most studies have been made with the bovine liver

enzyme and a striking manifestation of the subunit interactions is seen in detailed kinetic studies of the oxidation of L-glutamate by NAD^+ and NADP^+ (Engel & Dalziel, 1969), where Lineweaver–Burk plots with the coenzyme concentration as variable show linear sections with sharp changes of slope. Overall the plots are concave downwards. The basis for the explanation of these results has been laid by coenzyme-binding studies (Dalziel & Egan, 1972; Melzi-D'Eril & Dalziel, 1972), but the details are still rather obscure. Early work on dogfish liver glutamate dehydrogenase (Corman *et al.*, 1967) showed that this enzyme, unlike the bovine enzyme, utilizes NADP^+ much less efficiently than NAD^+ , but there was no evidence of coenzyme activation. Further, although the enzyme showed coenzyme activation with NAD^+ (Corman & Kaplan, 1967) it appeared less dramatic than with the bovine enzyme. The tuna liver enzyme showed the same disparity of activity with NAD^+ and NADP^+ with evidence of a limited degree of coenzyme activation with NAD^+ (Veronese *et al.*, 1976). It seemed to us that the dogfish enzyme might represent a more primitive form of glutamate dehydrogenase, exhibiting less well-developed allosteric properties than the mammalian enzyme.

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Materials and Methods

All solutions were prepared in glass-distilled water.

Coenzymes and substrates

NAD⁺ (grades I and II) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Initial rates with grade-I NAD⁺ were identical with those obtained by using highly purified NAD⁺ made by the method of Dalziel & Dickinson (1966), and so grade-I NAD⁺ was used in kinetic work. The concentration of NAD⁺ in solution was determined enzymically (Dalziel & Dickinson, 1966). NADP⁺ was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A., and from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K. The material was further purified for kinetic work by chromatography on DEAE-cellulose by using the method of Dalziel & Dickinson (1966). The concentration of NADP⁺ in solution was determined by the method of Ciotti & Kaplan (1957).

NADH (sodium salt) was prepared from NAD⁺ (grade I; Boehringer) and assayed enzymically. The residual A_{340} of this material after complete oxidation was 2%. NADPH (grade I) was purchased from Boehringer, and was used without further purification. This material was assayed by the method of Ciotti & Kaplan (1957) and found to contain 6% impurities as measured by the residual A_{340} .

L-Glutamic acid and L-glutaric acid were purchased from BDH Chemicals, Poole, Dorset, U.K. The glutamic acid was used without further purification, but the glutaric acid was recrystallized twice from benzene/ether without any change in the melting point (97°C, uncorrected). 2-Oxoglutarate was obtained from BDH and was recrystallized once from acetone/benzene (1:9, v/v). The melting point of the recrystallized material was 114°C (uncorrected). Pyridoxal 5'-phosphate was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Enzyme

Bovine glutamate dehydrogenase was obtained from Boehringer. The dogfish enzyme was prepared from acetone-dried powders of dogfish liver by a modification of the method of Corman *et al.* (1967). The enzyme was extracted from the acetone-dried powder in 50mM-phosphate buffer, pH 7.4, and was then precipitated by 20% (v/v) ethanol after adjustment to pH 5.8 by addition of 1M-sodium acetate buffer, pH 4.6. The material was then dissolved in 50mM-phosphate buffer, pH 7.4, and fractionated with (NH₄)₂SO₄, the fraction that precipitated between 34 and 44% saturation being collected and redissolved in phosphate buffer. After dialysis the preparation was chromatographed on DEAE-cellulose at pH 6.5 as described by Corman *et al.* (1967). The enzyme-containing fractions were com-

bined and the enzyme was concentrated by precipitation with (NH₄)₂SO₄. After dialysis the preparation was treated with calcium phosphate gel, which adsorbed some coloured impurities, and was then passed down a column of Sephadex G-200 as a final step. The highly purified preparation was stable for several months at -15°C. Occasionally the enzyme was crystallized, after concentration of the Sephadex G-200 column eluates, by slow addition of saturated (NH₄)₂SO₄. However, this did not increase the purity of the preparation as judged by specific-activity measurements, and so the crystallization was not done on a routine basis.

The purification procedure yielded 10–15mg of enzyme from 100g of acetone-dried powder, with a yield of about 10%. The specific activity of the preparation was 3.8 units/mg in the assay of Corman *et al.* (1967), which compares favourably with the value of 3.0 units/mg given by these authors. Protein concentrations were determined by A_{280} measurements by taking $A_{1\text{cm}}^{1\%} = 11.0$ for the dogfish enzyme (Malencik & Anderson, 1972) and $A_{1\text{cm}}^{1\%} = 9.7$ for the bovine enzyme (Olson & Anfinsen, 1952). For routine assays of the dogfish enzyme the assay of Corman *et al.* (1967) was modified to give much greater sensitivity by using an increased NAD⁺ concentration of 4mM. In this assay the purified preparation had a specific activity of 17.6 units/mg.

Initial-rate measurements

(a) *Glutamate-NAD(P)⁺ reactions.* These were performed fluorimetrically in a recording instrument based on the design of Dalziel (1962). Assays were performed in duplicate at 25°C in 0.11M-sodium phosphate buffer, pH 7.0. Progress curves were linear for at least 1min and initial rates were generally reproducible to within 5%, and to within 15% at the lowest substrate and coenzyme concentrations. The kinetic coefficients, ϕ'_0 etc., in the initial-rate eqn. (1)

$$\frac{e}{v_0} = \phi'_0 + \frac{\phi'_1}{[S'_1]} + \frac{\phi'_2}{[S'_2]} + \frac{\phi'_{12}}{[S'_1][S'_2]} \quad (1)$$

were obtained from the slopes and intercepts of Lineweaver-Burk plots as described by Dalziel (1957). In eqn. (1) e is the concentration of enzyme protomers (protomer mol.wt. = 55000), v_0 is the measured initial velocity and S'_1 and S'_2 are coenzyme and glutamate respectively.

(b) *2-Oxoglutarate-NAD(P)H-NH₄⁺ reactions.* These were performed fluorimetrically ([NAD(P)H] < 15 μ M) or spectrophotometrically ([NAD(P)H] > 15 μ M) in 0.11M-phosphate buffer, pH 7.0, at 25°C. Assays were performed in duplicate and were reproducible to within 5%, in general, and to within 15% at the lowest substrate and coenzyme concentrations. Progress curves were linear for at least 30s.

The kinetic coefficients, ϕ_0 etc., in the initial-rate eqn. (2)

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_3}{[S_3]} + \frac{\phi_{12}}{[S_1][S_2]} + \frac{\phi_{23}}{[S_2][S_3]} + \frac{\phi_{13}}{[S_1][S_3]} + \frac{\phi_{123}}{[S_1][S_2][S_3]} \quad (2)$$

were obtained from the slopes and intercepts of tertiary plots (Dalziel, 1969). In eqn. (2) e denotes the concentration of enzyme protomers, v_0 is the initial velocity and S_1 , S_2 and S_3 are 2-oxoglutarate, NH_4^+ and coenzyme respectively.

Transient studies

These were performed in a stopped-flow spectrophotometer based on the design of Gibson & Milnes (1964). The reactions were followed at 340nm with a 2.2cm path-length observation tube. Reactions took place at 25°C in 0.11 M-phosphate buffer, pH 7.0, and were initiated by mixing solutions of enzyme pre-mixed with NADP^+ with an equal volume of solutions of glutamate. The dead-time of the apparatus was approx. 5ms as measured by following the decolorization of 2,6-dichlorophenol-indophenol by ascorbate.

Inactivation reactions with pyridoxal 5'-phosphate

These were carried out essentially as described by Chen & Engel (1974). Enzyme (0.2mg/ml) was incubated in 0.1 M-phosphate buffer, pH 7.0, containing

0.5mM-pyridoxal 5'-phosphate and 50mM-sodium glutarate at 25°C in tubes protected from the light by aluminium foil. Reactions were complete within 60min.

Results

Glutamate- NAD(P)^+ reactions

Initial-rate studies of the oxidation of glutamate by NAD^+ catalysed by dogfish liver glutamate dehydrogenase were performed at 25°C and pH 7.0. The results are shown in Figs. 1–3. The concentrations of glutamate and NAD^+ used in the experiments were in the ranges 3–50mM and 2.5–1000 μM respectively. At 50mM-glutamate a plot of e/v_0 versus the reciprocal of the NAD^+ concentration (Fig. 1) shows sharp changes in slope at about 10, 60 and 300 μM - NAD^+ . The changes in slope of the plots and the NAD^+ concentrations at which they occur are similar to those reported for the bovine liver enzyme (Engel & Dalziel, 1969). Fig. 2 shows how the rates of glutamate oxidation varied in the NAD^+ concentration range 2.5–10 μM at several fixed glutamate concentrations. The plots are linear within experimental error. Entirely similar plots were obtained for the NAD^+ ranges 10–60 μM , 60–300 μM and 300–1000 μM . The secondary plots of the intercepts and slopes of the primary plots are shown in Figs. 3(a) and 3(b). Substrate inhibition occurs at concentrations of glutamate greater than 15mM, and the lines in Fig. 3 have been drawn to try

Fig. 1. Lineweaver-Burk plot showing the variation of the initial rate of glutamate oxidation with the NAD^+ concentration in the presence of 50mM-glutamate at pH 7.0 and 25°C

to ignore this effect. Figs. 3(a) and 3(b) show that, at the lower glutamate concentrations, the data conform to eqn. (1) and that the same values of ϕ'_2 and ϕ'_{12} apply throughout the NAD^+ concentration range (slopes independent of the NAD^+ concentration). On the other hand, four values of ϕ'_0 and ϕ'_1 are required. The values determined for the initial-rate parameters

Fig. 2. Primary plots showing the variation of the initial rate of glutamate oxidation with the NAD^+ concentration in the range 2.5–10 μM at pH 7.0 and 25°C

Glutamate concentrations: Δ , 3 mM; $\blacksquare$, 5 mM; $\square$, 8 mM; $\bullet$, 12 mM. For clarity results with 16 mM-glutamate have been omitted.

are found in Table 1. The precision of estimates for ϕ'_0 and ϕ'_1 was impaired by the lengthy extrapolations in the primary plots and by the substrate inhibition in the secondary plots. Nevertheless, complete duplicate experiments gave values for these parameters agreeing to within 15%. The substrate inhibition has not been studied in any detail, but it is probably due to the formation of an abortive enzyme– NADH –glutamate complex, as for the bovine enzyme.

Studies of the oxidation of glutamate by NADP^+ catalysed by dogfish liver glutamate dehydrogenase at pH 7.0 and 25°C led to the results shown in Fig. 4. The concentration ranges used here were glutamate, 3–200 mM, and NADP^+ , 5–2500 μM . At 50 mM-glutamate reciprocal plots were strictly linear over the entire range of NADP^+ concentrations, in contrast with the situation with NAD^+ . It was also apparent that the rates of glutamate oxidation were markedly slower when chromatographically purified NADP^+ was used instead of a commercial preparation. With 50 mM-glutamate and 100 μM - NADP^+ the rate was 30% slower with purified NADP^+ . The reason for this is probably that the latter was contaminated with a small amount of NAD^+ (Dalziel & Dickinson, 1965) and for this enzyme NAD^+ is a much better coenzyme than NADP^+ (Corman *et al.*, 1967) (see also Table 1). Reliable kinetic information can only be obtained by using purified NADP^+ , and this condition has been met in our experiments. It was not met in the earlier studies of the dogfish enzyme. Our data with NADP^+ in the range 25–2500 μM conform to eqn. (1) and the initial-rate parameters derived from the secondary plots of Fig. 4 are included in Table 1. All the initial-rate parameters are enormously increased on chang-

Fig. 3. Secondary plots showing the variation of the intercepts (a) and slopes (b) of primary plots (e.g. Fig. 2) with the glutamate concentration

The different symbols correspond to the different ranges of NAD^+ concentration used in the primary plots: (a): $\blacktriangle$, 360–1000 μM ; $\triangle$, 60–360 μM ; $\bullet$, 10–60 μM ; $\circ$, 2.5–10 μM ; (b): $\circ$, 360–1000 μM ; $\bullet$, 60–260 μM ; $\triangle$, 10–60 μM ; $\blacktriangle$, 2.5–10 μM .

Table 1. Kinetic coefficients for the oxidation of glutamate by NAD^+ and NADP^+ in 0.11 M-phosphate buffer, pH 7.0 at 25°C. The kinetic coefficients are those in eqn. (1) and were calculated from initial-rate data, as described in the text. Complete duplicate experiments indicated that parameters estimates were generally reproducible to within 15%.

Coenzyme	Concn. (μM)	ϕ'_0 (s)	ϕ'_1 ($\mu\text{M}\cdot\text{s}$)	ϕ'_2 ($\mu\text{M}\cdot\text{s}$)	ϕ'_{12} ($\mu\text{M}^2\cdot\text{s}$)	ϕ'_2/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (mm)	ϕ'_{12}/ϕ'_2 (μM)
NAD^+	2.5–10	0.85	1.1	1100	291 000	1.3	1.3	270
	10–60	0.42	3.3			8.6	2.9	
	60–300	0.25	6.9			27	4.3	
	300–1000	0.145	51			350	7.5	
NADP^+	1–2500	1.62	550	13 450	69 100 000	340	8.3	5140
NADP^{++}	28–500	2.4	200	210 000	28 000 000	83	8.0	142

* Data derived from results of Corman *et al.* (1967) obtained in 0.01 M-Tris/acetate buffer, pH 8.0. The calculations are on the basis of a subunit mol.wt. of 55000. For direct comparison with the data for the bovine enzyme (Engel & Dalziel, 1969) the parameters shown should be divided by 18.2.

Fig. 4. Secondary plots showing the variation of the intercepts (○) and slopes (●) of primary reciprocal plots of the initial rate of glutamate oxidation by NADP^+ at pH 7.0 and 25°C.

ing from NAD^+ to NADP^+ . There was no evidence of substrate inhibition by glutamate with NADP^+ as coenzyme. This suggests that the abortive complex E-NADPH-glutamate does not form readily. Also included in Table 1 are data derived from the results of Corman *et al.* (1967) with the dogfish enzyme in 0.07 M-Tris/acetate, pH 8.0; considering the changes in pH and the possible effects of contaminating NAD^+ , their values are not very different from ours.

It is possible that the change in kinetic characteristics of glutamate oxidation on changing from NAD^+ to NADP^+ could be associated with a change in rate-limiting step. Evidence on this point has been sought by using stopped-flow methods to study the oxidation of glutamate by NADP^+ catalysed by bovine and dogfish enzymes. For this 20 μM -protomer was mixed with 500–1800 μM - NADP^+ and 150 mM-

Fig. 5. Protection of dogfish liver glutamate dehydrogenase from inactivation by pyridoxal 5'-phosphate by incubation with NAD^+ or NADP^+ in the presence of 50 mM-sodium glutarate.

The incubation mixture contained: enzyme, 0.2 mg/ml; pyridoxal 5'-phosphate, 0.5 mM; sodium glutarate; indicated concentrations of NAD^+ (○) or NADP^+ (●). Each point represents the activity at equilibrium as measured in the standard assay.

glutamate at pH 7.0 and 25°C and the reaction was followed spectrophotometrically at 340 nm. With the bovine liver enzyme a transient increase in A_{340} was observed, which was completed within 150 ms, followed by a constant steady increase. The transient corresponded to 0.6 mol of NADPH/mol of enzyme protomer, by using $\epsilon_{340} = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ as for free NADPH. These results with the bovine enzyme are very similar to those described by di Franco (1974) and Iwatsubo & Pantaloni (1967). With the dogfish enzyme no detectable transient occurred over

Fig. 6. Primary plots showing the variation of the initial rate of reduction of 2-oxoglutarate by NADH and NH_4^+ at pH 7.0 and 25°C

NH_4^+ concentrations: $\blacktriangle$, 3 mM; $\triangle$, 10 mM; $\bullet$, 40 mM; $\circ$, 120 mM. For the primary plots the NADH concentrations were: (a) $5.7\ \mu\text{M}$; (b) $0.55\ \mu\text{M}$; (c) $0.3\ \mu\text{M}$.

the time period of measurement (2 s), thus indicating a clear difference between the two enzymes. The observed steady-state velocity (maintained over the 2 s period) agreed reasonably well (within 20%) with that calculated for these conditions from the initial-rate parameters shown in Table 1.

In an attempt to gain some information about the binding of NAD^+ and NADP^+ to dogfish liver glutamate dehydrogenase we have studied the protective effects of coenzyme against the inhibitory effects of pyridoxal 5'-phosphate. The method was as described by Chen & Engel (1974) in their studies with the bovine liver enzyme. It requires only modest amounts of enzymes, and our limited supplies of enzyme precluded the use of more direct methods for studying coenzyme binding. Fig. 5 shows how the equilibrium activity of the enzyme preparation varied with the NAD(P)^+ concentration after preincubation with 0.5 mM-pyridoxal 5'-phosphate for 60 min in the presence of 50 mM-glutarate. There was no protection afforded by coenzyme in the absence of glutarate, but glutarate alone afforded some protection (residual equilibrium activity = 20%); 20 mM-2-oxoglutarate alone provided the same modest degree of protection as 50 mM-glutarate. The results of Fig. 5 indicate that the binding of NADP^+ to the enzyme is much weaker than that of NAD^+ .

2-Oxoglutarate-NAD(P)H- NH_4^+ reactions

Some results of initial-rate measurements of the reductive amination of 2-oxoglutarate by NADH and NH_4^+ catalysed by dogfish liver glutamate dehydrogenase are shown in Figs. 6–8. The experiments were conducted at pH 7.0 and 25°C with independent variation of all three substrate concentrations. Figs. 6(a), 6(b) and 6(c) are representative primary double-reciprocal plots, each plot pertaining to a different NADH concentration and within which the concentration of 2-oxoglutarate is varied at a series of constant NH_4^+ concentrations. Secondary plots

Fig. 7. Secondary plots showing variation of the slopes and intercepts of primary plots (e.g. Figs. 6a, 6b and 6c) with the NADH concentration

(a) Variation of intercepts; (b) variation of slopes. NH_4^+ concentrations: $\blacktriangle$, 3 mM; $\triangle$, 10 mM; $\bullet$, 40 mM; $\circ$, 120 mM.

(Figs. 7a and 7b) of the slopes and intercepts of the primary plots were linear and Figs. 8a and 8b show the tertiary plots of the slopes and intercepts of the secondary plots. The ranges of substrate concentrations used in these experiments were: 2-oxoglutarate,

Fig. 8. Tertiary plots showing the variation of the slopes and intercepts of the secondary plots with the NH_4^+ concentration (a) Variation of the intercepts of Fig. 7(a) (O) and slopes of Fig. 7(b) (Δ); (b) variation of the slopes of Fig. 7(a) (▲) and intercepts of Fig. 7(b) (●).

Table 2. Kinetic coefficients for the reductive amination of 2-oxoglutarate by NADH and NADPH in 0.11 M-phosphate buffer, pH 7.0 at 25°C

The kinetic coefficients are those in eqn. (1) and were calculated from initial-rate data as described in the text. Complete duplicate experiments indicated that parameters were generally reproducible to within 15%, but as indicated in the text for ϕ_0 , and particularly ϕ_1 and ϕ_{12} , the precision of the estimates is probably a good deal less than this.

	ϕ_0 (s)	ϕ_1 (μm·s)	ϕ_2 (μm·s)	ϕ_3 (μm·s)	ϕ_{12} (μm ² ·s)	ϕ_{13} (μm ² ·s)	ϕ_{23} (μm ² ·s)	ϕ_{123} (μm ³ ·s)	ϕ_1/ϕ_0 (μm)	ϕ_2/ϕ_0 (mm)	ϕ_3/ϕ_0 (μm)
NADH kinetics	0.013	1.56	870	0.19	51000	94.5	1100	520000	120	66	14.6
NADPH kinetics	0.071 0.034*	31	650	10.4	145000	15800	65500	58200000	440 4500*	9.2 80*	146 400*

* Data of Corman *et al.* (1967) for 0.01 M-Tris/acetate, pH 8.0, with ϕ_0 calculated on the basis of a subunit mol.wt. of 55000. For these data the values are only apparent values, since systematic variation of the concentrations of all three substrates was not done in this work. To compare the present results with those for the bovine enzyme (Engel & Dalziel, 1970) the parameters shown should be divided by 18.2.

125–1000 μm; NH_4^+ , 3–120 mm; NADH, 0.3–6.0 μm. Substrate inhibition was observed with 2-oxoglutarate concentrations greater than 1 mm, and with NADH concentrations greater than 6.0 μm.

Inspection of Figs. 6–8 suggests that the data conform to eqn. (2). Evaluation of the initial-rate parameters from the tertiary plots yields the values given in Table 2. There are, however, aspects of the data that require comment. The substrate inhibition by 2-oxoglutarate at concentrations greater than 1 mm means that the extrapolation in estimating the intercepts of the primary plots results in lower precision of estimates for ϕ_0 and ϕ_2 . A much more serious problem is encountered in estimating the intercepts of Fig. 7(b). The substrate inhibition occurring with NADH concentrations greater than 6.0 μm means that there is a substantial extrapolation in obtaining these intercepts and there is uncertainty in the values of ϕ_1 and ϕ_{12} obtained from the tertiary plots. The uncertainties are, in fact, so large that the data do not prove that these parameters have finite positive values.

An entirely similar set of data have been obtained from studies of the reductive amination of 2-oxoglutarate by NADPH and NH_4^+ catalysed by the

dogfish enzyme. The primary, secondary and tertiary plots were very similar to those shown in Figs. 6, 7 and 8 and the parameters determined from the data are included in Table 2. The ranges of substrate concentrations used in the experiments were: 2-oxoglutarate, 100–500 μm; NH_4^+ , 3–120 mm; NADPH, 4–400 μm. In contrast with the situation with NADH as coenzyme, there was no inhibition by high (>6 μm) NADPH concentrations. On the other hand, 2-oxoglutarate showed significant substrate inhibition at concentrations greater than 500 μm. It may be noted that Corman *et al.* (1967) found no inhibition with this substrate at concentrations up to 15 mm in Tris/acetate buffer, pH 8.0. Unfortunately, the same uncertainties affected the NADPH and NADH data. The extrapolations in the primary reciprocal plots were substantial, owing to inhibition by 2-oxoglutarate, and the precision of ϕ_0 and ϕ_2 is low. The extrapolations in the plot corresponding to Fig. 7(b) were again considerable and the recorded positive values of ϕ_1 and ϕ_{12} may not be significantly different from zero.

Binding studies with NADH (Fig. 9) show that this coenzyme binds to the free enzyme (K_D approx. 50 μm).

Fig. 9. Demonstration of the binding of NADH to dogfish liver glutamate dehydrogenase at pH 7.0, 25°C

Enzyme (15mg) in 1ml of 0.11M-phosphate buffer containing 120 μ M-NADH was applied to a column (20cm $\times$ 1.2cm) of Sephadex G-25 equilibrated with the same buffer. The elution was continued with this buffer and the A_{340} of the effluent was recorded. Fractions of 2.2ml volume were collected with a flow rate of 1.2ml/min.

Under the same conditions there was very weak but detectable binding of NADPH ($K_D > 1000 \mu$ M). The results of the binding studies agree reasonably well with those of Malencik & Anderson (1972), who used fluorescence measurements. The weak binding of NADPH partly explains the poor effectiveness of NADPH as a coenzyme.

Discussion

Glutamate-NAD(P)⁺ reactions

Initial-rate parameters for the oxidation of glutamate by NAD⁺ with the dogfish enzyme (Table 1) may be compared with those of Engel & Dalziel (1969) for the bovine enzyme under the same experimental conditions as used here. The broad features of the kinetics and the absolute values of the coefficients are very similar. The largest differences are in ϕ'_1 at the higher ranges of NAD⁺ concentration. The somewhat smaller values of ϕ'_0 and ϕ'_1 mean that the dogfish enzyme is slightly more active than the bovine enzyme at pH 7.0. Since the dogfish enzyme does not display aggregation phenomena (Corman *et al.*, 1967), the present results confirmed the earlier findings (Engel & Dalziel, 1969) that the unusual kinetic behaviour of the bovine liver enzyme is not connected with the tendency of this enzyme to polymerize.

The most interesting aspect of the results with NADP⁺ is the strict linearity of the Lineweaver-Burk plots, confirming the previous report (Corman *et al.*, 1967). The behaviour contrasts sharply with the

situation for NAD⁺ and with that for the bovine liver enzyme, where both coenzymes are about equally effective and where with both coenzymes behaviour like that of Fig. 1 is observed (Engel & Dalziel, 1969). It is interesting that the maximum rate of glutamate oxidation by NADP⁺ ($1/\phi'_0$) is rather similar to that with NAD⁺ at the lowest range of NAD⁺ concentration, whereas the Michaelis constants for coenzyme and glutamate (ϕ'_1/ϕ'_0 and ϕ'_2/ϕ'_0) are very similar for NADP⁺ and NAD⁺ within the 300–1000 μ M-coenzyme concentration range. The mechanistic significance, if any, of these observations is not yet understood. Engel & Dalziel (1969) showed that the bovine liver enzyme exhibited linear Lineweaver-Burk plots when the alternative and poor substrate norvaline was used. Thus, with both dogfish and bovine liver enzymes, changes in reactants which result in much lower rates of catalysis lead to a loss of the characteristic features associated with the favoured substrate and coenzyme.

The stopped-flow experiments with the bovine and dogfish enzymes at high glutamate and NADP⁺ concentrations suggested that the rate-limiting step is different for the two enzymes. According to di Franco (1974) the initial transient seen with the bovine enzyme is due to the rapid formation of the E-NADPH-2-oxoglutarate complex, and this step is then followed by 2-oxoglutarate dissociation, combination of glutamate with the E-NADPH complex to give the abortive E-NADPH-glutamate complex and then dissociation of NADPH at the steady-state (inhibited) rate. With the dogfish enzyme at high substrate and coenzyme concentrations there is clearly no evidence for the accumulation of any NADPH-containing species in approaching the steady state. One would not have expected the formation of significant amounts of an E-NADPH-glutamate complex, because, as indicated, there is no substrate inhibition with glutamate. It appears that the rate-limiting step with the dogfish enzyme with NADP⁺ as coenzyme may be hydride transfer, in contrast with the bovine enzyme, where the rate-limiting step of the uninhibited reaction appears to be after E-NADPH-2-oxoglutarate formation (di Franco, 1974). It has been suggested that the lack of apparent coenzyme activation in the kinetics of norvaline oxidation by the bovine enzyme might be due to a change of rate-limiting step (Engel & Dalziel, 1969).

Studies of NAD⁺ and NADP⁺ binding to bovine liver glutamate dehydrogenase (Dalziel & Egan, 1972) have shown that reciprocal plots showing the binding of coenzymes to the enzyme-glutarate complex exhibit the same broad features as Lineweaver-Burk plots with variable coenzyme concentration, that is, they are concave downwards. Unfortunately, lack of adequate supplies of enzyme has meant that the experiments of Dalziel & Egan (1972) could not

be repeated with the dogfish enzyme. If it could be obtained in fairly large quantities the dogfish enzyme is probably more suitable for the investigation of NAD⁺ binding than is the bovine enzyme. The former is more soluble, solutions of 25 mg/ml being obtainable at pH 7.0, and it should, in principle, be easier to investigate the binding at high coenzyme concentrations with greater precision.

The results of Fig. 5 indicate that, in the presence of glutarate, NAD⁺ and NADP⁺ afford the same degree of protection for the dogfish enzyme from the inhibitory effects of pyridoxal 5'-phosphate. The results with NAD⁺ are very similar to those found for the bovine enzyme by Chen & Engel (1974). The present results indicate that NAD⁺ binds more firmly to the dogfish enzyme than NADP⁺, and this partly explains why NAD⁺ is a more effective coenzyme in kinetic studies. However, if the protection observed in the presence of glutarate is due to a coenzyme-induced conformational change, as suggested by Chen & Engel (1974), then it appears that both coenzymes can mediate the change. On this basis the kinetic differences of the dogfish enzyme towards NAD⁺ and NADP⁺ are not due to the inability of NADP⁺ to induce a conformational change, which is effected by NAD⁺ on binding to the enzyme-glutarate complex.

On the question of the catalytic mechanism of the dogfish enzyme, it seems certain that with NAD⁺ as coenzyme this is the same as for the bovine enzyme. The kinetics in the two cases are so similar. The precise details have yet to be worked out, but a rapid-equilibrium random mechanism has been proposed for the bovine enzyme (Engel & Dalziel, 1969) and has been supported by isotope-exchange data (Silverstein & Sulebele, 1973) and product-inhibition studies (Engel & Chen, 1975). With NADP⁺ as coenzyme, the kinetics are much simpler for the dogfish enzyme, and this allows a test of mechanism which cannot be applied with confidence to the bovine enzyme because of coenzyme activation. The maximum rate relationship $\phi'_1\phi'_2/\phi'_{12}\phi_0 = 1.5$ is consistent with a rapid-equilibrium random mechanism, but not with a compulsory mechanism (Dalziel, 1957). The test is not particularly convincing in the present case, because of the errors associated with ϕ_0 . However, on balance a random mechanism seems the more likely. The fact that hydride transfer, or a step preceding it, is rate limiting fits in with the suggested mechanism, and further support is given by the facts that NAD⁺, glutarate and 2-oxoglutarate can bind independently to the free enzyme. The binding of glutarate and 2-oxoglutarate is deduced from the facts that these two compounds protect the enzyme from modification by pyridoxal 5'-phosphate in exactly the same way as has been reported for the bovine enzyme (Chen & Engel, 1974).

The maximum rate relationships obtained with data derived from the results of Corman *et al.* (1967) at pH 8.0 (see Table 1) are not consistent with a compulsory mechanism. Although these authors proposed such a mechanism, they did not apply this test. Their results indicate that $\phi'_1\phi'_2/\phi'_{12}\phi_0 = 44$, which is not at all consistent with a compulsory mechanism. However, the data may be criticized, as indicated above, on the grounds that the NADP⁺ probably contained NAD⁺.

2-Oxoglutarate-NAD(P)H-NH₄⁺ reactions

The present results may be compared with those for the bovine enzyme under the same experimental conditions, obtained by Engel & Dalziel (1970). Difficulties experienced by us in the accurate estimation of ϕ_1 and ϕ_{12} were also encountered in work with the bovine enzyme. We had hoped that in changing to a different enzyme changes in the relative magnitude of the initial-rate parameters might have occurred, and that ϕ_1 and ϕ_{12} would have been easier to measure.

In comparing the dogfish and bovine enzymes with NADH as coenzyme, it is clear that, except for ϕ_{23} , the initial-rate parameters are similar. The substantially larger value for ϕ_{23} with the dogfish enzyme means that this parameter could be estimated more accurately than for the bovine enzyme and increases confidence that mechanisms which require $\phi_{23} = 0$ can be eliminated from consideration. On changing from NADH to NADPH with the dogfish enzyme there are big changes (Table 2). The data apparently conform to the same type of rate equation, but the individual parameters, except ϕ_2 , are substantially increased. The constancy of ϕ_2 is particularly striking. The large increases in most of the parameters with NADPH means that this is a much poorer coenzyme than NADH for the dogfish enzyme and confirms earlier work.

The information in Table 1 can be used to deduce something of the mechanism of reaction for the dogfish enzyme. The data are sufficiently accurate and detailed to establish that the parameters ϕ_0 , ϕ_2 , ϕ_3 , ϕ_{23} , ϕ_{13} and ϕ_{123} have finite positive values for both coenzymes, but, as indicated above, the situation is not clear with ϕ_1 and ϕ_{12} . It is difficult to think of a valid mechanism in which there would be no ϕ_1 term, since 2-oxoglutarate must react with some enzyme species and protection studies with pyridoxal 5'-phosphate show that 2-oxoglutarate binds to the free enzyme. We conclude that ϕ_1 does have finite positive values for both coenzymes, but that they are too small to be measured accurately in this analysis. The situation with ϕ_{12} cannot be resolved at present and the uncertainty unfortunately affects the choice of mechanism.

The theoretical work of Dalziel (1969) shows that the present data conform either to the requirements of a rapid random-order equilibrium mechanism, if $\phi_{12} > 0$, or to the requirements of one or other of the two compulsory-order mechanisms (i) and (ii), if $\phi_{12} = 0$.

All other compulsory and partially compulsory mechanisms may be discarded because $\phi_{23} > 0$ and $\phi_{13} > 0$, and all enzyme-substitution mechanisms because $\phi_{123} > 0$. The elimination of compulsory mechanism (iii)

is important because this mechanism was proposed for the dogfish enzyme by Corman *et al.* (1967) on the basis of much more limited data. It may be noted that the NAD(P)H data also fail to fulfil another requirement of this mechanism, that $\phi_{123}/\phi_{12} = \phi_{23}/\phi_2$. Values are $\phi_{123}/\phi_{12} = 10:1.3 = \phi_{23}/\phi_2$ with NADH as coenzyme and $\phi_{123}/\phi_{12} = 400; \sim 100 = \phi_{23}/\phi_2$ with NADPH as coenzyme. Further inspection of Table 2 indicates that, of the two possible compulsory mechanisms, (i) can be discarded. If mechanism (i) were correct $\phi_1 = 1/k_{+1}$ and this parameter should be independent of the nature of the coenzyme. This is clearly not so. The argument is not rigorous if there is a change in mechanism on changing coenzyme. However, the kinetic characteristics of the reactions with the two coenzymes are so similar (in contrast with the situation with NAD⁺ and NADP⁺; see above) that a fundamental change in mechanism seems unlikely. Consideration of mechanism (ii) shows that this is possible. For this mechanism ϕ_2 should be independent of the nature of the coenzyme (because $\phi_2 = 1/k_{+1}$) and within experimental error this probably is so. Further analysis shows that $\phi_{123}\phi_3/\phi_{13}\phi_{23} = 0.98$ for NADH and 0.59 for NADPH. Strict adherence to the mechanism requires that $\phi_{123}\phi_3/\phi_{13}\phi_{23} = 1$ (Dalziel, 1969). Testing of the maximum-rate relation-

ships by combining the data for NADPH with the maximum rate (ϕ_0) of glutamate oxidation by NADP⁺ shows that $15.6 = \phi_0'\phi_{23}/\phi_2\phi_3 > 1$ and $80 = \phi_0'\phi_{13}/\phi_1\phi_3 > 1$ and that the maximum-rate relationships are satisfied (Dalziel, 1969). A final requirement of the compulsory mechanism is that the relationship $\phi_{123}/\phi_{12} = K_{eq}/[\text{H}^+]$ where K_{eq} is the overall equilibrium constant for these reactions. The data with both coenzymes fit the relationship satisfactorily even though there is a very large increase in ϕ_{123} and ϕ_{12}' in changing from NAD(H) to NADP(H). For NAD⁺ and NADH as coenzymes $\phi_{123}/\phi_{12} = 1.8$ (cf. $K_{eq}/[\text{H}^+] = 1.28$) (Engel & Dalziel, 1967) and for NADP⁺ and NADPH as coenzymes $\phi_{123}/\phi_{12}' = 0.84$ (cf. $K_{eq}/[\text{H}^+] = 0.77$) (Engel & Dalziel, 1967). These observations confirm the accuracy of the ϕ_{123} measurements and the elimination of substitution mechanisms for 2-oxoglutarate reduction, which require $\phi_{123} = 0$.

The kinetic data apparently fulfil the requirements of mechanism (ii) rather well, and better than do data for the bovine enzyme (Engel & Dalziel, 1970). However, protection studies show that 2-oxoglutarate protects against the effects of pyridoxal phosphate and it must therefore bind to the free enzyme. Since the enzyme binds both NADH and 2-oxoglutarate independently it is unlikely that the mechanism could conform strictly to mechanism (ii). These findings indicate that the random rapid-equilibrium mechanism should be considered further. The finding that within the error $\phi_{123}\phi_3/\phi_{13}\phi_{23} = 1$ is consistent with this mechanism if 2-oxoglutarate and NH₄⁺ bind independently of one another (Dalziel, 1969). The mechanism requires the Haldane relationships $\phi_{123}[\text{H}^+]/\phi_{12}' = K_{eq}$, as for the compulsory mechanism. The findings that $\phi_0'\phi_{23}\phi_2\phi_3 > 1$ and $\phi_0'\phi_{13}/\phi_1\phi_3 > 1$ are not predicted by the mechanism, but they are not inconsistent with it.

At this stage the choice of mechanism rests on whether $\phi_{12} > 0$, and the data are not accurate enough to allow a firm decision. It seems unlikely that sufficiently accurate values of ϕ_{12} could be obtained in the near future because of the difficulties caused by inhibition at high substrate concentrations and because ϕ_{12} is much smaller than ϕ_{123} . Kinetic data for the bovine enzyme (Engel & Dalziel, 1970) suggest the rapid-equilibrium random-order mechanism, and this conclusion has been supported by product-inhibition studies (Engel & Chen, 1975) and by studies of isotope exchange at equilibrium (Silverstein & Sulebele, 1973). Mechanism (ii) is, after all, a special case of a random mechanism, and strict adherence to mechanism (ii) seems to be ruled out because we know that coenzyme and 2-oxoglutarate can bind to the free enzyme independently. On balance therefore the evidence seems to support the random rapid-equilibrium mechanism.

If a rapid-equilibrium random-order mechanism is accepted then the dissociation constants of substrates from binary, ternary and quaternary complexes with the enzyme may be calculated as indicated by Engel & Dalziel (1970). The fact that the dissociation constants of E-NADH ($\phi_{123}/\phi_{12} = 10\mu\text{M}$) and E-NADPH ($\phi_{123}/\phi_{12} = 400\mu\text{M}$) fit roughly with the approximate values derived from Fig. 9 provide some support for the mechanism. The support is rather weak, however, because values for ϕ_{12} are probably inaccurate. Better tests of the mechanism could be made if direct estimates for some of the other dissociation constants were available. The random mechanism does require that the dissociation constants for 2-oxoglutarate and NH_4^+ from binary complexes with the enzyme should be independent of the nature of the coenzyme. The values are $\phi_{123}/\phi_{23} = 480\mu\text{M}$ (NADH) and $880\mu\text{M}$ (NADPH) for the dissociation of 2-oxoglutarate from the binary complex and $\phi_{123}/\phi_{13} = 5.5\text{mM}$ (NADH) and 3.8mM (NADPH) for the dissociation of NH_4^+ from its complex with the enzyme. The dissociation constants for 2-oxoglutarate and NH_4^+ from the ternary complex enzyme- NH_4^+ -2-oxoglutarate should also be independent of the nature of the coenzyme. In these cases the values are $\phi_{13}/\phi_3 = 490\mu\text{M}$ (NADH) and $1500\mu\text{M}$ (NADPH) for the dissociation constants for 2-oxoglutarate and $\phi_{23}/\phi_3 = 5.6\text{mM}$ (NADH) and 6.3mM (NADPH) for the dissociation constants for NH_4^+ . The change in the ratio ϕ_{13}/ϕ_3 with coenzyme is rather large, but on the whole the data are perhaps consistent with the mechanism.

A. E. thanks the University of Hull for a Research Studentship. We thank Dr. P. C. Engel for helpful discussions.

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