The kinetic mechanism of ox liver glutamate dehydrogenase in the presence of the allosteric effector ADP

The oxidative deamination of L-glutamate

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1. In steady-state kinetic studies of ox liver glutamate dehydrogenase in 0.11 M-potassium phosphate buffer, pH 7, at 25°C, the concentration of ADP was varied from 0.5 to 1000 μ M. Inhibition was observed except when the concentrations of both glutamate and coenzyme were high, when activation was seen. With NAD⁺ or NADP⁺ as coenzyme, 200 μ M-ADP was sufficient to saturate the enzyme with respect to the major effect of this nucleotide. 2. In the presence of 210 μ M-ADP, widely varied concentrations of coenzyme give linear Lineweaver–Burk plots, in marked contrast with results obtained previously for kinetics without ADP. 3. This has allowed evaluation for the reaction with NAD⁺, NADP⁺ and acetylpyridine–adenine dinucleotide (315 μ M-ADP in the last case) of all four initial rate parameters, i.e. the ϕ coefficients in the equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]}$$

where A is coenzyme and B is glutamate. 4. The relative constancy of ϕ_B and of ϕ_{AB}/ϕ_A with the different coenzymes point to a compulsory-order mechanism with glutamate as the leading substrate. 5. This conclusion, though unexpected, agrees well with various previous observations on the binding of oxidized coenzyme.

The steady-state kinetic mechanism of the reaction catalysed by ox liver GDH (EC 1.4.1.3) remains controversial, despite the attention received in the literature (e.g. Frieden, 1959a,b,c; Engel & Dalziel, 1969, 1970; Barton & Fisher, 1972; Rife & Cleland, 1980). The kinetics of the oxidative deamination is both interesting and complicated to interpret in view of the associated ‘coenzyme activation’ (Olson & Anfinsen, 1953; Frieden, 1959a). Frieden (1959a) suggested that the non-linearity of primary plots, obtained with NAD⁺ as coenzyme in Tris buffer at pH 8, might be the result of coenzyme binding to a second, non-catalytic, site. With NADP⁺ over a restricted concentration range, the kinetic behaviour ap-

peared to be simpler, without this deviation from linearity (Frieden, 1959a). The data suggested that the mechanism was sequential, but no further conclusions could be drawn.

The sensitivity of a fluorimetric assay enabled Engel & Dalziel (1969) to investigate a much wider concentration range in great detail, and to establish that coenzyme activation occurred with both NAD⁺ and NADP⁺ in phosphate buffer. The pattern of non-linearity in Lineweaver–Burk plots of data obtained at pH 7 could be described by several apparently linear sections linked by ‘abrupt transitions’ (Engel & Dalziel, 1969), and Engel & Ferdinand (1973) discussed the way in which such a pattern might arise from a mixture of positive and negative co-operativity in the enzyme mechanism. The complex nature of the coenzyme primary plots prevented a definite assignment of a kinetic mechanism over the entire concentration range, although a rapid-equilibration random-order mechanism seemed most consistent with the

Abbreviations used: GDH, glutamate dehydrogenase; APAD⁺, oxidized acetylpyridine–adenine dinucleotide.

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data (Engel & Dalziel, 1969; Silverstein & Sulebele, 1974; Engel & Chen, 1975).

When the reaction mixture contains the allosteric effector ADP, at concentrations greater than 200 μM , the coenzyme primary plots are linearized (Engel, 1968; Godinot & Gautheron, 1971; Markau *et al.*, 1972), thus potentially simplifying analysis of the kinetic mechanism. One possible method for such analysis is the use of alternative substrates (Dalziel, 1957). If eqn. (1):

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \quad (1)$$

applies to a two-substrate reaction mechanism (Dalziel, 1957) then one may distinguish between the two ordered and the rapid-equilibrium random-order possibilities by examining the constancy of certain initial-rate parameters when different substrate pairs are used. If, for example, A is the leading substrate and two or more B-type substrates are used in separate experiments, then ϕ_A and ϕ_{AB}/ϕ_B should remain constant even though ϕ_0 , ϕ_B and ϕ_{AB} are likely to be changed substantially. If A is substituted then all four terms may be affected.

Such an approach has been employed successfully in the case of alcohol dehydrogenase (see, e.g., Dalziel, 1975b). Several alcohols were used to demonstrate the ordered binding of coenzyme before substrate. Indeed, most studies of nicotinamide nucleotide-linked dehydrogenases suggest that the coenzyme does bind first, often enabling the substrate to bind correctly (Dalziel, 1975a).

In the present paper we report the initial-rate parameters for glutamate oxidation with three different coenzymes in the presence of a fixed concentration of ADP. The conditions were chosen to facilitate comparison with the data of Engel & Dalziel (1969), and they lead to some unexpected conclusions about the order of substrate addition.

Materials and methods

Ox liver GDH was supplied by Biozyme Laboratories (Blaenavon, Gwent, Wales, U.K.) as a crystalline suspension in $(\text{NH}_4)_2\text{SO}_4$ solution, and was stored at 4°C. Samples of the GDH preparation gave a single band when run on polyacrylamide gels in the presence of sodium dodecyl sulphate. For each experiment a sample of the enzyme was centrifuged, the pellet resuspended in 0.11 M-potassium phosphate buffer, pH 7, containing 100 μM -EDTA, and dialysed against the same buffer. The GDH solution was clarified by centrifugation and the enzyme concentration cal-

culated from A_{280} measurements by using the absorption coefficient of 0.93 cm^2/mg (Egan & Dalziel, 1971).

ADP and all of the natural coenzymes used in the present study were purchased from Boehringer Corp., Lewes, E. Sussex, U.K., and were purified on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) followed by desalting on Bio-Gel P-2 (Bio-Rad Laboratories, Watford, Herts., U.K.) essentially by the method of Dickinson & Engel (1977). APAD⁺ was obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.), and its purity was confirmed by t.l.c. on cellulose plates with isobutyric acid/water/conc. NH_3 (66:33:1, by vol.) as solvent. Monosodium L-glutamate was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), and all other reagents were of analytical grade from BDH Chemicals, Poole, Dorset, U.K.

Initial rates, invariably at 25°C in 0.11 M-potassium phosphate buffer, pH 7, were measured on a recording fluorimeter similar to that described by Engel & Dalziel (1969), and specific activities are expressed as $\mu\text{M-NAD(P)H/s}$ per $\mu\text{M-GDH}$ subunit [with the subunit M_r taken as 56000 (Julliard & Smith, 1979)]. The fluorimeter was calibrated either by the method of Dalziel (1962) or by comparing a series of initial rates of NADPH formation in the yeast glucose-6-phosphate dehydrogenase (Biozyme Laboratories) reaction measured on the fluorimeter with those determined spectrophotometrically on a Cary 219 instrument. In the case of APAD⁺ calibration, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma Chemical Co.) was used in view of its broader coenzyme specificity. The concentrations of NAD⁺ and APAD⁺ were determined enzymically with yeast alcohol dehydrogenase (Biozyme Laboratories) by using molar absorption coefficients of 6.22 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ and 7.8 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ respectively, or with yeast glucose-6-phosphate dehydrogenase in the case of NADP⁺.

With the use of the nomenclature of Dalziel (1957), initial rates measured in the presence of ADP could be fitted to eqn. (1). The slopes and intercepts were obtained from initial-rate data, and secondary plots were used to evaluate the kinetic parameters by using a program written for the Wang 2200 desk-top computer by Dr. J. Kinderlerer (unpublished work).

Whereas eqn. (1) was used by Engel & Dalziel (1969) to determine kinetic constants for each pseudo-linear section of the double-reciprocal plots, in the present study it can be applied to the entire range of coenzyme and substrate concentrations. The stability of GDH in phosphate buffer and the sensitivity of the fluorimeter ensure

that duplicate rate measurements differ by no more than 5% and usually by less than 3%.

Results

Effect of ADP on the oxidative deamination of L-glutamate by ox liver GDH at pH 7

ADP is generally considered to be an activator of ox liver GDH (Frieden, 1959b). Although under some conditions it does activate, more often ADP inhibits glutamate oxidation (Engel, 1968; Markau *et al.*, 1972; George & Bell, 1980; Cook, 1982). Fig. 1 shows the effects of varying the ADP concentration (0.5–2400 μM) on the rate of glutamate oxidation at pH 7 under the assay conditions described, with NAD^+ and APAD^+ as coenzymes (NADP^+ gives a similar pattern to that seen with NAD^+). Although activation is obtained when the concentrations of both NAD(P)^+ (but not APAD^+) and substrate are high, under all other conditions examined ADP behaves as an inhibitor.

All four curves in Fig. 1 show a marked change in the initial rate as the ADP concentration is raised over the range 0–200 μM . In the case of NAD(P)^+ , ADP concentrations greater than 200 μM result in little or no further change in initial velocity. However, in the case of APAD^+ , the curves do not plateau until the concentration of ADP reaches at least 300 μM .

In the following kinetic studies ADP was used at

the lowest concentration that would ensure linearization of the double-reciprocal plot (Godinot & Gautheron, 1971) while minimizing the weak competitive inhibition by ADP with respect to coenzyme.

Reaction of glutamate and NAD^+ catalysed by ox liver GDH in the presence of ADP

In order to obtain good estimates of the kinetic constants and test linearity of the double-reciprocal plots (see Fig. 2), an NAD^+ concentration range of 0.2–509 μM was covered. Fig. 3 shows the initial-rate-dependence on NAD^+ over the range 50.9–509 μM at five fixed glutamate concentrations in the presence of 210 μM -ADP. The inset to Fig. 3 shows the secondary plots derived from these primary data.

In the absence of ADP one would expect deviation from linearity in the primary plots around 300 μM - NAD^+ (Engel & Dalziel, 1969); clearly no such deviation occurs even with the highest L-glutamate concentration used (40 mM). In a second set of experiments L-glutamate concentration was varied in the range 5–50 mM with NAD^+ at five fixed concentrations (9.2–102 μM). The double-reciprocal plots were again convincingly linear, as were secondary plots of slopes and intercepts. Mean values for the ϕ parameters are given for four sets of data from two experiments in Table 1.

Fig. 1. Effect of ADP on the initial rate of oxidative deamination of glutamate by ox liver GDH in 0.1 M-potassium phosphate, pH 7

The assay conditions were as follows: ●, 174 μM - NAD^+ , 40 mM-glutamate; △, 13 μM - NAD^+ , 3.75 μM -glutamate; ○, 100 μM - APAD^+ , 40 mM-glutamate; ■, 10 μM - APAD^+ , 4 mM-glutamate (right-hand scale).

Fig. 2. Linearization of the primary Eadie coenzyme plots for NAD^+ (1–60 μM) in the presence of 210 μM -ADP, with glutamate concentration fixed at 50 mM ($\circ$, top and right-hand scales) and 5 mM ($\bullet$, bottom and left-hand scales)

Reaction of glutamate and NADP^+ catalysed by ox liver GDH in the presence of ADP

As with NAD^+ , concentrations in the range 42.8–428 μM were chosen in order to evaluate the slopes and intercepts of NADP^+ and L-glutamate primary plots in the presence of ADP. Both primary and secondary plots were linear, unlike NADP^+ primary plots in the absence of ADP (Engel & Dalziel, 1969). Secondary plots were linear and gave estimates for all four ϕ parameters. Mean values from four sets of data are given in Table 1.

Reaction of glutamate and APAD^+ catalysed by ox liver GDH in the presence of ADP

With ADP^+ as coenzyme, estimates were obtained for the ϕ parameters under conditions identical with those used for NAD^+ and NADP^+ (i.e. with 210 μM -ADP) and also where the allosteric effects of ADP were saturating (315 μM). In both experiments linear plots were obtained,

although the data for 210 μM -ADP were not sufficiently detailed to exclude minor deviations from linearity. The two slightly different sets of kinetic parameters are given in Table 1. Fig. 4 shows the APAD^+ primary and secondary plots at 315 μM -ADP.

Discussion

The kinetic mechanism of the oxidative deamination of L-glutamate catalysed by ox liver GDH was first investigated in detail by Frieden (1959c). He suggested that either a rapid-equilibrium random-order mechanism or an ordered binding of coenzyme first and glutamate second would be consistent with the data for the two-substrate reaction in Tris buffer, pH 8, with NADP^+ as coenzyme. In the light of data for the three-substrate reductive amination reaction, however, the second possibility was considered more likely. A revised interpretation emerged from a more detailed study by Engel & Dalziel (1969, 1970), with phosphate buffers. The data for the three-substrate reaction could not in fact be reconciled with a compulsory-order mechanism with coenzyme leading, but were consistent with a rapid-equilibrium random-order mechanism. The initial-rate data for the two-substrate reaction, however, still did not allow a choice to be made between random-order and compulsory-order mechanisms. Studies of isotope exchange at equilibrium (Silverstein & Sulebele, 1974) and of product inhibition (Engel & Chen, 1975) have lent support to the view that the reaction follows a rapid-equilibrium random-order mechanism. This conclusion conflicts, however, with that drawn from rapid-reaction studies (Iwatsubo & Pantaloni, 1967), which seem to indicate rate-limiting steps in the product-release sequence. Although it may be argued that these studies are distorted by the use of high, and somewhat inhibitory, glutamate concentrations, it has to be admitted also that the interpretation of the two-substrate initial-rate data is in some doubt because of the complex pattern of coenzyme activation.

Against this background, it was hoped that the use of saturating concentrations of ADP might simplify the kinetic pattern by removing the superimposed effect of changing degrees of allosteric regulation. The effects exerted by ADP on ox liver GDH seem to be manifold. There is a weak competition between ADP and coenzyme for site I ($K_i \approx 4 \text{ mM}$ under these conditions; D. P. Hornby & P. C. Engel, unpublished work), and substrate inhibition seen at high glutamate concentrations is relieved by ADP, which may displace NADH from the enzyme–glutamate– NADH abortive complex (Markau *et al.*, 1972; Bailey *et al.*, 1982;

Fig. 3. Lineweaver-Burk plots for the oxidative deamination of glutamate by ox liver GDH in the presence of 210 μM -ADP. The NAD^+ concentration was varied at five fixed glutamate concentrations: ●, 40 mM; ▽, 20 mM; △, 10 mM; □, 5 mM; ○, 2.5 mM. The inset shows the derived secondary plots of slopes (●) and intercepts (○) against reciprocals of the glutamate concentrations.

Table 1. Initial-rate kinetic parameters for the oxidative deamination of L-glutamate by ox liver GDH in the presence of ADP. The ϕ constants listed are the four coefficients in eqn. (1), in which A represents the coenzyme and B represents glutamate.

[ADP] (μM)	Coenzyme	ϕ_0 (s)	ϕ_A ($\text{mM}\cdot\text{s}$)	ϕ_B ($\text{mM}\cdot\text{s}$)	ϕ_{AB} ($\text{mM}^2\cdot\text{s}$)	ϕ_{AB}/ϕ_B (mM)	ϕ_{AB}/ϕ_A (mM)	$\phi_{AB}/\phi_A\phi_B$ (s^{-1})
210	NAD^+	0.405 ± 0.023	0.051 ± 0.002	3.88 ± 0.45	1.40 ± 0.36	0.361	28.0	7.22
210	NADP^+	0.420 ± 0.008	0.047 ± 0.003	2.46 ± 1.31	1.50 ± 0.28	0.610	31.9	12.6
315	APAD^+	0.150 ± 0.017	0.143 ± 0.005	3.30 ± 0.61	5.81 ± 0.37	1.76	40.6	12.3
210	APAD^+	0.150 ± 0.021	0.250 ± 0.04	0.867 ± 0.352	2.27 ± 0.44	9.16	2.65	10.6

Cook, 1982). Perhaps most interesting, however, is the removal of the ‘abrupt transitions’ from co-enzyme primary plots in the oxidative deamination reaction.

As Figs. 3 and 4 show, the Lineweaver-Burk plots against the reciprocal of the coenzyme concentration are indeed linearized by sufficiently high concentrations of ADP, and the derived ϕ parameters are listed in Table 1. For APAD^+ two different ADP concentrations were used because 210 μM -ADP proved to be insufficient to saturate the enzyme in the presence of this coenzyme.

There is, of course, no reason why the same concentrations of ADP should prove to be saturating when different coenzymes are used. In making valid comparisons it seems more important that the enzyme should be in the same state than that the ADP concentrations should be identical. The ϕ parameters for 315 μM -ADP were therefore chosen for comparison with the sets for NAD^+ and NADP^+ .

Although good estimates of ϕ_A were readily obtained for all three coenzymes, estimates of ϕ_B are somewhat less accurate, as shown by the error

Fig. 4. Lineweaver-Burk plots for the oxidative deamination of glutamate by ox liver GDH in the presence of $315 \mu\text{M}$ -ADP. The APAD⁺ concentration was varied at five fixed glutamate concentrations: $\circ$, 40 mM; ∇ , 20 mM; $\triangle$, 8 mM; $\square$, 5 mM; $\bullet$, 4 mM. The inset shows the secondary plots of slopes ($\bullet$) and intercepts ($\triangle$) versus reciprocals of the glutamate concentration.

limits in Table 1. Despite this difficulty, ϕ_B remains constant within the estimated error. The ϕ_A values are similar in the case of NAD⁺ and NADP⁺ but about 3-fold higher with APAD⁺. The presence of a ϕ_{AB} term in each case rules out a ping-pong mechanism.

The relative constancy of the ϕ_B terms suggests that glutamate could be the first substrate to bind in an ordered mechanism. This order of binding is also implicit in the approximate constancy of the ratio ϕ_{AB}/ϕ_A (the dissociation constant for glutamate from an enzyme-glutamate complex if

glutamate leads). The constancy of the ϕ_{AB}/ϕ_A ratio above also would be consistent with a rapid-equilibrium random-order mechanism, but such a mechanism would not predict constancy of ϕ_B . The alternative compulsory-order mechanism, in which coenzyme has to bind first, likewise would not predict constancy of ϕ_B . Thus, despite the uncertainties of the analysis, these data point to a mechanism in which the amino acid binds first.

It is interesting to consider these findings in the light of the direct binding studies by Egan & Dalziel (1971), chemical modification experiments with pyridoxal 5'-phosphate (Chen & Engel, 1974, 1975a,b; Chen *et al.*, 1977) and the c.d. difference spectroscopy studies by Bayley & O'Neill (1980). All of these studies were performed under essentially the same conditions as the experiments reported in the present paper. Egan & Dalziel (1971) could detect no binding of oxidized coenzyme to ox liver GDH in the presence of ADP. The results of c.d. difference spectroscopy as applied to the binding of oxidized coenzyme to GDH (Bayley & O'Neill, 1980) suggest that ADP binds mainly to the regulatory site (site II) and that NAD⁺ in the absence of ADP also binds to site II; the presence of a dicarboxylate such as glutarate, or presumably glutamate itself, is necessary for coenzyme to bind at the active site (site I).

The observation (Chen & Engel, 1974) that concentrations of up to 15 mM-NAD⁺ are unable to protect against pyridoxal 5'-phosphate inactivation, whereas NAD⁺ together with glutarate confers substantial protection, also suggests that amino acid substrate binding may be necessary for site I binding of the coenzyme during the catalytic cycle. The combination of NAD⁺ and glutarate produces a considerable conformational change in ox liver GDH, as indicated by c.d. studies (Chen *et al.*, 1977). The source of this conformational change may be the binding of glutarate (or substrate) when site II is occupied by ADP (or saturating NAD⁺ concentrations).

Modification of ox liver GDH with diethyl pyrocarbonate seems to mimic some of the effects of ADP (George & Bell, 1980). Ethoxyformylation of one histidine residue per subunit with this reagent has the same effect of relieving inhibition due to the enzyme-NADH-glutamate complex. However, although diethyl pyrocarbonate activates ox liver GDH in a similar manner to ADP, the modified enzyme can still generate 'abrupt transitions' in coenzyme primary plots. The implications are that diethyl pyrocarbonate modifies ox liver GDH at site I but not at site II, and that ADP acts in the same way as saturation with coenzyme in locking the enzyme into its most activated form. The latter conclusion seems to be borne out by the kinetic data supplied in the present paper. The

values for ϕ_A in the presence of ADP are very close to those given by Engel & Dalziel (1969) (obtained without ADP) for the highest ranges of NAD⁺ and NADP⁺ concentrations. The value for ϕ_0 also seems to be decreased to those values for saturating coenzyme concentrations in the case of both NAD⁺ and NADP⁺.

The inability of norvaline in place of glutamate to generate 'abrupt transitions' in coenzyme primary plots (Engel & Dalziel, 1969) may also be of some relevance to this discussion. The use of norvaline at pH 8 gave linear primary plots with respect to coenzymes. The absence of a γ -carboxy group may be the reason for the removal of 'abrupt transitions' (which may be associated with a conformational change) (Chen *et al.*, 1977); furthermore the mechanism in the presence (and in the absence) of ADP is very sensitive to pH (Bailey *et al.*, 1982). The oxidative deamination of monocarboxylic amino acids is optimal at pH values around 10, where the possibility of charge interactions between substrate and enzyme is removed since the amino acid group with which the substrate γ -carboxy group interacts will not be protonated. The conformational change and the reaction pathway may be dependent on coenzyme binding first (at high pH values), and hence with monocarboxylic amino acids low rates are obtained (see Hornby *et al.*, 1983).

The suggestion that ADP induces a change in the order of substrate addition, together with removal of the apparent negative co-operativity, is interesting in view of the conflict arising from previous kinetic studies on ox liver GDH. It is clearly important with ox liver GDH to undertake all studies under the same conditions of buffer salt and pH, since this enzyme is extremely sensitive to such variables. The studies described in the present paper, and the corroborating evidence, were all achieved under the same experimental conditions, thus making comparisons more meaningful.

Although, for many dehydrogenases, the primary binding of coenzyme is a pre-requisite for substrate binding (see, e.g., Holbrook *et al.*, 1975), there is no reason *a priori* why this mechanism should apply to all dehydrogenases; Dalziel (1975a) has suggested that the preferred-pathway mechanism may be a general description of which compulsory-ordered pathways are special cases. Taken together, the present observations with ox liver GDH in the presence of ADP at pH 7 point to an ordered binding sequence but with glutamate rather than the coenzyme as the leading substrate.

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