Biochem. J. (1985) **230**, 95–99
Printed in Great Britain

Ox glutamate dehydrogenase

Comparison of the kinetic properties of native and proteolysed preparations

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(*Received 21 January 1985/15 April 1985; accepted 7 May 1985*)

Kinetic constants were determined for commercially available samples of ox liver glutamate dehydrogenase, which had previously been shown to have suffered limited proteolysis during preparation, with a range of substrates and effectors. These were compared with the values obtained with enzyme preparations purified in such a way as to prevent this proteolysis from occurring [McCarthy, Walker & Tipton (1980) *Biochem. J.* **191**, 605–611]. The K_m values and maximum velocities determined with different substrates revealed little difference between the two preparations although the proteolysed enzyme had lower K_m values for NH_4^+ and glutamate when the activities were determined with NADPH and $NADP^+$ respectively. This preparation was more sensitive to inhibition by Cl^- ions but less sensitive to inhibition by high concentrations of the substrate NADH. The two preparations also differed in their sensitivities to allosteric effectors, with the proteolysed enzyme being the less sensitive to inhibition by GTP. At high concentrations of NADH, this preparation was also more sensitive to activation by ADP and ATP.

Recently we have shown that commercially available preparations of ox liver glutamate dehydrogenase (EC 1.4.1.3) have suffered limited proteolysis during the purification procedures used (McCarthy *et al.*, 1980). A different purification procedure that does not result in this proteolysis has been devised (McCarthy *et al.*, 1980), and preparations obtained in this way are referred to below as being native. The sedimentation properties of native and proteolysed preparations of the enzymes have been compared (McCarthy *et al.*, 1981). Although the native preparations were found to associate to the same degree as the proteolysed preparations, they showed greater sensitivity to depolymerization by NADH plus GTP. As NADH is a substrate and GTP is a potent allosteric inhibitor of the enzyme [see Frieden (1968) for a review], these results suggested that there might also be differences in the kinetic properties of the native and the proteolysed enzyme preparations.

For the reaction in the direction of NAD(P)H oxidation, kinetic studies are hindered by high substrate inhibition by both NADH and 2-oxoglutarate (Frieden, 1959; Engel & Dalziel, 1970; Rife & Cleland, 1980) and by the fact that the enzyme has been found to exhibit different kinetic properties in different buffers (Engel & Dalziel, 1969). Also, the kinetics of the enzyme in the direction of oxidative deamination are complex in that, at fixed high concentrations of glutamate, double-reciprocal plots of velocity versus [NAD^+] take the form of up to four linear segments with fairly abrupt discontinuities between them (Engel & Dalziel, 1969). For these reasons complete kinetic analyses of the native and proteolysed glutamate dehydrogenase preparations were not undertaken in the present work. Instead a wide range of experiments was selected to enable apparent kinetic parameters of the native and the proteolysed preparations to be compared for a large number of enzymic properties.

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Materials and methods

Bovine glutamate dehydrogenase was purified from brain and liver tissues as previously described (McCarthy *et al.*, 1980). The liver enzyme was also obtained as a solution in 50% (v/v) glycerol from Boehringer–Mannheim, Mannheim, West Germany. All nucleotides used and dipotassium 2-oxoglutarate were also obtained from this source. All other reagents were from BDH Chemicals, Poole, Dorset, U.K.

In the direction of reductive amination, the enzyme reaction was monitored by measuring the

decrease of absorbance due to NAD(P)H at 340nm. In all these assays, the enzyme preparations were preincubated with the NAD(P)H for at least 2min, before initiation of reaction by adding the remaining substrates, to allow for the slow binding of NADH to the inhibitory site reported by Frieden (1973). Except where indicated, the concentrations of substrates used in the assay cuvettes were 80μM-NAD(P)H, 5mM-2-oxoglutarate and 100mM-NH_4Cl.

In the direction of oxidative deamination, the reduction of $NAD(P)^+$ was monitored by measuring the increase in fluorescence due to the produced NAD(P)H, with a Perkin–Elmer MPF-44B fluorimeter. Except where indicated, the concentrations of substrates were 1mM-$NAD(P)^+$ and 40mM-glutamate.

All the experiments to determine kinetic constants and the effects of allosteric nucleotides were carried out in the presence of 50mM-KH_2PO_4 adjusted to pH7.4 with NaOH, at 30°C. The experiments to investigate the effects of pH on the enzyme activity were carried out with four different buffer systems, to take account of any interactions of the buffer salts and the enzyme preparations, and in the presence of 450mM-KCl to minimize any effects due to differences in ionic strengths in the different buffers at different pH values. Although the protein concentration used in the assay cuvette (0.12μg/ml) was 10000 times less than estimates for the dissociation constant for enzyme aggregation (see Cohen *et al.*, 1976), and the initial velocity was found to be linearly dependent on enzyme concentration, indicating that concentration-dependent effects were negligible, all the assays were carried out at the same protein concentration. Assays were usually performed alternately for the two enzyme sources for each experimental condition and at least in duplicate. This allowed accurate comparisons of the responses of the two types of preparation. Data from inhibition by GTP were treated as described by Dixon (1953) and from activation by ATP and ADP as described by Wong (1975).

The enzymes obtained from bovine brain and liver purified as described in McCarthy *et al.* (1980) were identical in all physical, chemical and kinetic properties examined (see McCarthy *et al.*, 1980, 1981). Hence only the enzyme obtained from bovine brain was compared with the commercially obtained liver preparations in the experiments described in the present paper. Polyacrylamide-gel electrophoresis of the latter preparation in the presence of sodium dodecyl sulphate indicated it to be essentially completely converted into the lower-M_r, proteolysed, form, in that no higher-M_r form could be detected (see McCarthy *et al.*, 1980).

Preliminary experiments showed that several batches of disodium $NADP^+$ (98%) obtained from Boehringer–Mannheim showed no reaction with either of the enzyme preparations. The $NADP^+$ was purified by chromatography on a column (2.5cm × 42cm) of DEAE-cellulose equilibrated with 160mM-NH_4HCO_3. The eluted peaks were detected by their absorbance at 250nm. The major peak was freeze-dried, and, with this purified $NADP^+$, glutamate dehydrogenase activity could be detected with both enzyme preparations. In the presence of 40mM-glutamate, the activities of both preparations with 1mM-$NADP^+$ were 40% of those observed with 1mM-NAD^+. This is close to the value of 45% obtained by Engel & Dalziel (1969). The nature of the inhibitor originally present in the $NADP^+$ was not investigated.

Results and discussion

Comparison of apparent K_m and apparent V values

The results of these experiments are shown in Table 1, which lists the K_m values obtained under identical conditions for the two enzyme preparations. Under all these conditions, the apparent V values for the two preparations were identical within experimental error. Only the apparent K_m for NH_4Cl in the presence of NADPH and for glutamate in the presence of $NADP^+$ showed slight, although reproducible, differences. However, it is unlikely that such small differences in these apparent K_m values would be of significance in terms of the functioning of the enzyme *in vivo*.

The experiments on the variation of initial velocity with NAD^+ or $NADP^+$ concentration gave double-reciprocal plots, which took the form of apparently straight lines with fairly abrupt discontinuities, as reported by Engel & Dalziel (1969). The two enzyme preparations showed close agreement over the range of coenzyme concentrations (1–1000μM; results not shown).

When the NH_4Cl concentration was varied in the presence of NADH, inhibition was observed at high concentrations of this substrate. Since similar inhibition could be obtained by high (>600mM) concentrations of NaCl or KCl, but not Na_2SO_4, this inhibition may be attributed to Cl^- ions. The commercially available enzyme was found to be more sensitive to this inhibition (Fig. 1).

The native preparation from bovine brain was more susceptible to inhibition by high concentrations of NADH (Fig. 2). This agrees with the sedimentation results reported previously (McCarthy *et al.*, 1981), where the bovine brain preparation was found to be more susceptible to depolymerization by NADH and GTP.

Effect of pH on enzyme activity

The effect of pH was examined on NADH

Table 1. *Kinetic behaviour of native and proteolysed preparations of glutamate dehydrogenase*
Native glutamate dehydrogenase from ox brain and proteolysed preparations from ox liver were assayed at pH 7.4 and 30°C as described in the text.

Varied substrate	Fixed substrate (concentration)	K_m (mM) Native enzyme	K_m (mM) Proteolysed enzyme
NAD^+	Glutamate (40mM)	Complex	Complex
$NADP^+$	Glutamate (40mM)	Complex	Complex
Glutamate	NAD^+ (1mM)	1	1
Glutamate	NAD^+ (4μM)	25	25
Glutamate	$NADP^+$ (1mM)	3.0±0.1*	2.5±0.1*
NADH	NH_4Cl (100mM)+2-oxoglutarate (5mM)	0.02	0.02
NADPH	NH_4Cl (100mM)+2-oxoglutarate (5mM)	0.02	0.02
2-Oxoglutarate	NADPH (80μM)+NH_4Cl (100mM)	0.47	0.47
2-Oxoglutarate	NADH (80mM)+NH_4Cl (100mM)	0.36	0.36
NH_4Cl	NADPH (80μM)+2-oxoglutarate (5mM)	44±2*	36±2*
NH_4Cl	NADH (80μM)+2-oxoglutarate (5mM)	50	50

* Mean ± S.E.M.

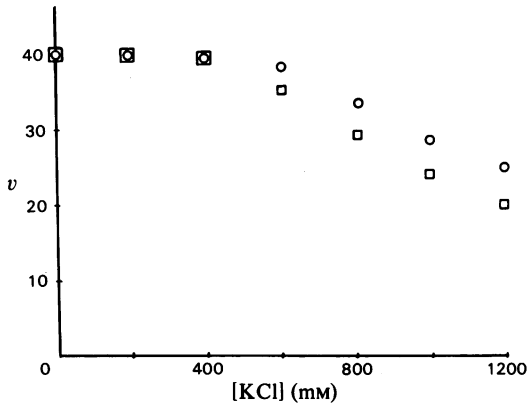

Fig. 1. *Inhibition of native* (○) *and proteolysed* (□) *preparations of glutamate dehydrogenase by KCl*
Initial velocities, v, expressed as μmol of NADH oxidized/min per mg of enzyme, were determined at pH 7.4 at 30°C as described in the text in the presence of 80μM-NADH, 5mM-2-oxoglutarate and 100mM-NH_4Cl.

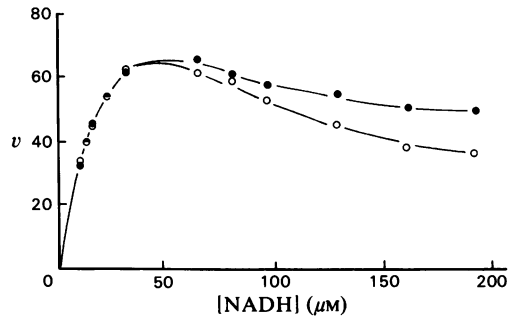

Fig. 2. *High-substrate inhibition of native* (○) *and proteolysed* (●) *preparations of glutamate dehydrogenase by NADH*
Initial velocities, v, expressed as μmol of NADH oxidized/min per mg of enzyme, were determined at pH 7.4 at 30°C as described in the text in the presence of 80μM-NADH, 5mM-2-oxoglutarate and 100mM-NH_4Cl.

oxidation over the pH range 5.5–9.0 and on NAD^+ reduction over the pH range 6.0–8.3 with citrate, phosphate, triethanolamine and bicarbonate buffers each at 50mM. Control experiments where the enzymes were incubated at pH 5.5 or pH 9.9, and assayed at pH 7.4, showed that neither enzyme preparation was irreversibly inhibited by the exposure to these pH values. The enzyme preparations were compared for a range of substrate conditions, and the results obtained revealed no difference in the effect of pH on the different preparations.

Effects of GTP, ADP and ATP on the enzyme activity

It has previously been shown that the native preparation from ox brain was more sensitive to inhibition by GTP than was the proteolysed preparation from ox liver when assayed with NAD^+ and glutamate as the substrates (McCarthy & Tipton, 1984). The K_i values, shown in Table 2, indicate that this difference in sensitivities is found under all assay conditions used. It occurs both under conditions of high-substrate inhibition by NADH and under conditions where this is not detectable. Thus the observation that GTP promotes NADH binding (Frieden, 1962), which would also imply that high NADH binding would affect GTP binding,

Table 2. *Inhibition of native and proteolysed preparations of glutamate dehydrogenase by GTP*
The activities of native glutamate dehydrogenase from ox brain and proteolysed preparations from ox liver were assayed at pH 7.4 and 30°C as described in the text. The reduction of $NAD(P)^+$ was studied in the presence of 40mM-glutamate, and the oxidation of NAD(P)H was studied in the presence of 5mM-2-oxoglutarate and 100mM-NH_4Cl. Where the two enzyme preparations are shown to have different K_i values, these differed by at least twice the sum of their standard errors.

	K_i (μM)	
Coenzyme	Native enzyme	Proteolysed enzyme
NAD^+ (1.0mM)	6.5	9.5
$NADP^+$ (1.0mM)	18	30
NADH (80mM)	6.5	9
NADH (16μM)	12	18
NADH (80μM)	9	16

might provide an explanation for this difference at inhibitory concentrations of the coenzyme, but not at lower concentrations, where the two enzyme preparations respond similarly to NADH. Fisher (1973) suggested that tightly bound nucleotide present in glutamate dehydrogenase preparations could affect the binding of nucleotide modifiers. Both enzyme preparations used in these studies gave an A_{280}/A_{260} ratio of 1.90:1, suggesting that the observed differences in nucleotide binding were not due to the presence of tightly bound nucleotides.

The effects of ADP on the enzyme activity are shown in Table 3. Reductive amination with NADPH and oxidative deamination with either NAD^+ or $NADP^+$ showed no difference in response to activation by ADP by the two enzyme preparations. Reductive amination in the presence of 80μM-NADH showed that both the K_a and the maximum activation differed for the two preparations (Fig. 3), but that the difference was much diminished in the presence of 16μM-NADH. In this case, the difference between the two preparations might be explained on the basis of the different inhibitory NADH binding observed for the two preparations.

Under the conditions used, ATP was a much weaker activator than ADP, and measurements in the presence of $NADP^+$ or 16μM-NADH could not be accurately quantified. The activation of the enzyme assayed with NAD^+ is shown in Fig. 4, and the activation constants are given in Table 3. Although the difference observed in the effect of ATP on the oxidation of NADH by the two preparations might be explained by differences in

Table 3. *Activation of native and proteolysed preparations of glutamate dehydrogenase by ATP and ADP*
The activities of the native ox brain and proteolysed ox liver enzyme preparations were determined at pH 7.4 at 30°C as described in the text. The reduction of $NAD(P)^+$ was determined in the presence of 40mM-glutamate, whereas the oxidation of NAD(P)H was determined in the presence of 5mM-2-oxoglutarate and 100mM-NH_4Cl. Where different activation constants (K_a values) are shown for the two preparations, these differed by at least twice the sum of their standard errors.

		K_a (μM)	
Effector	Coenzyme	Native enzyme	Proteolysed enzyme
ADP	NAD^+ (1.0mM)	32	32
	$NADP^+$ (1.0mM)	33	33
	NADH (80μM)	154	85
	NADH (16μM)	18	16
	NADPH (80μM)	20	20
ATP	NAD^+ (1.0mM)	85	130
	NADH (80μM)	480	250
	NADPH (80μM)	130	130

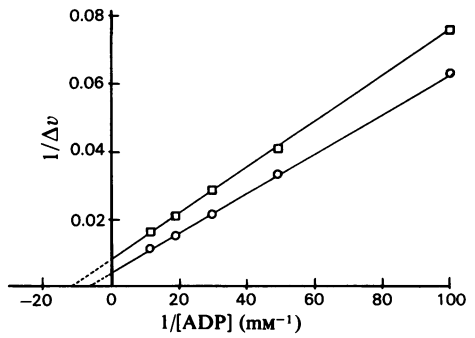

Fig. 3. *Activation of glutamate dehydrogenase preparations by ADP, assayed in the presence of 80μM-NADH*
Initial velocities were determined as described in the text, and the increase in velocity (Δv) over that determined in the absence of ADP (Wong, 1975) is expressed in μmol of NADH oxidized/min per mg of enzyme. ○, Native enzyme from ox brain; □, proteolysed enzyme from ox liver.

NADH binding, the causes of the greater sensitivity of native ox brain enzyme to activation by this nucleotide cannot be explained in this way.

The experiments presented here suggest that the proteolysis apparent in commercially obtained preparations of glutamate dehydrogenase seems to have only slight effects on the active-site properties of the enzyme, as judged by measurements of the apparent K_m and V values or by measurements of the effects of pH. However, the 'allosteric' pro-

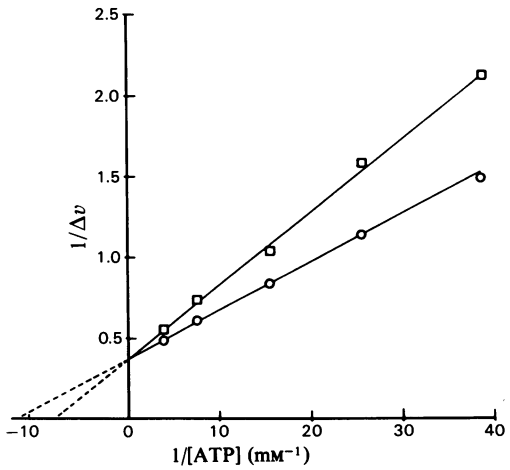

Fig. 4. *Activation of glutamate dehydrogenase preparations by ATP, assayed in the presence of 1.0mM-NAD+* Initial velocities were determined as described in the text, and the increase in velocity (Δv) over that determined in the absence of ATP (Wong, 1975) is expressed as μmol of NADH formed/min per mg of enzyme. ○, Native enzyme from ox brain; □, proteolysed enzyme from ox liver.

perties of the enzyme (high-substrate inhibition by NADH, activation by ATP, inhibition by GTP and, indirectly, activation by ADP) are affected by the proteolysis. It is tempting to ascribe these differences to the proteolytic removal of the *N*-terminal peptide, since Hucho *et al.* (1975) have produced evidence implicating this region of the enzyme molecule in its interactions with allosteric nucleotides.

We are grateful to the Trinity College Development Fund and the Medical Research Council for Grants in support of this work.

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