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Purification of glutamate dehydrogenase from ox brain and liver

Evidence that commercially available preparations of the enzyme from ox liver have suffered proteolytic cleavage

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1. A rapid procedure, involving ion-exchange chromatography on DEAE-cellulose and affinity chromatography on GTP–Sepharose, was used to purify glutamate dehydrogenase from ox brain and liver. 2. Preparations purified in this way differed from those of the ox liver enzyme that were obtained from commercial suppliers in their mobilities on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. This difference appears to result from the occurrence of limited proteolysis during the preparation of the latter enzyme samples. 3. *N*-Terminal sequence analysis showed the presence of four amino acid residues in the enzyme prepared in this study that were not present in those obtained from the commercial sources and which have not been reported in previous studies on the sequence of the ox liver enzyme. 4. A preliminary examination of the enzyme prepared in this way indicated that the Michaelis constants for the substrates are similar to those obtained from the commercial preparation, but that the response to allosteric effectors was modified.

Despite extensive studies of the properties of liver glutamate dehydrogenase [L-glutamate–$NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3] and its interactions with allosteric effectors [for reviews, see, e.g., Sund *et al.* (1975) and Eisenberg *et al.* (1976)], its functions within the cell are far from clear. Studies of the metabolism of glutamate in liver have also failed to clarify the situation, and there is no agreement on whether the reaction is close to equilibrium *in vivo* or, if it is not, in which direction it is displaced (see, e.g., Mendes-Mourao *et al.*, 1975; Krebs *et al.*, 1978).

The situation concerning the role of the enzyme in the brain is further complicated because glutamate is believed to act as a neurotransmitter as well as playing a major role as a precursor of the neurotransmitter γ-aminobutyrate in that organ. Studies with radioactively labelled glutamate have suggested that there may be two functionally distinct pools of this compound in brain (see, e.g., Van den Berg, 1973). The importance of glutamate dehydrogenase in these systems is, however, uncertain. Studies of the metabolism of glutamate by isolated brain mitochondria have also failed to indicate a major role of glutamate dehydrogenase in the processes involved (Brand & Chappell, 1974; Dennis *et al.*, 1976).

The properties of the enzyme from brain have not been studied in detail. It is present in that organ at about one-tenth of the activity of the enzyme in liver (Schmidt, 1963), and immunological studies have suggested that the enzymes from the two sources may be similar (Talal & Tomkins, 1964). In attempts to study the properties of the enzyme from ox brain in relation to its function, we have investigated methods for its purification. The procedures that have commonly been used to purify the enzyme from liver [see, e.g., Olson & Anfinsen (1952) and Fahien *et al.* (1969)] were found to be unsuitable with the brain as a starting material, and a new method, which is considerably less time-consuming, has been devised. This method is also applicable to the purification of the enzyme from ox liver, and the homogeneous enzyme prepared from both these sources has been shown to differ from purified preparations of the enzyme that are available from commercial sources both structurally and in response to allosteric affectors.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and methods

Materials

Ox liver glutamate dehydrogenase preparations were obtained from Boehringer–Mannheim G.m.b.H., Mannheim, West Germany [as a crystalline suspension in $(NH_4)_2SO_4$ and as a solution in 50% glycerol], P-L Biochemicals, Milwaukee, WI, U.S.A. (as a solution in 50% glycerol), Sigma (London) Chemical Co., Poole, Dorset, U.K., United States Biochemical Corp., Cleveland, OH, U.S.A., and Calbiochem, La Jolla, CA, U.S.A. [as crystalline suspensions in $(NH_4)_2SO_4$]. Sephadex G-25 and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden, and Whatman DEAE-cellulose DE-52 was from Whatman, Springfield Mill, Maidstone, Kent, U.K. [8-^{14}C]GTP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Nucleotides were obtained from Boehringer–Mannheim G.m.b.H., L-glutamic acid γ-methyl ester, Tris, trypsin (diphenylcarbamoyl chloride-treated), dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, *N*-ethylmorpholine and monopotassium α-oxoglutarate from Sigma, and *NN'*-methylenebisacrylamide was from Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

Assays

Glutamate dehydrogenase was routinely assayed spectrophotometrically at 30°C by following the decrease in A_{340} as NADH is oxidized. The assay mixture contained, in a total volume of 2.5ml, 0.16mM-NADH, 5mM-2-oxoglutarate, 50mM-$(NH_4)_2SO_4$, 50mM-sodium/potassium phosphate buffer, pH7.4, and enzyme. For the assay of crude enzyme solutions, the assay mixture also contained 0.4mM-KCN to decrease NADH oxidase activity and 0.4% (v/v) Triton X-100 to disrupt the tissue. Neither of these compounds affected the activity of the purified enzyme at the concentrations indicated. One unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of 1μmol of NADH/min under these conditions, and thus corresponds to 16.7nkat.

In impure preparations of the enzyme, protein concentration was determined by using the microbiuret method (Itzhaki & Gill, 1964) with bovine serum albumin as the standard. The protein concentrations of purer solutions were determined by measuring the A_{280}, by using the value of $A^{1\%}_{1cm}$ of 9.7 that has been reported for the pure enzyme from ox liver (Olson & Anfinsen, 1952).

Electrophoresis in the presence of SDS

Polyacrylamide-gel electrophoresis was carried out in a slab-gel apparatus (Studier, 1973) with gels prepared with 0.1% (w/v) SDS and with acrylamide and *NN'*-methylenebisacrylamide at concentrations of either 13 and 0.07% respectively or of 20 and 0.06% as indicated. A stacking gel of 6% acrylamide and 0.17% *NN'*-methylenebisacrylamide was used in both cases. The resolving gel contained 375mM-Tris/HCl buffer, pH8.5, and the stacking gel contained 150mM-Tris/HCl buffer, pH6.8. Both electrode compartments contained 400mM-glycine/50mM-Tris/HCl buffer, pH8.3.

Protein samples were prepared for electrophoresis by dissolving them in a mixture containing 250mM-Tris/HCl buffer, pH6.8, 2% SDS, 100mM-2-mercaptoethanol, 0.0015% Bromophenol Blue and 10% glycerol. Where indicated, the solutions were heated rapidly to 100°C for 2–3min and allowed to cool before application to the gel. Electrophoresis was carried out at 80V for 16h, after which time the band of Bromophenol Blue had travelled most of the length of the gel. After electrophoresis the gels were fixed by soaking in 50% (v/v) methanol/10% (v/v) acetic acid for 30min and then stained for protein by soaking for 30min in the same mixture also containing 1.25% (w/v) Coomassie Brilliant Blue R. Excess stain was then removed in 10% (v/v) methanol/7% (v/v) acetic acid. Fixing, staining and destaining were carried out at 50°C, with shaking to speed the processes.

Subunit molecular weights were estimated from a calibration curve prepared by plotting log(subunit molecular weight) versus mobility relative to Bromophenol Blue for proteins of known subunit molecular weight. The standard proteins used were: β-galactosidase (135000), bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000) and lysozyme (14300).

Partial tryptic digestion

Samples (100μg) of protein in 100μl of 20mM-sodium/potassium phosphate buffer, pH7.4, were denatured by incubation with 10μl of 1% SDS at 37°C for 30min. Proteolysis was initiated by adding 10μl of a freshly prepared trypsin solution (0.1mg/ml in the same buffer). The course of the reaction was followed by removing 20μl samples at known times and quenching the proteolysis by addition of 80μl of ice-cold acetone containing 5mM-phenylmethanesulphonyl fluoride. The precipitated protein was isolated by centrifugation and the fragments were examined by electrophoresis in the presence of SDS.

Preparation of GTP–Sepharose

GTP was coupled to Sepharose 4B by the method of Jackson *et al.* (1973) as modified by Godinot *et al.* (1974), where L-glutamic acid γ-methyl ester is used as the spacer instead of ε-aminohexanoic acid methyl ester. After the spacer was coupled to the

activated Sepharose and the ester group was treated with hydrazine (Godinot *et al.*, 1974), a portion of the gel was retained and used as the hydrazide gel in the purification procedure.

Incorporation of GTP into 5ml of the hydrazide gel was measured by adding 2.7μCi of [8-^{14}C]GTP to the unlabelled GTP before the periodate oxidation. The radioactivity in a sample of the gel washings was determined by scintillation counting in a mixture containing Triton X-100, toluene and 2,5-diphenyloxazole in the ratio 250:500:1 (v/v/w). About 35% of the added GTP was tightly bound to the gel, a value similar to those reported by Jackson *et al.* (1973) and Godinot *et al.* (1974), corresponding to 0.8μmol of GTP/ml of settled gel.

Purification of ox brain glutamate dehydrogenase

One ox brain, obtained from a freshly slaughtered animal, was transported to the laboratory in ice and was used as the starting material for the purification. All steps were carried out at 0–4°C.

Step 1: *homogenization.* The brain was divided into three equal portions of 120–150g and each was suspended in 500ml of 4mM-sodium/potassium phosphate buffer, pH7.4, containing 0.5mM-EDTA and 0.1mM-phenymethanesulphonyl fluoride. The last compound had been added to the buffer immediately before use by adding a suitable volume of a 100mM solution in acetone to a small quantity (approximately one-tenth of the final volume) of boiling buffer, and immediately mixing this solution with the remainder of the ice-cold buffer (H. P. Voorheis, unpublished work). The brain portions were homogenized in a Kenwood liquidizer at full speed for 1.5min.

Step 2: *$(NH_4)_2SO_4$ precipitation.* Solid $(NH_4)_2SO_4$ was added to the homogenates (while in the liquidizer) to give a 20%-saturated solution (113g/litre) and homogenization was continued for another 30s at full speed. The homogenates were then combined and stirred for 20min before being centrifuged at 10000*g* for 30min. The pellet was discarded and solid $(NH_4)_2SO_4$ was added slowly with continuous stirring to give 50% saturation (188g/litre). The mixture was stirred for a further 20min, and the precipitate that formed was removed by centrifugation as above and resuspended in 20mM-sodium/potassium phosphate buffer, pH7.4, to give a total volume of 200–250ml. This solution was dialysed overnight against 20vol. of the same buffer with at least two changes of buffer.

Step 3: *chromatography on DEAE-cellulose.* The dialysed sample was applied to a column (4cm × 12cm) of DEAE-cellulose (Whatman DE-52) that had been equilibrated with the same buffer. The column was washed with this buffer until the A_{280} of the effluent was less than 0.1, and then elution of the enzyme was effected with a 2-litre linear gradient of 20–150mM-sodium/potassium phosphate buffer, pH7.4. Fractions (approx. 25ml) were collected, and those containing glutamate dehydrogenase activity were pooled.

Step 4: *chromatography on GTP–Sepharose.* The combined active fractions from the previous step were concentrated to a volume of approx. 20ml either by ultrafiltration in an Amicon apparatus with a Diaflo XM-50 membrane or by precipitation with $(NH_4)_2SO_4$. In the latter procedure, solid $(NH_4)_2SO_4$ was added slowly to the stirred solution to make it 50% saturated (312g/litre). Stirring was then continued for a further 20min before the precipitate was removed by centrifugation at 10000*g* for 20min and resuspended in 20mM-sodium/potassium phosphate buffer, pH7.4. The concentrated enzyme solution was passed through a column (2.5cm × 20cm) of Sephadex G-25 that had been equilibrated with 100mM-Tris/acetate buffer, pH7.15, containing 1mM-KH_2PO_4 and 0.1mM-EDTA.

The solution was then applied to a column (2cm × 4cm) of the hydrazide gel, prepared as described above, that had been equilibrated in the same buffer: this removed any components which bound to the Sepharose or the spacer arm. Glutamate dehydrogenase activity was not appreciably retarded by this material, and essentially complete recovery of the applied activity was obtained when the applied volume plus a further 50ml of buffer had been passed through the column. The enzyme preparation was then applied to a 2cm × 4cm column of GTP–Sepharose which had been equilibrated in the same buffer, and the column was washed with about 150ml of that buffer until the A_{280} of the effluent had fallen to less than 0.01. Enzyme activity was then eluted with a linear 400ml gradient of 0–400mM-KCl in the same buffer. Fractions (approx. 10ml) were collected and those containing glutamate dehydrogenase activity were combined and concentrated by ultrafiltration, as described above, to give a protein concentration of at least 2mg/ml. The enzyme solution was then dialysed overnight against 3 litres of 20mM-sodium/potassium phosphate buffer, pH7.4. Glycerol was added to the dialysed enzyme solution to give a final concentration of 30% (v/v) and this was stored at 4°C.

The chromatography on GTP–Sepharose was carried out as quickly as possible, as preliminary work showed that the enzyme was slightly unstable in the Tris/acetate buffer.

Purification of liver glutamate dehydrogenase

The procedure described above was applied to the purification of the enzyme from rat and ox liver, with the only change being that the initial homogenate was prepared in 10vol. of buffer.

Purification of commercially purchased ox liver glutamate dehydrogenase

Samples of ox liver glutamate dehydrogenase from Boehringer–Mannheim or Sigma, which showed a number of minor protein bands after electrophoresis in the presence of SDS as described above, were further purified by absorption to and elution from GTP–Sepharose as described above.

N-Terminal sequence analysis

Sequence analysis of 3mg of protein was carried out on a Beckman 890 C Protein Sequenator by using a 0.1M-Quadrol [*NNN'N'*-tetrakis(2-hydroxypropyl)ethylenediamine] programme essentially as described by Hunkapiller & Hood (1978). Phenylthiohydantoin derivatives of released amino acids were determined by high-performance liquid chromatography on a Dupont 830 Liquid Chromatogram with a Whatman Partisil PX5 ODS column. Amino acid phenylthiohydantoins were eluted with a linear gradient of acetonitrile from 15 to 48% in 0.01M-sodium acetate buffer, pH4.5, over a period of 7min and then eluting with 48% acetonitrile for a further 4min. Eluted phenylthiohydantoin derivatives were identified by their absorbance at 269nm. Where indicated, back-hydrolysis of phenylthiohydantoin derivatives was carried out in 65% (v/v) HI at 110°C for 24h. Liberated amino acids were identified with a Rank–Hilger Chromaspek amino acid analyser.

Results and discussion

The results of typical purification procedures of the enzymes from ox brain and liver are shown in Table 1. The entire purification could easily be accomplished in 3 days, compared with the several weeks required for the method of Fahien *et al.* (1969), and the final specific activities are similar to that reported for the ox liver enzyme purified by the longer method.

The allosteric effector GTP (Frieden, 1963) formed the basis of an effective affinity resin for the purification of the enzyme. Such an affinity column was shown by Godinot *et al.* (1974) to be useful for the selective adsorption and subsequent elution of glutamate dehydrogenase from a number of mammalian sources, but, to our knowledge, it has not since been used as part of a purification procedure. There was some variation between different preparations of the GTP–Sepharose in the sharpness of the emergent peak of activity and in the salt concentration necessary to elute the enzyme. This had no apparent effect on the degree of purification obtained, and the reasons for this variability are unknown. Concentration-dependent effects on the ability of the enzyme to bind to GTP–Sepharose have been reported (Godinot *et al.*, 1974), but we could not detect any such behaviour in these studies with either the ox liver or brain enzyme.

The concentration-dependent association of glutamate dehydrogenase (see, e.g., Sund *et al.*, 1975) prevents the use of polyacrylamide-gel electrophoresis, except in the presence of denaturing agents, for assessing the homogeneity of the preparation. In the presence of SDS, polyacrylamide-gel electrophoresis of 2–15μg of protein per track (10mm wide) revealed a single band that stained for protein when the preparations from either ox brain or liver were applied. Comparison of the mobility of this band with those of the proteins of known subunit molecular weight gave a value of 55000 ± 5000 for the subunit weight of both the brain and liver enzyme. This value agrees with values reported for the ox liver enzyme (see, e.g., Cassman & Schachman, 1971).

In gels that were loaded with larger amounts of protein (20–30μg per track), three minor bands with subunit molecular weights corresponding to approx. 18000, 22000 and 33000 could be detected in the purified preparations from both ox liver and brain. Bands staining for protein in these positions were also detected when preparations of the purified ox liver enzyme obtained from Boehringer–Mannheim or Sigma were subjected to electrophoresis in the same way. A number of other minor protein bands

Table 1. *Purification of ox brain glutamate dehydrogenase*

Values in parentheses refer to the purification of ox liver glutamate dehydrogenase by the same procedure, starting from 40g of fresh ox liver.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
1	1500 (500)	75000 (6000)*	2280 (1800)	0.03 (0.3)	[1] ([1])	[100] ([100])
2	250 (160)	6200 (880)*	925 (1410)	0.15 (1.6)	5 (5)	41 (75)
3	600 (500)	320 (150)†	775 (1200)	2.4 (8.0)	80 (25)	33 (67)
4	90 (90)	12.5 (25)†	500 (1000)	40 (40)	1300 (120)	22 (55)

* Measured by micro-biuret assay.

† Measured by A_{280}.

could also be detected after electrophoresis of these preparations, but these additional protein-staining bands could be removed by chromatography of these enzyme samples on GTP-Sepharose as described above. The three minor protein bands described above were not removed by this process. The significance of these bands is not clear: their appearance in preparations from different ox tissues that have been prepared by different methods makes it perhaps unlikely that they are identical contaminants. It is unlikely that they are proteolytic fragments of glutamate dehydrogenase arising during the preparation of the enzyme, since phenylmethanesulphonyl fluoride and EDTA were present in the initial stage of the purification used here. The formation of these minor bands by proteolysis during denaturation of the sample before electrophoresis would also appear to be unlikely, as denaturing the samples at 100°C as recommended by Pringle (1970) made no difference to minor bands. The possibility that these polypeptides are specifically associated with the glutamate dehydrogenase hexamer and perhaps have some functional significance cannot be excluded.

Although the glutamate dehydrogenase preparations from ox liver and brain had identical mobilities, within experimental uncertainty, on electrophoresis in the presence of SDS, these were slightly, but reproducibly, lower than for the purified ox liver enzyme preparations purchased from Boehringer–Mannheim, Sigma, P-L Biochemicals, Calbiochem or United States Biochemical Corp. (Fig. 1).

Electrophoresis of a mixture of any one of the preparations obtained from a commercial source with either of those purified by the method described above resulted in a diffuse protein-staining band with leading and trailing edges that corresponded respectively to those given by the two samples when they were subjected to electrophoresis separately. If several samples of each preparation were run on the same polyacrylamide gel, the difference in the mobilities of the different preparations was statistically significant, as shown in Table 2 for the preparation from Sigma.

If the first two stages of the purification were carried out as described above, but without the addition of phenylmethanesulphonyl fluoride, and the preparation was stored at room temperature for 2 weeks before the remaining stages of the purification procedure were completed, the purified enzyme gave a single band on polyacrylamide-gel electrophoresis in the presence of SDS that corresponded in mobility to that of the commercially obtained samples. The omission of phenylmethanesulphonyl fluoride resulted, however, in no change in the mobility of the purified enzyme if the entire procedure was completed in 3–4 days.

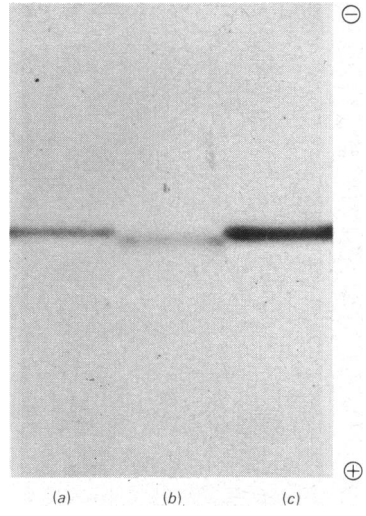

Fig. 1. *Polyacrylamide-gel electrophoresis in the presence of SDS of ox glutamate dehydrogenase samples*
For this, 13% polyacrylamide gels were used, and the proteins were stained with Coomassie Brilliant Blue R. (*a*) Glutamate dehydrogenase purified from fresh ox brain (3 μg). (*b*) Ox liver glutamate dehydrogenase purchased from Sigma (3 μg). (*c*) Glutamate dehydrogenase purified from fresh ox liver (3 μg). Only part of the gel is shown, to accentuate the difference in protein mobility.

Table 2. *Relative mobilities of glutamate dehydrogenase samples on polyacrylamide-gel electrophoresis in the presence of SDS*
Electrophoresis was carried out on a 13% polyacrylamide gel. The mobilities are calculated relative to the migration of Bromophenol Blue (=1), and are expressed as means ± S.E.M.

Glutamate dehydrogenase source	Relative mobility
Sigma	0.453 ± 0.002 ($n = 4$)
Fresh ox brain	0.445 ± 0.001 ($n = 4$)*
Fresh ox liver	0.443 ± 0.001 ($n = 3$)*

* $P < 0.003$ (t test) compared with enzyme preparation from Sigma.

Table 3. *N-Terminal sequence analysis of ox glutamate dehydrogenase samples*

Source	Sequence
Boehringer–Mannheim ox liver enzyme	H_2N-Ala-Asp-(X)-Glu
Fresh ox liver	H_2N-(X)-Asp-Ala-Ala-Ala-Asp-(X)-Glu
Fresh ox brain	H_2N-(X)-Asp-Ala-Ala-Ala-Asp-(X)-Glu

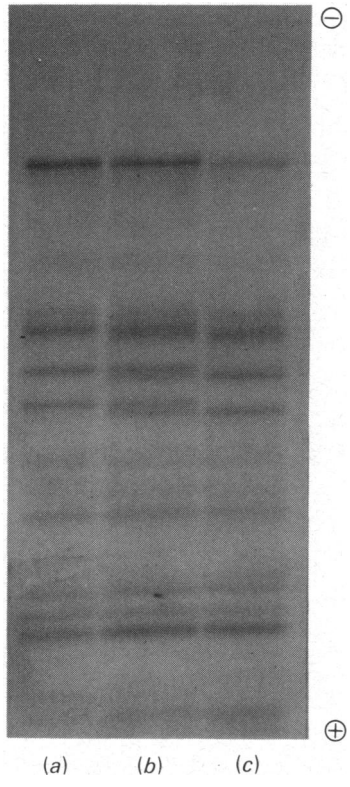

Fig. 2. *Polyacrylamide-gel electrophoresis in the presence of SDS of partial proteolytic fragments of ox glutamate dehydrogenase samples on 20% polyacrylamide gels*

(*a*) Separation of fragments from 10μg of glutamate dehydrogenase purified from fresh ox liver, digested for 10min as described. (*b*) Separation of a mixture of (*a*) and (*c*), showing resolution of the larger fragments. (*c*) Fragments from 10μg of ox liver glutamate dehydrogenase purchased from Boehringer–Mannheim, digested for 10min.

If the difference in mobility were due to the loss of a portion of the polypeptide chain (by proteolysis occurring during the preparation of the samples obtained from the commercial sources), it should be possible to detect the differences more easily if the mobilities of the fragments produced by partial proteolysis are compared. This is shown to be the case by the results shown in Fig. 2, where the fragments produced by tryptic digestion of the ox liver enzyme prepared in this work are compared with those obtained from identical treatment of the enzyme purchased from Boehringer–Mannheim. Some of the larger fragments can clearly be resolved in the track where both samples have been applied.

The brain and liver preparations purified as described above gave identical proteolytic fragments when compared in this way. This partial proteolytic peptide 'map' shows that enzyme purchased from Boehringer–Mannheim differs significantly from the enzyme preparations obtained by the method described here.

In studies with the ox liver enzyme obtained from Boehringer–Mannheim, Hucho *et al.* (1975) have shown that the *N*-terminus is on the surface of that protein, and it is thus a possible site for limited proteolysis to occur during a long purification procedure. The results of the *N*-terminal sequence analysis are shown in Table 3. The sequence for the enzyme from Boehringer–Mannheim agrees with the published sequence data (Moon *et al.*, 1972). The third residue was not positively identified, since the phenylthiohydantoin derivatives of some amino acids (e.g. arginine, cysteine) do not chromatograph well on this high-performance liquid chromatography system. The published sequence (Moon *et al.*, 1972) places arginine at this position, and the result shown in Table 3 is consistent with this. Both the ox brain and liver enzymes purified by the method described in this paper show the same sequence of Ala-Asp-(X)-Glu-.., but this is not the *N*-terminus. Instead, four additional residues were found in both samples. The *N*-terminal residue could not be identified by high-performance liquid chromatography of the phenylthiohydantoin derivative in either sample. Neither could it be identified after back-hydrolysis of the phenylthiohydantoin derivative. Attempts to determine the *N*-terminus by the dansylation technique were hampered by the insolubility of the proteins even in the SDS/*N*-ethylmorpholine method of Gray (1972). This resulted in a low degree of dansylation, and the excess of unlabelled material prevented the separation of dansyl derivatives on polyamide t.l.c. This difference in *N*-terminus of four residues corresponds to a subunit molecular-weight difference of approx. 500. This is smaller than that predicted by the difference in gel mobilities (2%, or approx. 1200), but the findings of Noel *et al.* (1979) show

that small differences in mobilities on electrophoresis in the presence of SDS may not correlate with differences in subunit molecular weight. Therefore it is uncertain whether these extra residues at the *N*-terminus are the only differences between the protein samples.

If, as seems likely, these protein cleavages are due to the method of purification of the ox liver enzyme used by the commercial suppliers, it may be necessary to re-examine many of the properties of this enzyme that have been obtained from studies that used such preparations.

The purified enzyme from ox brain and liver prepared by the above method have behaved identically when compared in several ways: mobility during electrophoresis in the presence of SDS, both of the native proteins and of their partial proteolytic fragments; *N*-terminal sequence analysis; sedimentation-velocity experiments; and inhibition of NADH oxidation by GTP. This suggests that these proteins were identical. For a comparison of some of the kinetic properties of this unproteolysed enzyme, purified ox brain enzyme and a preparation from ox liver obtained from Boehringer–Mannheim were assayed by following the reduction of NAD^+ fluorimetrically in the presence of L-glutamate at 30°C in 50mM-sodium/potassium phosphate buffer, pH 7.4. Assays were performed alternately for the two enzyme sources for each experimental condition, and at least in duplicate, to allow accurate comparison of the responses. The K_m values of the two preparations with respect to L-glutamate were identical at both 4μM- and 1mM-NAD^+ (23 and 1mM respectively), and the dependence of the initial velocity on the NAD^+ concentration at 40mM-L-glutamate gave a complex non-Michaelian curve (see Dalziel & Engel, 1968), with no detectable differences between the two enzyme sources.

The responses to allosteric effectors were studied at fixed concentrations of 40mM-L-glutamate and 1mM-NAD^+. Although there was no detectable difference between the K_a value determined for activation by ADP, a difference was detected when the activation by ATP was studied, with K_a values of $156 \pm 15\mu$M and $86 \pm 7\mu$M being determined for the commercial preparation and the brain enzyme respectively (results combined from three separate determinations). In both cases the maximum degree of activation obtained was similar. Differences in the response to inhibition by GTP were also found, with the brain enzyme again being the more sensitive, with a K_i value of $6.5 \pm 1.0\mu$M compared with a value of $10 \pm 1\mu$M for the commercial preparation (data from three separate determinations). These differences in the responses to allosteric effectors are consistent with the proposal that the *N*-terminus of the enzyme is involved in its allosteric regulation (Hucho *et al.*, 1975). Preliminary studies have shown that differences in the response to allosteric effectors can also be detected when the enzyme is assayed in the direction of glutamate formation.

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