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Catalytic activity of bovine glutamate dehydrogenase requires a hexamer structure

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Previous workers have shown that the hexamers of glutamate dehydrogenase are dissociated first into trimers and subsequently into monomers by increasing guanidinium chloride concentrations. In renaturation experiments it is shown that trimers of glutamate dehydrogenase can be reassociated to give the hexamer form of the enzyme, with full regain of activity. Monomeric subunits produced at high guanidinium chloride concentrations cannot be renatured. The trimer form of the enzyme is shown to have no catalytic activity, although the hexamer form in guanidinium chloride has full activity.

Bovine glutamate dehydrogenase (EC 1.4.1.3) is an allosteric enzyme consisting of six apparently identical subunits of M_r 56000 each (Cassman & Schachman, 1971). The enzyme has been shown to exhibit complex initial-rate kinetics (Dalziel & Engel, 1968; Engel & Dalziel, 1969), which have been attributed to negative homotropic interactions between the subunits. Subsequent ligand-binding studies with oxidized (Dalziel & Egan, 1972) and reduced coenzymes (Melzi D'Eril & Dalziel, 1973; George & Bell, 1980) showed similar behaviour. Further evidence for induced conformational changes between subunits and their effects on the catalytic and regulatory behaviours of the enzyme have been presented (Bell & Dalziel, 1973; Alex & Bell, 1980; Smith & Bell, 1982), and it has been suggested that, under some circumstances at least, the enzyme may operate via a reciprocating-subunit model (Smith & Bell, 1982) of the type first proposed to account for anomalous behaviour in alkaline phosphatase (Lazdunski *et al.*, 1971). Such a model clearly invokes the integrity of the quaternary structure of the enzyme for the catalytic function.

It is well established that the assembly of the quaternary structure of oligomeric enzymes is a multi-stage process, with polypeptide-chain folding preceding assembly into quaternary structure, which itself may proceed through intermediate stages (Ghélis & Yon, 1982). With glutamate dehydrogenase, several studies of denaturation, where polypeptide-chain unfolding has been monitored, have been reported (Sugrabova *et al.*, 1979; Muller & Jaenicke, 1980), and more recent elegant studies (Tashiro *et al.*, 1982) have shown that the hexamer form of the enzyme dissociated (in guanidinium chloride) to first a trimer stage, with little loss of 'native' conformation as judged by c.d. spectroscopy, followed by dissociation into monomers, with subsequent unfolding of the polypeptide chain.

The aims of the study reported here were to examine (i) the activity of trimers of glutamate dehydrogenase and (ii) the ability of the various molecular species produced in guanidinium chloride solutions to renature to give active hexamers of glutamate dehydrogenase.

Experimental

Glutamate dehydrogenase was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., as a glycerol solution. Before use, enzyme solutions were extensively dialysed against 0.1 M-sodium phosphate, pH 7, containing 10 μM-EDTA. Guanidinium chloride, buffer salts, coenzymes and substrates were all obtained from Sigma and were the highest grade available. All solutions were made up in 18 MΩ water obtained from a 4-Bowl Milli-Q system (Continental Water Systems, El Paso, TX, U.S.A.). The pH of all solutions was carefully adjusted to pH 7.0 before use. Concentrations of glutamate dehydrogenase were determined spectrophotometrically, by using an A_{280} of 0.93 cm^{-1} for a 1 mg/ml solution (Egan & Dalziel, 1971). Activity was measured in an assay mixture containing 50 mM-glutamate and 250 μM-NAD^+ in 0.1 M-phosphate, pH 7.0, containing 10 μM-EDTA.

Results

Preliminary experiments showed that, after incubation with certain guanidinium chloride concentrations, in 0.1 M-phosphate at pH 7.0, full activity was regained on dilution into assay mixture, and that this was an essentially instantaneous process. In the experiments shown in Table 1, glutamate dehydrogenase (final concn. 16.5 μM) was incubated in 0.1 M-phosphate, pH 7.0, with guanidinium chloride concentrations of 0.3–3 M for 30 min, and the ability to regain enzymic activity on dilution into assay mixture was measured.

The time course of the effects shown in Table 1 was examined by incubating enzyme with various fixed concentrations of guanidinium chloride and testing re-activation on dilution into the assay mixture as a function of time of incubation. Fig. 1 shows the results of such experiments with 1.5 M-, 2.25 M- and 4.5 M-guanidinium chloride, and incubation times up to 60 min.

In addition to testing for re-activation of the enzyme by dilution into assay mixture, we have also removed guanidinium chloride by dialysis against 0.1 M-phosphate buffer containing 1.0 μM-EDTA, pH 7.0, for 24 h. This procedure produced no further activation of enzyme that had been incubated with guanidinium chloride in any case.

Finally, we assayed glutamate dehydrogenase in the same guanidinium chloride concentration in which it had been incubated. In these experiments, the results of which are shown in Table 2, enzyme was incubated for 30 min in various guanidinium chloride concentrations as described above, and samples were assayed in reaction mixtures containing the same guanidinium chloride concentration

Table 1. *Re-activation of glutamate dehydrogenase after incubation in guanidinium chloride*

Glutamate dehydrogenase (16.5 μM) was incubated in different guanidinium chloride concentrations in 0.1 M-phosphate, pH 7.0, for 30 min. Re-activation was measured by diluting a sample of the incubation mixture into assay mixture to give a final enzyme concentration of 55 nM. Values in parentheses are standard deviations for a total of six determinations.

[Guanidinium chloride] (M)	Re-activation (%)
4.5	0
3.0	3.4 (±3.6%)
2.25	38.9 (±3.2%)
1.5	100 (±1.1%)
0.75	100 (±0.6%)
0.3	100 (±1.2%)

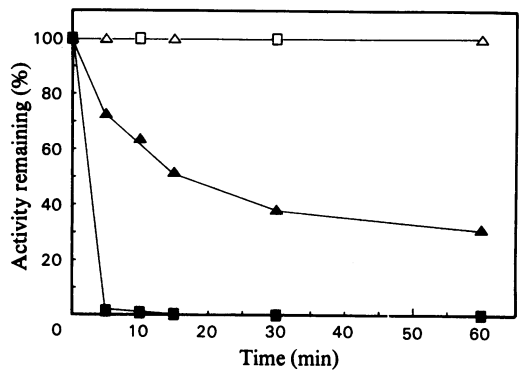

Fig. 1. *Time course of inactivation of glutamate dehydrogenase by guanidinium chloride*

Enzyme (16.5 μM) was incubated in 0.1 M-phosphate, pH 7.0, containing (△) no guanidinium chloride, (□) 1.5 M-guanidinium chloride, (▲) 2.25 M-guanidinium chloride or (■) 4.5 M-guanidinium chloride for times up to 60 min. Samples were removed and assayed in 0.1 M-phosphate, pH 7.0, containing 50 mM-glutamate and 250 μM-NAD^+.

Table 2. *Activity of glutamate dehydrogenase in guanidinium chloride*

Glutamate dehydrogenase (16.5 μM) was incubated in different guanidinium chloride concentrations for 30 min. Samples were then removed and assayed in assay mixture containing the same concentration of guanidinium chloride used in the original incubation. Control experiments involved the incubation and assay of enzyme under similar conditions except that NaCl replaced the guanidinium chloride. Other conditions were as in Table 1.

[Guanidinium chloride] or [NaCl] (M)	Activity measured in NaCl (min^{-1})	Activity measured in guanidinium chloride (min^{-1})	Percentage of expected activity when assayed in guanidinium chloride
4.5	5.9 (±6.2%)	0	0
3.0	9.1 (±5.3%)	0	0
2.25	18.6 (±4.4%)	0	0
1.5	35.3 (±1.9%)	0	0
0.75	62.4 (±1.3%)	8.6 (±4.7%)	13.8
0.3	92.0 (±0.9%)	83.0 (±0.8%)	90.2
0.0	113.1 (±0.4%)	113.1 (±0.4%)	100

as used in the incubation, in addition to the substrates. To control for ionic-strength effects, the activities were compared with those obtained at the equivalent ionic strengths by using NaCl. Although 3M-NaCl produced 92% inhibition, owing to ionic-strength effects, activities at the enzyme concentrations used in our assays (55nM) were easily and reproducibly measured.

Discussion

As discussed in the introduction, glutamate dehydrogenase is a hexameric enzyme which has been demonstrated to exhibit a number of subunit–subunit interactions affecting both the catalytic activity of the enzyme and the regulatory properties of the enzyme. It is clearly of considerable importance to establish whether the hexamer form of the enzyme is of necessity required for enzymic activity, for regulatory effects, or for both in this complex enzyme. In view of the demonstration (Tashiro *et al.*, 1982) that the hexamer can be dissociated by varied concentrations of guanidinium chloride, first into trimers and subsequently into monomers, and that, as judged by c.d. measurements, the trimer retains considerable 'native' conformation, we have investigated the catalytic activity of the trimer form of the enzyme, and studied renaturation of the various forms of the enzyme.

From the experiments shown in Table 1 it is quite apparent that when glutamate dehydrogenase is incubated with guanidinium chloride concentrations up to 1.5M, full activity can be regained on dilution into phosphate buffer containing glutamate and NAD^+. At higher guanidinium chloride concentrations decreasing amounts of activity can be recovered, and at 4.5M-guanidinium chloride no activity is recovered. Tashiro *et al.* (1982) have shown that at guanidinium chloride concentrations below 0.6M the enzyme retains its hexamer form, at concentrations between 0.6M and approx. 2M there are varying amounts of trimer and hexamer present, with predominantly trimers above 1M, and that at higher concentrations monomers are obtained.

It has not escaped our notice that these transitions between oligomeric forms of glutamate dehydrogenase correlate with the concentrations of guanidinium chloride from which we find re-activation of the enzyme possible. When glutamate dehydrogenase is incubated in guanidinium chloride concentrations where trimers are formed, full activity is regained on dilution into phosphate. When, however, the enzyme is incubated with concentrations that result in the formation of monomers, no re-activation is possible, even with prolonged incubation. Clearly, the trimer form produced under these conditions cannot regain activity. From the time-course experiments shown in Fig. 1, it is apparent that the dissociation of the trimers is a slow process, and dependent on the guanidinium chloride concentration.

From the experiments shown in Table 2 another equally important conclusion can be drawn, that is, that the trimer form of the enzyme has no catalytic activity, although, as discussed previously (Tashiro *et al.*, 1982), the trimer appears to have lost little, if any, of its native conformation as judged by c.d. This conclusion is based on our experiments where we have preincubated the enzyme in fixed concentrations of guanidinium chloride and subsequently determined enzymic activity in assay mixtures containing the same guanidinium chloride concentration to prevent reassociation. Table 2 shows that, at concentrations where the enzyme retains its hexamer form, it retains full activity. However, at guanidinium chloride concentrations where the enzyme is predominantly (0.75M) or completely (1.5M) trimeric, there is considerable or complete loss of activity, respectively, even though the trimers can be reassociated to give full regain of activity. The trimeric form of glutamate dehydrogenase has no catalytic activity, although it can, under the appropriate conditions, be reassociated to give a hexamer with complete regain of activity. This effect is unlikely to be a specific inhibitory effect of guanidinium chloride, since, as shown in Table 2, we measure essentially full activity in guanidinium chloride concentrations where the hexamer form of the enzyme is retained.

In summary, we have shown that glutamate dehydrogenase trimers can be reassociated to give full regain of activity, but that the trimers themselves have no catalytic activity. This observation implies that the hexamer form of glutamate dehydrogenase is required for the activity of the enzyme, and emphasizes the importance of interactions between the two trimers that constitute the active hexamer.

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