The unfolding and refolding of glutamate dehydrogenases from bovine liver, baker's yeast and *Clostridium symbosium*

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The unfolding behaviour of the hexameric glutamate dehydrogenases from bovine liver, *Clostridium symbosium* and baker's yeast in solutions of guanidinium chloride (GdnHCl) was studied. Changes in M_r studied by light-scattering indicate that, in each case, the hexamer dissociates to form trimers, which then dissociate to monomers at higher concentrations of GdnHCl. Dissociation to trimers is accompanied by a reversible loss of enzyme activity, but no gross structural changes can be detected by fluorescence or c.d. Dissociation to monomers is accompanied by large structural changes, and the loss of activity cannot be reversed by dilution. The parallel behaviour of all three enzymes shows that the previously noted inability of the isolated subunits of the bovine liver enzyme to refold [Bell & Bell (1984) *Biochem. J.* **217**, 327–330] is not a result of any modification of the enzyme as a result of import into mitochondria, since the *C. symbosium* and baker's-yeast enzymes do not undergo any such post-translational translocation.

INTRODUCTION

Glutamate dehydrogenase (EC 1.4.1.3) from bovine liver is an allosteric enzyme consisting of six identical subunits, each of M_r 56000 (Smith *et al.*, 1975). Although the amino acid sequence of the enzyme is known (Smith *et al.*, 1975), there is at present no X-ray crystal structure available. Recently, the low-resolution crystal structure of the hexameric NAD⁺-dependent glutamate dehydrogenase from *Clostridium symbosium* has been published (Rice *et al.*, 1987). Comparisons made with the previous low-angle X-ray-scattering (Sund *et al.*, 1969) and electron-microscope (Fiskin *et al.*, 1971) studies of the bovine liver enzyme suggest that the two enzymes have similar structures, with the six subunits essentially arranged as two sets of trimers (Rice *et al.*, 1987).

A number of studies of the unfolding of bovine liver glutamate dehydrogenase in the presence of GdnHCl have been made (Tashiro *et al.*, 1982; Bell & Bell, 1984). These studies have shown that the hexameric enzyme dissociates in two distinct stages. At concentrations of GdnHCl between approx. 0.5 M and 1.5 M, dissociation into trimers occurs, with little or no change in secondary structure as detected by c.d. Although the trimer is inactive, the dissociation can be reversed on dilution of the denaturant, and essentially full activity can be restored. At concentrations of GdnHCl above about 2 M, the trimer dissociates into its constituent subunits, with an accompanying loss of secondary structure. Activity cannot now be restored by dilution of the denaturing agent.

In animal cells, glutamate dehydrogenase is known to be exclusively located in the mitochondrial matrix (de Duve *et al.*, 1962). The enzyme is imported into the mitochondria after synthesis on the microsomal membranes (Godinot & Lardy, 1973). A number of proteins imported into mitochondria are known to undergo post-translational proteolytic processing during import (Miura *et al.*, 1986). It therefore appeared possible that,

if such processing occurred in the case of liver glutamate dehydrogenase, it might (at least in part) explain the inability of isolated subunits of the enzyme to refold to the active hexameric enzyme. In order to explore this possibility, we have performed comparative studies on the unfolding and refolding of bovine liver glutamate dehydrogenase and two other hexameric glutamate dehydrogenases of similar subunit M_r , which do not undergo an import process. These last two enzymes are the NADP⁺-dependent glutamate dehydrogenase from baker's yeast, which is located in the cytoplasm (Smith *et al.*, 1975), and the NAD⁺-dependent enzyme from *Clostridium symbosium* (Rice *et al.*, 1985, 1987).

Our results indicate that, although there are differences in detail, the pattern of dissociation and loss of ability to refold is similar for all three enzymes. The inability of the bovine liver enzyme to refold from its isolated subunits is therefore unlikely to be a consequence of any modification concomitant with the import process.

EXPERIMENTAL

Glutamate dehydrogenase from bovine liver was purchased as an (NH₄)₂SO₄ suspension from Sigma Chemical Co. Glutamate dehydrogenase from *C. symbosium* (Rice *et al.*, 1985) was generously given by Dr. P. Engel, Department of Biochemistry, University of Sheffield, Sheffield, U.K. The enzymes were at least 95% homogeneous on SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), with subunit M_r values of 56000 ± 2000 (bovine liver) and 49000 ± 2000 (*C. symbosium*), in agreement with previous results (Smith *et al.*, 1975; Rice *et al.*, 1985).

The NADP⁺-dependent from baker's yeast was isolated by the method of Doherty (1970), with the following modifications: (i) the initial extract was prepared by plasmolysis of yeast with 1 M-NH₃ (de la Morena *et al.*, 1968); (ii) the extract was subjected to two heat-treatment

Fig. 1. Activity of glutamate dehydrogenase in the presence of GdnHCl

Enzyme (25 $\mu\text{g}/\text{ml}$) was incubated in the presence of GdnHCl at 25 °C in 0.1 M-potassium phosphate buffer, pH 7.0, for 30 min, then diluted into an assay mixture containing the same concentration of GdnHCl, as described in the Experimental section. On the ordinate the activity is expressed as a percentage of that of a control in which NaCl replaced GdnHCl. ○, △ and □ represent the enzymes from bovine liver, baker's yeast and *C. symbosium* respectively.

steps as described in the preparation of Fraction III by Grisolia *et al.* (1964), before the two ion-exchange chromatography steps on DEAE-cellulose (Doherty, 1970); (iii) gel filtration on Sephacryl S-300 was used instead of sucrose gradient centrifugation.

With these modifications, 10 mg of enzyme, of specific activity 110 $\mu\text{mol}/\text{min}$ per mg of protein, could be isolated from 500 g of yeast cake. The preparation was at least 95% homogeneous on SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and the subunit M_r was $50\,000 \pm 2000$. By gel filtration on Sephacryl S-300, the M_r of native enzyme was determined as $270\,000 \pm 20\,000$, in agreement with previous work (Venard & Fourcade, 1972), thus confirming the hexameric nature of the baker's-yeast enzyme. The standard proteins used in the gel-filtration experiment were: ferritin (M_r 450 000), bovine liver glutamate dehydrogenase (M_r 336 000), yeast alcohol dehydrogenase (M_r 145 000) and bovine serum albumin (M_r 66 000).

The protein concentrations of the bovine liver and *C. symbosium* enzymes were determined spectrophotometrically at 280 nm by using values of $A_{1\text{cm}}^{1\%}$ of 0.93 and 1.05 respectively (Egan & Dalziel, 1971; P. Engel, personal communication). The protein concentration of the baker's-yeast enzyme was determined by the method of Sedmak & Grossberg (1977), with bovine serum albumin as standard.

Assays of enzyme activity were performed at 25 °C as described by Doherty (1970). In each case the concentrations of 2-oxoglutarate and NH_4Cl in the assay mixture were 3 mM and 50 mM respectively. For the *C. symbosium* enzyme the NADH concentration was 150 μM and the buffer was 0.1 M-potassium phosphate,

pH 7.0. For the bovine liver and baker's-yeast enzymes the NADPH concentration was 150 μM and the buffer was 0.1 M-potassium phosphate, pH 7.8.

GdnHCl (AristaR grade) was obtained from BDH Chemicals. The effect of GdnHCl on the activities of the enzymes was determined as described previously (Bell & Bell, 1984). Enzyme (25 $\mu\text{g}/\text{ml}$) was incubated at 25 °C in 0.1 M-potassium phosphate buffer, pH 7.0, containing a certain concentration of GdnHCl. After 30 min incubation, a sample was diluted 50-fold (bovine liver and *C. symbosium* enzymes) or 100-fold (baker's-yeast enzyme) into the assay mixture containing the same concentration of GdnHCl. In control experiments NaCl replaced GdnHCl.

Fluorescence studies were performed at 20 °C in a Perkin-Elmer MPF3A fluorimeter. Spectra were recorded 15 min after addition of GdnHCl; no further changes occurred after this time.

C.d. spectra were recorded at 20 °C in a Jobin-Yvon Dichrographe IV, in cells of 0.1 mm path-length. Spectra were recorded 15 min after addition of GdnHCl.

Changes in M_r of the enzymes on addition of GdnHCl were determined by light-scattering as described by Tashiro *et al.* (1982). The wavelength used was 360 nm, and the experiment was performed at 20 °C in the fluorimeter described above. Solutions were filtered through a 0.22 μm -pore-size filter before measurements were made. The data were analysed by the method of Parr & Hammes (1975), by using the values for refractive-index increments quoted by Tashiro *et al.* (1982).

RESULTS

Effect of GdnHCl on enzyme activity

The three glutamate dehydrogenases were inactivated in the presence of increasing concentrations of GdnHCl. In order to correct for effects of ionic strength on the enzymes, control incubations and assays were performed in the presence of identical concentrations of NaCl. The corrected activities of the enzymes as a function of the concentration of GdnHCl are shown in Fig. 1. All three enzymes are inactivated by incubation in 2 M-GdnHCl, with the baker's-yeast enzyme apparently the most sensitive. The GdnHCl concentrations leading to 50% inactivation are 0.50 M, 0.70 M and 0.80 M for the enzymes from baker's yeast, *C. symbosium* and bovine liver respectively.

It is worthwhile noting that, although the data for the bovine liver enzyme show the same pattern as those reported by Bell & Bell (1984), there are significant differences in detail. In our hands, the bovine liver enzyme is somewhat more stable; thus at 0.75 M-GdnHCl we observe 58% activity retained, compared with 14% reported in the previous work. These differences arise primarily from differences in the activities measured in the presence of GdnHCl rather than those measured in the control solutions (in the presence of NaCl). It is possible that the differences could be due to the quality of the GdnHCl used. The Sigma Grade I GdnHCl used by Bell & Bell (1984) contains a significant amount of material which absorbs in the far u.v.; thus 6 M aqueous solutions of BDH (Aristar) and Sigma (Grade I) material without further purification show $A_{1\text{cm},230}$ values of 0.16 and 2.12 respectively. The nature of such u.v.-absorbing impurities and their

Fig. 2. Reactivation of glutamate dehydrogenase after incubation in GdnHCl

Enzyme was incubated in the presence of GdnHCl at 25 °C in 0.1 M-potassium phosphate buffer, pH 7.0, for 30 min, then diluted 100-fold into an assay mixture. On the ordinate the reactivation is expressed as a percentage of that of a control incubated in the absence of GdnHCl. The enzyme concentrations during the initial incubation were 50, 15 and 32 µg/ml for the bovine liver (○), baker's yeast (△) and *C. symbosium* (□) enzymes respectively.

Fig. 3. Changes in M_r of glutamate dehydrogenase in the presence of GdnHCl

The changes in M_r were determined by light-scattering as described in the text. Experiments were performed in 0.1 M-potassium phosphate buffer, pH 7.0, at 20 °C. The wavelength was 360 nm. The ordinate represents the ratio of M_r in the presence of GdnHCl to that in the absence of GdnHCl. The enzyme concentrations were 100, 100 and 200 µg/ml for the bovine liver (○), baker's yeast (△) and *C. symbosium* (□) enzymes respectively.

possible effects on enzymes have been discussed previously (Fridovich, 1965; Nozaki, 1972).

Reversibility of inactivation by GdnHCl

After incubation at 25 °C for 30 min in the presence of various concentrations of GdnHCl, the glutamate dehydrogenases were diluted 50- or 100-fold into the appropriate assay mixtures. The residual concentration of GdnHCl was thus ≤ 0.06 M and had no significant effect on enzyme activity. As shown in Fig. 2, for all three enzymes there is a critical threshold concentration of the denaturing agent above which the degree of reactivation declines rapidly. There was no effect on the profiles shown in Fig. 2 of including dithiothreitol (1 mM) in the incubation or assay mixtures, thereby excluding the possibility that oxidation of thiol groups might account for the incomplete regain of activity after incubation in high concentrations of GdnHCl.

The data for the bovine liver are comparable with those obtained by Bell & Bell (1984), although there are some differences in detail, possibly for the reasons discussed above. Fig. 2 shows that the critical threshold concentration for the baker's-yeast enzyme is much higher than that for the *C. symbosium* or bovine liver enzymes.

Changes in M_r on addition of GdnHCl

The changes in M_r of the three glutamate dehydrogenases on addition of GdnHCl as detected by light-scattering are shown in Fig. 3. The ordinate represents the ratio of M_r at a given concentration of GdnHCl to the M_r in the absence of GdnHCl. The data for the bovine liver enzyme are similar to those reported by Tashiro *et al.* (1982), and confirm that there is an intermediate range of concentrations of GdnHCl, approx.

1–1.5 M, at which the hexamer has dissociated to trimers. At concentrations of GdnHCl above about 1.5 M, dissociation to monomers occurs. Similar patterns of dissociation (hexamer $\rightarrow$ trimer $\rightarrow$ monomer) are observed for the *C. symbosium* and baker's-yeast enzymes. For the latter enzyme, it is clear that the value of m_1/m_0 at 6 M-GdnHCl is rather higher than that (0.17) expected for dissociation of hexamer to monomers. This could arise either from incomplete dissociation of the enzyme or from the use of inappropriate values for the refractive-index increments in the presence of GdnHCl [the values used in the calculations were those quoted by Tashiro *et al.* (1982) and are based on data for solutions of bovine serum albumin]. It is also possible that the higher than expected 'plateau' value of m_1/m_0 for the baker's-yeast enzyme at intermediate concentrations of GdnHCl is due to the latter cause. However, the data for the baker's-yeast enzyme strongly suggest that the intersubunit forces in this enzyme are considerably greater than in the bovine liver or *C. symbosium* enzymes.

Structural changes on addition of GdnHCl

Structural changes in the glutamate dehydrogenases on addition of GdnHCl were monitored by fluorescence (which detects changes in the environment of aromatic side chains, predominantly tryptophan) and by far-u.v. c.d. (which detects changes in secondary structure).

The fluorescence emission spectra of all three enzymes (excited at 290 nm) showed a peak at approx. 325 nm. On addition of 5 M-GdnHCl there was a shift in the emission maximum to approx. 345 nm and a marked decline in the fluorescence intensity, the fluorescence at 325 nm being decreased to approx. 25% of its value in the absence of GdnHCl. These changes are consistent with the exposure of previously buried tryptophan side

Fig. 4. Structural changes in glutamate dehydrogenase in the presence of GdnHCl

Experiments were performed in 0.1 M-potassium phosphate buffer, pH 7.0, at 20 °C. $\circ$, $\triangle$ and $\square$ represent the enzymes from bovine liver, baker's yeast and *C. symbosium* respectively. (a) Changes in fluorescence expressed relative to the total change between 0 and 5 M-GdnHCl. Excitation and emission wavelengths were 290 and 325 nm respectively. The enzyme concentrations were 50, 40 and 45 $\mu\text{g/ml}$ for the bovine liver, baker's yeast and *C. symbosium* enzymes respectively. (b) C.d. spectra over the range 205–250 nm. Spectra were recorded in 0.1 mm-path-length cells. The enzyme concentrations were 0.75 mg/ml in each case; $\blacktriangle$ represents the spectrum of the baker's-yeast enzyme in the presence of 5 M-GdnHCl. (c) Changes in θ_{225} expressed relative to the total change between 0 and 5 M-GdnHCl. Conditions were as in (b).

Fig. 5. Comparison of the degree of reactivation of glutamate dehydrogenase with structural changes

$\blacktriangle$, $\bullet$, and $\blacksquare$ represent the percentage reactivation (data from Fig. 2), percentage of total change in θ_{225} (data from Fig. 4c) and percentage of total change in fluorescence (data from Fig. 4a) respectively. (a), (b) and (c) show the data for the enzymes from bovine liver, baker's yeast and *C. symbosium* respectively.

chains on addition of GdnHCl. Fig. 4(a) shows the changes in fluorescence at 325 nm for the three enzymes.

The c.d. spectra of the enzymes over the range 205–250 nm are shown in Fig. 4(b). These spectra are characteristic of proteins with a significant content of helical structure. By using the reference values of θ_{208} and θ_{225} (Chen *et al.*, 1974; Chang *et al.*, 1978), the helical content for the enzymes from bovine liver, *C. symbosium* and baker's yeast can be estimated as $47 \pm 4\%$, $39 \pm 2\%$ and $62 \pm 3\%$ respectively. The estimate for the *C. symbosium* enzyme can be compared with the value of 55% obtained by X-ray crystallography (Rice *et al.*, 1987).

On addition of 5 M-GdnHCl, there is effectively a complete loss of secondary structure of the enzymes [see Fig. 4(b) for the example of the baker's-yeast enzyme]. The changes in θ_{225} , which reflect the changes in helical

content (Chen *et al.*, 1974), on addition of GdnHCl are shown in Fig. 4(c). It is clear that for each enzyme the changes in fluorescence and c.d. show the same pattern.

DISCUSSION

A comparison of the various changes in the structure and activity brought about by GdnHCl shows that, for each of the three glutamate dehydrogenases examined, the decline in percentage reactivation after denaturation closely parallels the structural changes detected by fluorescence and c.d. (Fig. 5). An exact correspondence would not necessarily be expected, because the different measurements were performed at different enzyme concentrations, and this might have some effect on the dissociation equilibria involved. However, the fact that our data on the reactivation and c.d. changes of the

bovine liver enzyme are very similar to those reported previously (Bell & Bell, 1984; Tashiro *et al.*, 1982) at considerably different enzyme concentrations suggests that concentration has only a relatively small effect on the denaturation profiles reported here.

When taken together with the data on the changes in M_r (Fig. 3), it is clear that these structural changes and the decline in percentage reactivation occur over the same range of GdnHCl concentrations as that at which the trimeric species dissociates to monomers.

Thus, for each enzyme, the dissociation from hexamer to trimers involved a reversible loss of activity (Figs. 1 and 2), but is not associated with gross structural changes (Figs. 4a and 4c). Tsou (1986) has emphasized that the loss of enzyme activity on addition of denaturants can be caused by subtle changes at the active site, and does not necessarily parallel the more widespread structural changes detected by techniques such as c.d. and fluorescence. The dissociation from hexamer to trimer is reminiscent of the behaviour of the catalytic unit of aspartate carbamoyltransferase (Gerhart & Schachman, 1965) and is consistent with the observations of 32 symmetry in the bovine liver and *C. symbiosum* glutamate dehydrogenases (Sund *et al.*, 1969; Fiskin *et al.*, 1971; Rice *et al.*, 1987).

By contrast, for each enzyme the further dissociation from trimer to monomers is accompanied by considerable structural changes (Figs. 4a and 4c) and by the loss of reactivation (Fig. 2). The changes occur at considerably higher concentrations of GdnHCl for the baker's-yeast enzyme (Fig. 5), suggesting that in this case the intersubunit contacts in the trimer are much stronger.

Since all three glutamate dehydrogenases show a qualitatively similar pattern of structural changes and loss of reactivation, it is clear that the previously noted inability of the subunits of the bovine liver enzyme to refold after dissociation cannot be due to any special structural changes occurring as a result of import into the mitochondria. Instead it is probable that the inability of the isolated glutamate dehydrogenase subunits to refold under the conditions used in this work can be correlated with their size. Jaenicke (1984) and Teschner *et al.* (1987) have drawn attention to the low extents of reactivation of many multidomain protein chains of > 300 amino acids, which is thought to be a result of lack of proper co-ordination between the processes of chain folding and domain pairing. It remains a task for future work to establish conditions under which such large chains can be efficiently refolded after denaturation.

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