Urea-induced inactivation and denaturation of clostridial glutamate dehydrogenase: the absence of stable dimeric or trimeric intermediates

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Urea-induced effects in clostridial glutamate dehydrogenase (GDH, EC 1.4.1.2) were studied by spectrophotometry, circular dichroism, FPLC, affinity chromatography and PAGE. Denaturation of enzyme occurred over a narrow range of urea concentrations (2.5–3.5 M), accompanied by inactivation of enzyme with a similar rate constant. The contribution of instantaneous inhibition by urea was also ascertained. FPLC studies of urea-treated GDH gave no evidence for dissociated oligomeric fragments of the hexamer in the presence of sub-denaturing concentrations of urea. Likewise a mixture of fully 5,5'-dithiobis-(2-nitrobenzoic acid)-modified GDH hexamers and unmodified enzyme in 2 M urea failed to give rise to hybrid

molecules. Exposure of unmodified GDH to high concentrations of urea led to the dissociation of hexamers to denatured monomers followed by association to form non-specific high-*M_r* aggregates. This conclusion was confirmed by native gradient PAGE experiments. Various specific ligands stabilized the enzyme against urea-induced inactivation, succinate and 2-oxoglutarate being particularly effective. This protection of the native state was enhanced in ternary complexes, and the complex most resistant to urea-induced inactivation was the productive ternary complex GDH–NADH–2-oxoglutarate. Native gradient PAGE experiments indicate that these protecting ligands preserve the native hexameric structure of GDH.

INTRODUCTION

Glutamate dehydrogenase (GDH) catalyses the reversible oxidative deamination of L-glutamate with NAD(P)⁺ as coenzyme. Structural studies have shown that most GDHs are hexamers composed of identical subunits arranged as a dimer of trimers [1–8]. A number of studies on the unfolding of various GDHs in the presence of denaturing agents such as guanidium chloride (GdnHCl) or urea have been made [9–15]. There are two different opinions concerning the dissociation of hexameric GDH during denaturation. Light-scattering studies have suggested that, at concentrations of GdnHCl insufficient to cause denaturation, hexameric molecules dissociate into trimers [12,15]. Moreover, from these data and studies of activity of the enzyme in the presence of GdnHCl, it was concluded that dissociated trimers are inactive [13,15]. On the other hand, ultracentrifugation studies have yielded no significant evidence for dimeric or trimeric intermediates [10,14].

Another approach to the investigation of protein dissociation processes is immobilization of the oligomers on insoluble matrices [16–26], with the advantage that the insoluble matrix may stabilize the dissociated fragments which are often unstable in solution [18,24]. Using this method it was found that hexamers of bovine liver GDH under different conditions can dissociate into inactive monomers (at neutral pH) or dimers (at alkaline pH or at neutral pH in the presence of NADH), or into active trimers (at acidic pH or at neutral pH in the simultaneous presence of NADH and 2-oxoglutarate) [25,26]. Moreover, dissociated active trimer showed allosteric regulation by GTP similar to that seen with the hexamer.

The crystal structure of clostridial GDH has been solved by high-resolution X-ray analysis (0.196 nm) [27], providing detailed insight into the structural relationship of the subunits within the hexamer, and the gene encoding this enzyme has also been cloned and overexpressed [28], making site-directed mutagenesis

possible. Unfolding of clostridial GDH in the presence of GdnHCl has been investigated in parallel with similar studies of bovine liver and bakers' yeast GDHs [15]. It was concluded in all three cases that the GDH hexamer could be dissociated into inactive trimers followed by dissociation into denatured monomers.

The question of whether folded trimers or dimers are formed under mildly denaturing conditions is of importance in the context of our own experimental approach to subunit interactions in GDH, which involves the construction of hybrid oligomers. The present paper reports a detailed investigation of urea-induced dissociation of clostridial GDH and of the resulting changes in its structure and activity. In our hands and with this denaturant, GDH does not appear to give rise to stable trimeric or dimeric intermediates. The various effects of specific ligands on the dissociation process are also described. Some of this work has been briefly presented elsewhere [29].

MATERIALS AND METHODS

Clostridial GDH was purified from *Escherichia coli* TG1 pGS516 cell paste by a modification of the single-step affinity-chromatographic procedure [30]. Bacteria were harvested, suspended in 0.1 M Tris/HCl buffer, pH 7.0, containing 1 mM EDTA (1:10, w/v), sonicated and centrifuged at 27000 *g* for 30 min. Supernatant was loaded on a dye-affinity column of Remazol Brilliant Red GG–Sepharose CL-6B, that had been equilibrated with the 0.1 M Tris/HCl buffer, pH 7.0, and washed with the same buffer. For elution the enzyme's property of reversible inactivation at high pH [31,32] was exploited. The bound enzyme was selectively eluted from the Remazol Red dye column with 0.1 M Tris/HCl, pH 8.8. This procedure excludes contamination of GDH samples by small amounts of proteins

non-specifically bound to the Sepharose (these could elute using the less specific salt procedure) [30]. Active fractions were pooled and GDH was precipitated with ammonium sulphate at a final concentration of 60% saturation. After centrifugation the enzyme was resuspended in a small volume of 0.1 M Tris/HCl buffer, pH 7.0, and stored in 60% ammonium sulphate at 4 °C. Before use, the enzyme solution was desalted on a Sephadex G-25 (fine) column.

The concentration of clostridial GDH was determined spectrophotometrically at 280 nm by using a value of the absorption coefficient of $1.05 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ [33].

Grade-II NAD⁺ (free acid), grade-II NADH (disodium salt) and 2-oxoglutarate (disodium salt) were obtained from Boehringer Mannheim UK (Lewes, East Sussex, U.K.). Tris [tris(hydroxymethyl)aminomethane], L-glutamate (monosodium salt), L-alanine, NAD⁺-agarose (NAD⁺ immobilized through the N-6 atom of the adenine ring to cross-linked 4% beaded agarose), and M_r markers for gel-filtration chromatography and for non-denaturing PAGE were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sepharose CL-6B, Sephadex G-25 (fine) and the FPLC pre-packed column of Superose 6 HR 10/30 were from Pharmacia Biotech Ltd. (St. Albans, U.K.). All other chemicals were analytical reagent grade and supplied by BDH Chemicals Ltd. (Atherstone, Warwickshire, U.K.) or Fisons Scientific Ltd. (Loughborough, Leics., U.K.).

GDH activity was measured spectrophotometrically at 25 °C (Uvikon 941 Plus, Kontron Instruments) by recording the change in absorbance at 340 nm due to the production of coenzymes (NAD⁺ or NADH). Assay solutions contained the following concentrations of ligands: (a) forward reaction (oxidative deamination of L-glutamate) 1 mM NAD⁺ and 40 mM L-glutamate in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA; (b) reverse reaction (reductive amination of 2-oxoglutarate) 0.1 mM NADH, 10 mM 2-oxoglutarate and 50 mM NH₄Cl in the same buffer as (a), pH 7.0.

Studies of the inactivation of clostridial GDH were carried out at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and various concentrations of urea. After 60 min incubation the residual activities of samples (in both directions of GDH reaction) were measured by dilution of 10 μl aliquots into 1 ml of assay solution in the presence and absence of the same concentration of urea as in the incubation mixture. For the study of kinetics of inactivation, enzyme was incubated in a fixed concentration of urea. Aliquots (5–10 μl) were withdrawn at the indicated times of incubation and assayed as described above. Studies of the effect of ligands on the urea-induced inactivation were performed in 0.1 M Tris/HCl buffer, pH 7.0, containing 1 mM EDTA. In these experiments the specific compounds were included in the incubation mixture at appropriate concentrations.

CD spectra of samples were recorded in 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C using a Jasco J600 spectropolarimeter. Cells path lengths were 0.1 and 1.0 mm for protein concentrations of 1.5 and 0.3 mg/ml respectively.

Apparent M_r s of samples were estimated by using a Pharmacia FPLC system with a Superose 6 HR 10/30 pre-packed column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl. This column was calibrated with gel-filtration relative-molecular-mass-marker proteins: thyroglobulin (669 000), apoferritin (443 000), β -amylase (200 000), alcohol dehydrogenase (150 000) and albumin (66 000). Samples (100 μl ; 1 mg/ml each) were loaded separately. The flow rate was 0.5 ml/min.

Native PAGE was carried out on the PhastSystem at 15 °C (Pharmacia Biotech Ltd.) using PhastGel gradient 8-25. Non-denaturing PAGE relative-molecular-mass-marker proteins were

used for calibration: urease (hexamer 545 000; trimer 272 000), alcohol dehydrogenase (150 000), BSA (dimer 132 000; monomer 66 000), chicken egg albumin (45 000).

Modification of clostridial GDH (10 mg/ml) with 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [34], which reacts with residue Cys-320 [35,36], was carried out in 0.1 M potassium phosphate buffer, pH 7.0. After 30 min incubation at 20 °C, modified enzyme was separated from the low- M_r compounds by gel-filtration on a Sepharose G-25 (fine) column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. Studies of urea-induced denaturation of DTNB-modified GDH were carried out under the same conditions as for unmodified enzyme. Recoverable enzyme activity was measured after 5-fold dilution and 10–15 min incubation at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 50 mM 2-mercaptoethanol. In the absence of urea treatment this procedure releases the TNB⁻ residues from DTNB-treated GDH with quantitative recovery of enzyme activity. On the other hand, after urea treatment, the unmodified enzyme gives identical activity measurements with or without pre-incubation with 2-mercaptoethanol. It can be assumed, therefore, that the treatment removes TNB⁻ without refolding of denatured enzyme, and that the measurement of recoverable activity after this procedure accurately indicates the fraction of DTNB-modified enzyme still retaining its native structure after urea treatment.

For hybridization experiments equal amounts of DTNB-modified and unmodified enzymes (final concentrations approx. 2 mg/ml each) were mixed and incubated in the presence and absence of 2 M urea. After 30 min incubation at 20 °C (with urea) or 16 h at 4 °C (without urea) 200 μl samples were loaded on to an NAD⁺-agarose affinity column (6 ml) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Bound proteins were eluted with a 0–1 M gradient of NaCl in the same buffer using a Pharmacia FPLC system. The flow rate was 0.25 ml/min. As a control modified and unmodified samples of enzyme were treated separately in the same way.

RESULTS AND DISCUSSION

Figure 1 shows the results of incubation of clostridial GDH in the presence of various concentrations of urea. After 60 min incubation at 20 °C the CD spectra and residual activities of samples were examined. As in the case of GdnHCl [15] the results for urea show a decrease of the CD signal at 220 nm (Figure 1a) over a narrow concentration range (2.5–3.5 M urea). [Note that the 100% change value in Figure 1(a) corresponds to a residual CD signal of about 20% of the initial value persisting even in 5.4 M urea. This presumably reflects a core of secondary structure resistant to denaturation.] The data in Figure 1(b) show that in the absence of urea in assay solutions there is no significant difference in the inactivation profile according to which (forward or reverse) reaction was used for determination of activity. Comparison of the results in Figures 1(a) and 1(b) shows practically identical dependence of the change of CD signal at 220 nm and the change of activity on urea concentration, suggesting that inactivation is a direct consequence of denaturation. In support of this view, kinetic studies of denaturation and inactivation in 4 M urea also show (Figure 2) that both processes follow pseudo-first-order kinetics with similar rate constants of about $3 \times 10^{-3} \cdot \text{s}^{-1}$.

In contrast to the results of Figure 1(b) (assayed without urea), when samples, after 60 min incubation in various concentrations of urea, were assayed in reaction mixtures containing the same concentration of urea it was found that residual activities depended on which reaction was used for the assay (Figure 1c).

Figure 1 Urea-induced denaturation and inactivation of clostridial GDH

After 60 min incubation of enzyme in the presence of various concentrations of urea the CD spectra [changes in CD signal at 220 nm ($\Delta\Theta_{220}$)] shown in (a) and residual activities (b and c) of samples were measured. The scale for the CD measurements extends from 0% change in the absence of urea to 100% change, representing the residual CD signal in 5.4 M urea. For activity measurement 5–10 μ l aliquots were assayed in the presence (c) and absence (b) of the same concentrations of urea as in incubation mixture. Residual activities were determined by using forward (○) and reverse (●) reactions. (d) represents data of (c) corrected by using the activities of native GDH measured in the presence of urea (Figure 3). Experiments were performed at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. Concentrations of GDH in incubation mixtures were 1.5 and 0.05 mg/ml for CD and inactivation experiments respectively.

Moreover, regardless of the direction of assay, activity measurements in the presence of urea in the assay solution (Figure 1c) gave a different inactivation profile from the corresponding measurements without urea (Figure 1b). Qualitatively similar results were obtained previously for clostridial as well as for bovine liver GDHs but using GdnHCl as a denaturing reagent [13,15]. Based on the results of light-scattering experiments it was concluded that at an intermediate range of GdnHCl concentrations (1.0–1.5 M) GDH hexamers dissociate into inactive

Figure 2 Kinetics of denaturation and inactivation of GDH induced by 4 M urea

GDH was incubated at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 4 M urea. The changes in CD signal at 220 nm (●) and activity (in reverse reaction without urea in assay solution) (○) were measured after the indicated times of incubation. The results are presented in linear (a) and semi-logarithmic (b) form. Concentrations of GDH in the incubation mixtures were 1.5 and 0.05 mg/ml for CD and inactivation experiments respectively.

trimers [13,15]. In the case of urea as a denaturing reagent the results (Figures 1b and 1c) could be interpreted in two ways, either: (a) there is a dissociation of GDH hexamers into smaller units, e.g. trimers, but these possess catalytic activity which responds to urea differently according to the direction of reaction; or, more simply, (b) there is no dissociation of hexamers in the range of urea concentrations 1.5–2.5 M and the observed differences are the results of a pure instantaneous, classical inhibition effect of urea on the catalytic activity of hexameric GDH. A similar dependence of the observed extent of inhibition on the direction of reaction was observed in the case of native GDH untreated with urea (Figure 3). It is important to note that up to 3.5 M urea there was no marked deviation from linearity during the time course of activity measurements in the presence of urea (typically 30 s after addition of enzyme, with mixing taking no more than 5 s). Thus the slower loss of activity through denaturation illustrated in Figure 2 does not significantly impinge upon the measurement of initial rates for Figures 1(c) and 3. Recalculated data of Figure 1(c), taking into consideration the effect of urea on the activity of native GDH (Figure 3), are given in Figure 1(d). Comparison of these results with the data of Figure 1(b) shows no significant differences (no more than 5%). Thus, for example, after correction for the contribution of inhibition, the figures of 29% (forward reaction) and 51% (reverse reaction) for the samples in 3.15 M urea (final concn.) (Figure 1c) are amended to 69% and 75% (Figure 1d). The

Figure 3 Instantaneous inhibitory effect of urea on the GDH activity

Activities of native GDH (0.05 mg/ml) were measured in forward (○) and reverse (●) reactions. Assay solutions (both at pH 7.0, details in the Materials and methods section) were supplemented with the concentrations of urea indicated.

Figure 4 Kinetics of GDH inactivation in the presence of 3.5 M urea

Enzyme (0.05 mg/ml) was incubated at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 3.5 M urea. Aliquots (5–10 μ l) of sample were withdrawn after the indicated times of incubation and assayed in forward (open symbols) and reverse (filled symbols) reactions in the presence (circles) and absence (triangles) of 3.5 M urea.

corresponding data points in Figure 1(b) for assay in the absence of urea are 70 % and 73 %. All the observed differences in the results are therefore attributable to urea inhibition.

Another experiment confirming this conclusion was a study of the kinetics of urea-induced inactivation (Figure 4). After the indicated time of incubation of enzyme with 3.5 M urea, samples were diluted into assay solutions (in both directions) containing 0 M and 3.5 M urea. Although the absolute values of GDH activity at zero time of incubation depend on the reaction direction and the presence or absence of urea (Figure 3), this does not influence the normalized time course of inactivation. Taking into consideration these results and those of Figure 2, we can confirm that all the performed measurements follow the

Figure 5 Elution profiles of native and urea-treated GDH on a Superose 6 column

Native GDH (0.3 mg/ml) in 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl was loaded on the FPLC column of Superose 6 HR 10/30 equilibrated with the same buffer (a). Samples treated with 4 M urea were run under the same conditions. Samples (50 μ l) of GDH (1.5 mg/ml) after 10 and 60 min incubation in 4 M urea at 20 °C (0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA) were withdrawn, diluted 5-fold with elution buffer and immediately loaded on the column (b and c respectively after 10 and 60 min incubation). Another sample of enzyme, treated for 60 min at 20 °C in 2 M urea, was diluted 5-fold with elution buffer containing in addition 2 M urea and loaded on to the column, equilibrated with the same concentration of urea in the buffer (d). For all samples the volume loaded was 200 μ l, and flow rate 0.5 and 0.3 ml/min for elution without and with urea respectively.

same process, namely unfolding of the native structure of GDH. None of the experiments make it necessary to invoke an intermediate dissociation of hexamers into smaller stable oligomers, e.g. trimers, although such a process is not ruled out by these results.

To obtain more information about the structural changes of GDH induced by urea, FPLC and native PAGE were used. Samples of GDH after 10 and 60 min incubation at 20 °C with 4 M urea in 0.1 M potassium phosphate buffer, pH 7.0, were diluted 5-fold in 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl and loaded on the FPLC pre-packed column of Superose 6 HR 10/30, equilibrated with the same buffer (Figure 5). When loaded after 10 min incubation, clostridial GDH (residual activity about 30 %) eluted from the column in two peaks, the first in the GDH native hexamer position (M_r 300 000) (cf. Figure 5a) and the second in a position corresponding, in the case of a native globular protein, to an M_r of about 80 000–90 000 (Figure 5b). This second elution position, intermediate between those expected for a folded monomer and folded dimer, could be explained by the presence either of partially unfolded monomers or, much less likely, of exceptionally compact dimers. Since this sample retained about 30 % of its

Figure 6 Native gradient PAGE of GDH samples treated with urea

Lanes represent: 1, non-denaturing PAGE M_r marker proteins; 2, native GDH; 3–8, GDH after 60 min incubation in 4 M urea in the absence (3) and presence of 100 mM 2-oxoglutarate (4), 5 mM NAD^+ (5), 5 mM NADH (6), 100 mM 2-oxoglutarate and 5 mM NAD^+ (7), 100 mM 2-oxoglutarate and 5 mM NADH (8). The urea-treated samples of enzyme (1.5 mg/ml) were loaded on 8–25% native gradient PhastGel without dilution.

initial enzyme activity, it seems most likely that the peak in the hexamer position is indeed native hexameric GDH. Incubation for 60 min in 4 M urea led to the simultaneous disappearance of the hexamer peak and the formation of a very spread shoulder with apparent M_r higher than that of the hexamer (Figure 5c). These results suggest that prolonged treatment with 4 M urea leads to the dissociation of the GDH hexamers into denatured monomers followed by association into non-specific high- M_r aggregates. This conclusion was confirmed by using native gradient PAGE (Figure 6). Use of 2-mercaptoethanol in all experiments changed neither the PAGE patterns nor the FPLC elution profiles, showing that aggregation is not merely the result of the formation of disulphide bonds between denatured fragments of enzyme. Also noticeable in Figures 5(a–d) is a small peak of apparently much lower M_r . A calibration plot suggests an M_r value of approx. 7000, although this must be regarded as very imprecise in view of the separation range of Superose 6. This peak is present in the original native sample (Figure 5a), but also appears to increase upon prolonged urea treatment (Figure 5c). The nature of the material under this peak is not clear, but more recent experiments with another batch of enzyme show much smaller amounts of this material and denaturation behaviour closely similar in other respects to that described here.

As mentioned above, none of these experiments provided significant evidence for dissociation of GDH hexamers into trimers or dimers in sub-denaturing concentrations of urea. After 60 min incubation with 2 M urea in 0.1 M potassium phosphate buffer, pH 7.0, the sample of GDH was loaded on the FPLC column of Superose 6 HR 10/30 equilibrated with the same buffer, pH 7.0, containing in addition 2 M urea. The elution profile (Figure 5d) shows that the treated enzyme is eluted from the Superose 6 column as a single peak with molecular size equivalent to the native hexamer.

Figure 7 Affinity chromatography of native and DTNB-modified enzyme and a mixture of these on NAD^+ -agarose

Samples (200 μ l) [native (a), DTNB-modified (b) and a mixture of these (c)], after 30 min incubation at 20 °C with 2 M urea in 50 mM potassium phosphate buffer (pH 7.0), were loaded on an NAD^+ -agarose column equilibrated with the same buffer. The final concentrations of enzymes were approx. 2 mg/ml each. Elution flow rate was 0.25 ml/min. Broken lines represent the NaCl gradient.

As a final direct test for the formation of trimers or dimers, use was made of the presence of a reactive cysteine group close to the coenzyme binding site. It has been shown that reaction of residue Cys-320 with Ellman's reagent, DTNB, abolishes activity [35,36]. The fully modified enzyme is unable to bind coenzyme. An affinity column of NAD^+ -agarose is able, therefore, to separate modified enzyme from unmodified enzyme. Comparison studies of unmodified GDH and DTNB-modified enzyme have shown no significant differences in the pattern of denaturation by urea (results not shown). The experiment shown in Figure 7 is based on the assumption that, if dissociation to dimers or trimers occurs to any extent in 2 M urea, a mixture of unmodified and DTNB-modified enzyme should generate hybrids with a corresponding alteration in the chromatographic properties. No detectable hybridization occurs in either the presence (Figure 7c) or absence (results not shown) of 2 M urea.

In the case of immobilized bovine liver GDH [26] it was found that certain specific ligands (coenzymes and substrates) promoted urea-induced denaturation. Moreover it was found that in the presence of only NADH, urea caused dissociation of enzyme hexamers into inactive dimers, whereas the simultaneous presence of NADH and 2-oxoglutarate led to the dissociation of hexamers into active trimers (although the dissociated trimers were less stable to urea-induced denaturation than hexamers). In this context the effect of specific ligands on the urea-induced inactivation of clostridial GDH was studied (Table 1). To exclude the stabilizing effect of phosphate buffer on enzyme activity these experiments were carried out in 0.1 M Tris/HCl buffer, pH 7.0, containing 1 mM EDTA. After 60 min incubation of enzyme at 20 °C in the presence of different concentrations of urea and the concentrations of ligands indicated in Table 1, 10 μ l aliquots

Table 1 Effect of ligands on inactivation of clostridial GDH induced by urea

Enzyme (0.05 mg/ml) was incubated at 20 °C in 0.1 M Tris/HCl buffer, pH 7.0, in the presence of various concentrations of urea. In each case the incubation mixture contained appropriate ligands at the final concentrations indicated in the Table. Aliquots (10 µl) were withdrawn after 60 min incubation and diluted 100-fold with assay solution (reverse reaction in 0.1 M Tris/HCl buffer, pH 7.0, without urea). The urea concentration causing 50% inactivation ($[C]_{0.5}$) in 1 h was determined from the plot of residual activities against urea concentration.

Ligand(s) added		Concentration of ligand(s) (mM)		$[C]_{0.5}$ (M)
1	2	1	2	
No ligands		—		2.4
NaCl		50		2.7
L-Alanine		50		2.7
L-Glutamate		12.5		2.7
Succinate		12.5		3.4
		25		3.7
		50		4.0
		92		4.4
2-Oxoglutarate		12.5		3.3
		25		3.6
		50		3.9
		92		4.3
NAD ⁺		0.625		2.65
NADH		0.25		2.85
		0.5		3.2
		2.5		3.45
		5		3.5
NAD ⁺	2-Oxoglutarate	0.625	12.5	3.5
NADH	L-Glutamate	0.25	12.5	3.2
NADH	Succinate	0.25	12.5	3.7
		2.5	50	4.65
		2.5	75	4.8
NADH	2-Oxoglutarate	0.25	12.5	4.5
		2.5	50	5.25
		2.5	75	5.4

were diluted into 1 ml of assay solution containing 0.1 mM NADH, 10 mM 2-oxoglutarate and 50 mM NH_4Cl in the same buffer. For each ligand residual activity was plotted against urea concentration. These plots showed no significant differences in the shape of curves, but the concentrations of urea resulting in 50% inactivation ($[C]_{0.5}$) varied according to the ligands added. All the ligands investigated stabilized the native enzyme, although this effect varied with the type and the concentration of ligand. NaCl, L-alanine, L-glutamate, NAD⁺ and NADH individually gave moderate stabilization, whereas the effects of succinate or 2-oxoglutarate were much stronger. Stabilization was increased by the formation of ternary complexes, and the complex most resistant to urea-induced inactivation was the productive ternary complex GDH-NADH-2-oxoglutarate ($[C]_{0.5} = 5.4$ M). These results are thus quite different from those found with immobilized bovine GDH.

To find out whether the stabilizing effect of specific ligands on GDH activity (Table 1) was the result of preservation of the hexameric structure or possibly of smaller fragments, the samples after 60 min incubation with 4 M urea were loaded (without dilution) on to a native gradient polyacrylamide gel (Figure 6). There were no bands corresponding to native trimer or dimer. The individual ligands (NAD⁺, NADH or 2-oxoglutarate) partially stabilized the native hexamer structure of the enzyme, while their combined presence almost completely prevented enzyme dissociation.

Overall, the results reported here lead us to the conclusion that, in the presence of sub-denaturing concentrations of urea, the hexamer of clostridial GDH does not dissociate into smaller oligomers but retains its native hexamer structure. On the time-scale of these experiments dissociation to the monomeric state appears to be simultaneous with unfolding and inactivation. If there is a folded monomeric state it must be a transient intermediate.

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