Re-activation of *Clostridium symbiosum* glutamate dehydrogenase from subunits denatured by urea

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In a study of the re-activation of urea-denatured clostridial glutamate dehydrogenase (GDH) the maximum re-activation achieved without any added ligands was about 6%, but with NAD⁺ and 2-oxoglutarate in combination about 70%. NAD⁺ alone was also effective but 2-oxoglutarate was not, in striking contrast with the opposite pattern for protection of this enzyme against unfolding in urea [Aghajanian, Martin and Engel (1995) *Biochem. J.* **311**, 905–910]. The extent of re-activation was not increased by raising the incubation temperature to 37 °C and was independent of the time of enzyme denaturation. CD and fluorimetric studies showed that dilution of denatured enzyme into potassium phosphate buffer led to rapid (half-time < 3–5 s) formation of ‘structured’ intermediates with secondary structure similar to that of native enzyme. These intermediate molecules were inactive, behaved as monomers on a size-exclusion column,

and were unable to associate to give the native hexameric structure. Addition of NAD⁺ facilitated isomerization of these ‘structured’ monomers into a form(s) capable of re-activation. A side effect in the refolding process was non-specific aggregation, depending on final enzyme concentration. The hexamer fraction from re-activated samples, however, showed the same specific activity as native enzyme. The portion of the enzyme that is not lost through aggregation thus appears to regain the native structure fully. Detailed time-course studies showed that re-activation follows second-order kinetics, suggesting that formation of a dimer may be the rate-limiting step. The possible mechanism for the unfolding and refolding processes of clostridial GDH and effects of coenzyme and substrate on these are discussed in relation to the known crystal structure.

INTRODUCTION

Glutamate dehydrogenases (GDHs) (EC 1.4.1.2–4) catalyse the reversible oxidative deamination of L-glutamate with NAD(P)⁺ as a coenzyme. Most GDHs are hexamers composed of identical subunits arranged as a dimer of trimers [1–12]. A number of studies of the unfolding of both soluble and immobilized GDHs in the presence of denaturing agents such as guanidinium chloride (GdmCl) and urea have been made [13–26]. For the clostridial enzyme, for which a high-resolution structure is available [10], it was concluded that, at concentrations of GdmHCl insufficient to cause denaturation, the hexamer dissociates into inactive trimers without any gross structural changes that could be detected by fluorescence or CD [20]. This process could be reversed by dilution of denaturant, and full activity was restored. Increasing the GdmCl concentration led to further dissociation into monomers and was accompanied by large structural changes in secondary structure, and the loss of activity could not be reversed by removing the denaturant. In contrast, size-exclusion chromatography and hybridization studies of clostridial enzyme in the presence of sub-denaturing concentrations of urea [26] gave no evidence for intermediate dissociation of hexamers into smaller oligomeric fragments. Treatment of enzyme with higher concentrations of urea led to the direct dissociation of hexamers into denatured monomers. We now report that unfolded clostridial GDH under certain conditions is able to refold to form active enzyme. This has previously been difficult to achieve for hexameric GDHs [5,14,15,17,18,20,24] and, although briefly reported elsewhere [25], is now documented in detail.

MATERIALS AND METHODS

Clostridial GDH was purified from *Escherichia coli* TG1 pGS516 cell paste [27] by a single-step affinity-chromatographic procedure

[28] modified as described elsewhere [26]. The enzyme, stored in 60%–satd. (NH₄)₂SO₄ at 4 °C, was desalted on a Sephadex G-25 (fine) column before use.

The concentration of clostridial GDH was determined spectrophotometrically at 280 nm by using an absorption coefficient of 1.05 litre·g^{−1}·cm^{−1} [27].

Grade-II NAD⁺ (free acid), grade-II NADH (disodium salt) and 2-oxoglutarate (disodium salt) were obtained from Boehringer-Mannheim (U.K.). L-Glutamate (monosodium salt) and Tris were purchased from Sigma Chemical Co., and Sepharose CL-6B, Sephadex G-25 (fine) and FPLC prepacked column Superose 6 HR 10/30 from Pharmacia Biotech Ltd. All other chemicals were analytical reagent grade and supplied by BDH Chemicals Ltd. (U.K.) or Fisons Scientific Ltd. (U.K.).

GDH activity was measured spectrophotometrically at 25 °C (Uvikon 941 Plus; Kontron Instruments) by recording the change in A₃₄₀ due to the production or removal of 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, pH 7.0.

Denaturation of clostridial GDH by 4 M urea was carried out at 20 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. After 60 min incubation with urea, re-activation was attempted: aliquots were withdrawn, diluted to various extents (20–100-fold) in the same buffer containing different combinations of ligands and incubated for various times at either room temperature or 37 °C before assay as above. Clostridial GDH has two cysteine residues per subunit [27], raising the potential problem of inter- or intra-subunit disulphide bridges arising through oxidation. However, a number of experiments were performed with and without the inclusion of 1 mM 2-

Abbreviations used: GDH, glutamate dehydrogenase; GdmCl, guanidinium chloride.

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mercaptoethanol and no significant difference in the extent of re-activation was detected. 2-Mercaptoethanol was not routinely used therefore.

Specific activity of certain refolded samples was determined as follows: 100 μ l enzyme samples (1.5 mg/ml) after 60 min incubation with 4 M urea were diluted 50- and 25-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ . After 24 h incubation at room temperature, samples were dialysed against 0.1 M potassium phosphate buffer, pH 7.0, with four changes over 2 days and subsequent filtration through 0.2 μ m Acrodisc syringe filters (Gelman Ltd) to remove undissolved denatured protein. Enzyme concentration and activity were determined as above.

CD spectra of samples were recorded in 0.1 M potassium phosphate buffer, pH 7.0, at 20 °C using a Jasco J600 spectropolarimeter. Cell path length was 1.0 mm.

Fluorescence emission spectra of the enzyme ($\lambda_{\text{ex}} = 290$ nm) were recorded at 20 °C using a Hitachi F-4500 fluorescence spectrophotometer.

Size-exclusion chromatography of denatured and re-activated samples of GDH was carried out on a Pharmacia FPLC system with a Superose 6 HR 10/30 prepacked column. This was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl, and was calibrated with gel-filtration molecular-mass marker proteins: thyroglobulin (669 kDa), apo-ferritin (443 kDa), β -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa) from Sigma Chemical Co.; ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa) and hen egg albumin (45 kDa) from Boehringer-Mannheim (U.K.). Samples of volume 100 μ l (1 mg/ml each) were loaded separately. The flow rate was 0.5 ml/min.

Native PAGE was carried out in general as described by Garfin [29]. Gels were stained with Coomassie Brilliant Blue R-250.

RESULTS

Initial demonstration of re-activation

As a rule, measurement of GDH activity took no more than 1–2 min. Measured in this way, with initiation of reaction by the enzyme, the activity of clostridial GDH determined after 60 min incubation with 4 M urea was less than 0.2% of that of native enzyme. However, when such an assay reaction was monitored over 10 min, a progressive regain of activity was observed (Figure 1). The rate of this re-activation increased remarkably with increasing final protein concentration in the assay solution. Over a longer period of assay, the measured rates for samples with the higher final protein concentrations declined as expected with accumulation of reaction products (Figure 1B). The above result suggests that unfolded clostridial GDH is able to refold into the active form under certain conditions and that the re-activation is a protein-concentration-dependent process.

Dependence of re-activation on incubation conditions and on addition of specific ligands

Aliquots (50 μ l) of clostridial GDH (1.3 mg/ml) after 60 min incubation with 4 M urea (residual activity < 0.2%) were diluted 50-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing different combinations of specific ligands. The activities were monitored over 3 days of incubation at either room temperature or 37 °C. For most samples relatively little further

Figure 1 Re-activation of denatured clostridial GDH in assay solution

(A) Time courses for changes in A_{340} are shown for the samples with final protein concentrations 30 (1), 22.5 (2), 15 (3) and 7.5 μ g/ml (4) in assay solution. (B) First derivatives of curves presented in (A). Samples of clostridial GDH (1.5 mg/ml) denatured with 4 M urea were diluted into 1 ml of assay solution containing 1 mM NAD^+ and 40 mM L-glutamate in 0.1 M potassium phosphate buffer, pH 7.0. The final urea concentration in the assay solution was kept constant at 0.08 M. The temperature was 25 °C.

re-activation occurred after 24–30 h. The results obtained after 24 h incubation at room temperature are given in Table 1. As a reference native enzyme was diluted and incubated under the same conditions. It is important to note that native GDH was quite stable at room temperature in the presence or absence of most ligands (no more than 5–10% activity loss). The only exception was incubation with NADH which caused approx. 15–20% loss of activity at room temperature and more than 60% at 37 °C (results not shown). The addition of NaCl only partially overcame the destabilizing influence of NADH. However, enzyme in the presence of L-glutamate and NADH showed similar stability to the other mixtures without NADH.

In the re-activation experiments, there was only slight recovery of activity (3–6%) in the absence of ligands or with addition of 2-oxoglutarate alone (Table 1). Re-activation was relatively higher in the presence of L-glutamate (20%) or NADH (30%) or both in combination (36%). Maximum re-activation was found in the presence of the oxidized coenzyme NAD^+ , either alone (63%) or with NaCl (65%) or 2-oxoglutarate (68%) at room temperature. Increasing the temperature to 37 °C did not enhance the re-activation of denatured GDH, but rather destabilized the native enzyme (results not shown).

In the case of added NAD^+ , the same final activity (to within 5%) was achieved after 24 h incubation regardless of whether

Table 1 Activities of urea-denatured samples of clostridial GDH after 24 h incubation in the presence of specific ligands

Samples (50 μ l) of clostridial GDH (1.3 mg/ml) denatured with 4 M urea were diluted 50-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing specific ligands at the concentrations indicated. The activities of samples after 24 h incubation at room temperature were assayed in the forward reaction and are expressed as a percentage of the activity of native enzyme.

Added ligands	Activity (%)
1. No ligands	5.6
2. NAD ⁺ (2 mM)	63
3. NADH (2 mM)	30
4. 2-Oxoglutarate (50 mM)	3.3
5. L-Glutamate (50 mM)	20
6. NAD ⁺ (2 mM) + NaCl (50 mM)	65
7. NAD ⁺ (2 mM) + 2-oxoglutarate (50 mM)	68
8. NADH (2 mM) + NaCl (50 mM)	39
9. NADH (2 mM) + L-glutamate (50 mM)	36

2 mM NAD⁺ was present in the initial dilution mixture or added subsequently after 15 or 60 min.

An important issue in assessing these results and those that follow is the reproducibility between experiments. It was found that variations in the detailed handling procedure at the dilution stage could result in considerable variation in the re-activation achieved, perhaps owing to aggregation in the early stages of refolding. With precise replication of procedure, results of duplicate dilutions usually gave results within about $\pm 5\%$.

Spectral characteristics of the refolding process

GDH (1.5 mg/ml) denatured as above was diluted 50-fold in 0.1 M potassium phosphate buffer, pH 7.0, and the fluorescence and CD spectra of the sample were measured. As a reference the same spectra of enzyme in native (N) and denatured (D) states were monitored. The results are summarized in Figure 2. As described elsewhere [26] the inactivation of GDH in the presence of 4 M urea follows first-order kinetics, with the same rate

constant for inactivation and for loss of the native CD signal, implying that the loss of the two properties is essentially the result of a single event. According to the data of Figure 2(A), denaturation of the enzyme is also accompanied by a decrease in the fluorescence signal and a shift of the peak in the protein emission spectrum (emission peaks are at 340 and 358 nm for the native and denatured states of enzyme respectively, excitation at 290 nm). Dilution of the denatured enzyme into the buffer containing no additives results in recovery of enzyme secondary structure with regain of most of the native CD signal (approx. 85 % of the ellipticity at 220 nm) and fluorescence (approx. 75 % of intensity at 340 nm and shift of emission peak back to 342 nm). However, according to the data of Table 1, very little re-activation accompanies the recovery of native spectrophotometric characteristics even after 24 h incubation of enzyme without additives. The mismatch between the low extent of re-activation and the regain of other properties implies the formation of inactive 'structured' intermediate(s). To follow this process, samples of denatured GDH were diluted directly into quartz cuvettes and ellipticity of enzyme at 220 nm and protein emission at 340 nm (excitation at 290 nm) were monitored. Detection was started no more than 3–5 s after dilution of samples. The results (not shown) indicate that the regain of enzyme secondary structure is a fast process, with a half-life less than 5 s, and goes to completion in less than 1.5 min. Detection of the enzyme refolding in the presence of NAD⁺ by these methods was limited owing to the very high absorbancy of solutions.

Kinetic measurements

Figure 3 shows kinetics of re-activation of GDH (1.5 mg/ml) denatured as described above and diluted 50-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD⁺. The continuous line is calculated by assuming a second-order reaction with a rate constant of $1.87 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a final re-activation level of 50 % (the value that was detected after 3 days of re-activation). This assumption clearly gives a good fit to the data over most of the time course. A similar attempt based on the assumption of first-order re-activation gave a poor fit and, although the fit for a third-order process was reasonable, it was

Figure 2 Spectral characteristics of native and urea-denatured samples of clostridial GDH

(A) Protein fluorescence, excitation wavelength 290 nm; (B) far-UV CD. Samples of clostridial GDH (1.5 mg/ml) denatured over 60 min in 4 M urea were diluted 50-fold into 0.1 M potassium phosphate buffer, pH 7.0 (curve S) also containing 4 M urea (curve D). Native enzyme at the same concentration was diluted into the buffer without urea (curve N). All spectra were measured at 20 °C at least 5 min after dilution of samples. The emission intensity of native enzyme at 340 nm in the fluorescence spectra (A) was taken as 100%.

Figure 3 Time course of re-activation of clostridial GDH denatured with 4 M urea

Clostridial GDH (1.5 mg/ml) after 60 min incubation with 4 M urea was diluted 50-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ . The final urea concentration was 0.08 M. Aliquots (10–20 μl) were withdrawn after the indicated times of incubation and activities were measured. The continuous line is calculated by assuming a second-order reaction with rate constant of $1.87 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a final re-activation level of 50%. Inset: the data for the first 40 min of re-activation on an expanded time scale.

Figure 4 Effect of residual urea concentration on time course of re-activation of denatured clostridial GDH by NAD^+

Clostridial GDH (1.4 mg/ml) denatured in 4 M urea was diluted 100-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ and also urea (added if necessary) with the final concentrations of 0.04 (curve 1), 0.08 (curve 2), 0.16 (curve 3) and 0.4 (curve 4) M. Aliquots (10–20 μl) were withdrawn after the indicated times of incubation and activities were measured. The continuous lines are calculated by assuming a second-order reaction with rate constants of 4.31×10^3 (curve 1), 1.45×10^3 (curve 2), 5.02×10^2 (curve 3) and 3.85×10^2 (curve 4) $\text{M}^{-1} \cdot \text{s}^{-1}$ and final levels of re-activation of 47% (curves 1, 2 and 3) and 17% (curve 4).

evidently inferior to that shown in Figure 3. The inset of the graph, however, shows the data for the first 40 min of re-activation on an expanded time scale; it is clear that enzyme re-activation is delayed over the first 2–3 min of incubation. This could be due to recovery of the secondary structure of denatured GDH, followed by isomerization of ‘structured’ intermediate(s) by NAD^+ present in the solution. However, the further re-

activation (approx. 5 min after initiation) can be described as a second-order process with a half-life of 29.7 min.

Effect of residual urea concentration on re-activation

To find out the effect of residual urea concentration on the process of enzyme re-activation, GDH (1.4 mg/ml) denatured as above (residual activity 0.2%) was diluted 100-fold in 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ and also urea (added if necessary) to give final concentrations of 0.04, 0.08, 0.16 and 0.4 M. The time courses of re-activation are shown in Figure 4. The continuous lines in the Figure are calculated by assuming a second-order reaction with rate constants of 4.31×10^3 , 1.45×10^3 , 5.02×10^2 and $3.85 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively for 0.04, 0.08, 0.16 and 0.4 M urea concentrations. The final levels of re-activation were 17% for 0.4 M and 47% for other urea concentrations. The results show that, in spite of the presence of residual urea, the re-activation processes follow second-order kinetics. Even though an increase in the residual urea concentration depresses the rate of re-activation, 40–45% re-activation could be achieved even in the presence of 0.16 M urea over 30–40 h incubation. With lower concentrations of urea (0.04 M), the re-activation process is practically complete after 7 h.

Effect of protein concentration on re-activation

As mentioned above, the rate of re-activation of urea-denatured GDH in assay solution depends on protein concentration (Figure 1). The results of enzyme re-activation in the presence of 2 mM NAD^+ for samples with the same residual urea concentration (0.08 M) and different final enzyme concentrations (30 $\mu\text{g}/\text{ml}$ in Figure 3 and 14 $\mu\text{g}/\text{ml}$ in Figure 4 curve 2) showed that re-activation is much faster for the sample with the higher protein concentration, although the final re-activation levels differed slightly (50 and 47% respectively). The half-lives of the re-activation processes were 29.7 and 87.3 min respectively for the samples with 30 and 14 $\mu\text{g}/\text{ml}$ protein concentrations. The increase in rate of re-activation with increasing enzyme concentration suggests that association is a rate-determining step in the formation of active enzyme, consistent with a second-order process. Strictly speaking, if the only process occurring in the re-activation mixture is re-activation limited by a second-order association process, both re-activations should yield the same second-order rate constant. Failure to achieve perfect agreement ($1.87 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 30 $\mu\text{g}/\text{ml}$ and $1.45 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 14 $\mu\text{g}/\text{ml}$) implies that there may be competing processes, as also suggested by the incomplete extents of re-activation in the two cases.

To find the protein concentrations giving maximal re-activation, different starting protein concentrations (0.25–5.6 mg/ml) were used. After 60 min incubation with 4 M urea, samples were diluted 50- and 25-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ . As a reference, native GDH at the same concentrations but untreated with urea was diluted similarly. The results (Table 2) show that there is an optimal range of enzyme concentration over which re-activation is maximal. More than 60% re-activation was achieved with starting concentrations of 1 and 1.5 mg/ml protein and both 50- and 25-fold dilutions. Also comparatively high re-activation was obtained with 0.5 and 2 mg/ml. Outside of this concentration range the extent of re-activation is considerably lower. In the case of the lowest protein concentrations, 25-fold dilution gave a higher re-activation level than 50-fold dilution. This could be a result of competition in dilute solution between productive association of refolded enzyme subunits and other

Table 2 Re-activation of samples of clostridial GDH denatured by urea at different protein concentrations

Samples of enzyme (50 μ l) with indicated protein concentrations after 60 min incubation with 4 M urea (residual activities < 0.2%) were diluted 25- and 50-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD⁺. As a control, identical samples of denatured enzyme and also native GDH were diluted 50-fold into the same buffer without NAD⁺. Activities of re-activated samples, after 20 and 120 h incubation, are given as a percentage of that of native enzyme.

Enzyme concentration (mg/ml)	Incubation time (h)	Activity of urea-treated GDH (%)		
		Diluted 50-fold in buffer	Diluted 50-fold in NAD ⁺	Diluted 25-fold in NAD ⁺
0.25	20	0.1	24	36
	120	0.4	29	41
0.5	20	0.3	48	53
	120	2.0	54	59
1.0	20	3.8	54	61
	120	11	61	63
1.5	20	6.7	61	63
	120	19	71	72
2.0	20	7.2	50	50
	120	19	56	53
3.0	20	5.5	39	34
	120	15	43	41
5.6	20	5.0	19	20
	120	12	28	26

possibly first-order processes. In contrast, at high enzyme concentrations non-specific aggregation of denatured molecules could be the reason for the low level of re-activation. Even for identical final concentrations, the highest yield of active enzyme is achieved with the procedure involving the lowest intermediate protein concentration (in 4 M urea).

It is important also to note that, in the case of high protein concentrations (≥ 1.5 mg/ml), dilution of urea-treated samples into the buffer without NAD⁺ gave a remarkably high re-activation (15–20%; Table 2). However, this process is much slower (over 5 days) than in the presence of NAD⁺. In the case of 0.5 or 0.25 mg/ml protein concentrations, there is practically no re-activation in the absence of NAD⁺.

Effect of initial extent of denaturation on achievable re-activation

In all experiments described thus far, re-activation of GDH was carried out after 60 min incubation of enzyme with 4 M urea, conditions sufficient to cause almost complete inactivation [26]. To find out whether the potential re-activation depends on the extent of denaturation, the re-activation was started after different times of enzyme incubation with 4 M urea. Aliquots of enzyme (50 μ l; approx. 0.2 mg/ml) were withdrawn after 10, 15, 20, 30 and 60 min incubation with 4 M urea and diluted 20-fold in 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD⁺. Residual activities of samples after the indicated times of incubation of enzyme with urea and activities recovered after 1 and 2 days incubation of samples in the presence of NAD⁺ are given in Table 3. The results show that re-activation of GDH from the denatured state does not depend on the time of incubation with urea (e.g. samples 3, 4 and 5). The final activity of samples with substantial residual activity before re-activation (samples 1 and 2) appears to be equal to the sum of activities remaining (after urea treatment) and recovered (from the denatured state).

Table 3 Re-activation of clostridial GDH with different levels of denaturation

Aliquots of enzyme (50 μ l; approx. 0.2 mg/ml) after the indicated times of incubation with 4 M urea were diluted 20-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD⁺. Activities of samples were determined after 24 and 48 h incubation and are given as a percentage of original activity of the native enzyme.

Sample	Time of incubation with 4 M urea (min)	Residual activity of samples (%)	Recovered activity of samples after incubation with NAD ⁺ (%)	
			24 h	48 h
0	0	100	100	98
1	10	15	41	44
2	15	4.4	28	34
3	20	2.0	26	30
4	30	0.8	25	28
5	60	< 0.2	25	29

Figure 5 Elution profiles of native (1), denatured (2) and refolded (3) samples of GDH on a Superose 6 column

Samples of enzyme (1.5 mg/ml) after 60 min incubation with 4 M urea (residual activity was approx. 0.2% of that of native enzyme) were diluted 25-fold into 0.1 M potassium phosphate buffer, pH 7.0, without any additives (2) and with 2 mM NAD⁺ (3). After 20–24 h incubation at room temperature, 200 μ l samples were loaded on a prepacked FPLC column of Superose 6 HR 10/30, equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing also 0.15 M NaCl. As a reference, native enzyme (1) at a concentration of 0.06 mg/ml was run in the same conditions. Elution flow rate was 0.5 ml/min.

Characterization of the refolded enzyme

To obtain information about the structure of refolded samples of GDH, size-exclusion chromatography and native PAGE were carried out. The samples of GDH (1.5 mg/ml) after 60 min incubation with 4 M urea in 0.1 M potassium phosphate buffer, pH 7.0 (residual activity 0.2%) were diluted 25-fold into 0.1 M potassium phosphate buffer, pH 7.0, either (a) without any additives or (b) with 2 mM NAD⁺. Elution profiles of native GDH and urea-treated samples of enzyme after 20–24 h incubation in the presence or absence of NAD⁺ are given in Figure 5. Dilution of urea-treated enzyme into buffer without NAD⁺, followed by 24 h incubation (curve 2), gave a main peak in the monomer (partially unfolded) position, with a very small amount of material in the native hexamer position (consistent with the low extent of re-activation; see Table 2) and somewhat more running in the position expected for high-molecular-mass aggre-

Figure 6 Elution profiles of re-activated samples of clostridial GDH with different starting protein concentrations on a Superose 6 column

Samples of GDH with different starting protein concentrations (1.5–5.6 mg/ml) after 60 min incubation with 4 M urea were diluted 25-fold into 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM NAD^+ , and incubated over 5 days at room temperature. Samples (200 μl) were loaded on a prepacked FPLC column of Superose 6 HR 10/30, equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl. For comparison, native clostridial GDH (1.5 mg/ml) was diluted 25-fold into the same buffer without NAD^+ before loading on to the column. Elution profiles are as follows: native GDH (A) and re-activated samples with starting protein concentrations 1.5 mg/ml (B), 2.0 mg/ml (C), 3.0 mg/ml (D) and 5.6 mg/ml (E). Elution flow rate was 0.5 ml/min. Each division on the y-axis represents 0.01 absorbance unit.

gates. The second sample, incubated in the presence of NAD^+ , gave no monomer peak and showed instead a peak in the native hexamer position (curve 3). The material eluted at about 20 ml in curve 3 is NAD^+ . These results suggest that incubation with NAD^+ leads to the refolding of GDH monomers into the native hexameric structure. This conclusion was confirmed by native PAGE experiments (not shown) in which refolded samples gave a sharp band in the characteristic position for native hexameric clostridial GDH.

The effect of protein concentration on re-activation (Table 2) was also investigated by size-exclusion chromatography on a pre-packed FPLC column of Superose 6 HR 10/30 equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl. Samples (200 μl) with different protein concentrations, re-activated by 25-fold dilution of denatured enzyme into 0.1 M potassium phosphate buffer containing 2 mM NAD^+ , and followed by incubation at room temperature over 5 days (recovered activities are given in Table 2), were ultimately loaded on the FPLC column. The elution profiles (Figure 6) show that, in the case of the 1.5 mg/ml sample, for which re-activation was maximal (72%), there was only a small amount of high-molecular-mass material. Increasing the protein concentration resulted in an increase in the size and amount of such material,

again confirming that aggregation is the major reason for the low level of re-activation in concentrated protein solutions.

Fractions of refolded samples eluted at the position expected for native enzyme were collected, and specific activities were measured. Although re-activation levels differed widely among the refolded samples from which they were derived, the specific activities of these native fractions varied insignificantly (24.8 ± 0.5 units/mg of protein).

The specific activities of samples re-activated in the presence of NAD^+ were determined also after extensive dialysis and subsequent removal of insoluble denatured enzyme as described in the Materials and methods section and were almost the same (24.5 ± 0.2 units/mg of protein) as that of native GDH not treated with urea (25.0 units/mg of protein).

DISCUSSION

Overall these results show that clostridial GDH, denatured by urea, is able to refold spontaneously into the native hexameric structure with regain of the original catalytic activity. This is the first hexameric GDH successfully refolded *in vitro* [25]. In spite of the persistent difficulty in achieving refolding of GDHs from vertebrate sources [5,14,15,17,18,20], there is clearly no intrinsic problem about refolding such large enzyme molecules. This is confirmed by recent reports of the refolding of two other bacterial GDHs, from the thermophiles *Sulfolobus solfataricus* [30] and *Bacillus acidocaldarius* [31]. The failure to refold GDHs from animal sources may relate to post-translational cleavage of their leader sequences and contrasts with the success achieved in reversible denaturation of other soluble oligomeric dehydrogenases, including, for example, lactate dehydrogenase from pig heart and skeletal muscle [32–34], mitochondrial and cytoplasmic malate dehydrogenases from pig heart [35,36], alcohol dehydrogenase from horse liver [37] and uridine diphosphoglucose dehydrogenase from bovine liver [38].

Under the experimental conditions employed for denaturation, the clostridial GDH apparently retains a low residual activity (less than 0.2%) after 1 h. More important, as previously shown [26], the enzyme also retains 15–20% of its native CD signal after 1 h even at a higher urea concentration (5.4 M). It has been argued [26] that this reflects a structural core, resistant to denaturation, and it is possible that the persistence of such a core is essential for the reversibility of unfolding.

According to the data from CD and fluorescence studies, the denatured enzyme regained its secondary structure, after dilution of denaturant, in a process with a half-life less than 5 s. However, the 'structured' intermediates thus formed remained as inactive monomers and are apparently unable to form the native hexameric structure without further assistance (Table 1 and Figure 5, curve 2). It is important to note that this conclusion applied for the samples with a final protein concentration of less than 0.05 mg/ml. At higher protein concentrations the 'structured' monomers showed a strong tendency to associate into non-specific aggregates (Figure 6) depending on the time of incubation (results not shown).

Of all the ligands investigated, the coenzymes NAD^+ and NADH produced the most striking re-activation effects on denatured enzyme (Table 1). Our recent investigations on the effects of coenzyme fragments and different analogues on the re-activation process have shown that ADP-ribose is the smallest fragment of NAD^+ able to promote re-activation of denatured enzyme [39].

Re-activation by NAD^+ or NADH has also been reported for other enzymes such as NAD^+ -specific GDH from *Neurospora crassa* [13], lactate dehydrogenase from pig heart [32],

glyceraldehyde-3-phosphate dehydrogenase from yeast [40] and UDP-galactose-4-epimerase from *Kluyveromyces fragilis* [41]. According to these reports, the coenzyme is involved in either a nucleation step in the process of refolding and/or reassociation [32,40] or an isomerization step of subunits already refolded and associated into dimers [41]. However, the fact that refolded monomers of clostridial GDH appear to be unable to associate into dimers or other correctly associated oligomers without assistance of coenzyme suggests that the effect of nucleotides on the refolding of clostridial GDH is an isomerization of 'structured' monomers into a form(s) capable of associating to yield active hexamers, and this would account for the deviation from strict second-order kinetics observed over the first 5 min of re-activation (Figure 3, inset). Since, however, the re-activation process follows second-order kinetics thereafter (Figure 3), it can be concluded that the formation of dimers is a rate-limiting step in re-activation. Further rapid association of dimers into active hexamers presumably then occurs either directly or perhaps more likely via formation of other oligomeric intermediates such as tetramer or trimer. However, the results presented here shed no light on this last question.

Thus the re-activation process of clostridial GDH from the denatured state can be summarized as:

where M_d is denatured monomer, M_s is 'structured' inactive monomer after dilution of enzyme into the buffer, M_s^* is 'structured' monomer after isomerization in the presence of NAD^+ , D is dimer and H is active hexamer.

In comparing the present study of refolding with our earlier experiments on unfolding [26], a striking contrast emerges in the effects of ligands on the two processes. As discussed above, NAD^+ is a potent promoter of refolding, whereas 2-oxoglutarate on its own does not promote folding. Precisely the opposite pattern is seen for unfolding [26], with 2-oxoglutarate shifting the urea concentration required for 50% inactivation from 2.4 M to 3.9 M, whereas NAD^+ alone achieves only a modest shift to 2.65 M. According to the crystal structure of GDH [42], substrate- (but not coenzyme) binding domains are involved in the intersubunit contacts that form the hexamer. Therefore NAD^+ may offer little protection at intermediate urea concentrations because on its own it cannot stabilize the quaternary structure. 2-Oxoglutarate, on the other hand, should do so, as is observed. Turning to the refolding process, binding of NAD^+ to the 'structured' intermediate presumably facilitates rearrangement to give correctly folded monomers. Dimerization of these is followed by further fast association into active hexamers. Taken together therefore the two sets of results lead to a model for folding and subunit association in clostridial GDH that should be amenable to further test.

Although the influence of various parameters on refolding of clostridial GDH has been explored in the present study, 72% recovery of activity is presumably not the ultimate limit. Other variables are involved (choice of buffers, methods and rate of removal of denaturant, etc.) and optimization of these may increase the achievable level of re-activation.

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