The unfolding and refolding of cytoplasmic aspartate aminotransferase from pig heart

Shauna M. WEST and Nicholas C. PRICE
School of Molecular and Biological Sciences, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

The unfolding of cytoplasmic aspartate aminotransferase from pig heart in solutions of guanidinium chloride (GdnHCl) was studied. Data from protein fluorescence, c.d. and thiol-group reactivity indicated that the enzyme was unfolded in 6 M-GdnHCl. Spectroscopic studies showed that this unfolding was accompanied by dissociation of the pyridoxal 5'-phosphate cofactor. On dilution of the GdnHCl, re-activation of the enzyme occurred in reasonable yield, provided that dithiothreitol and pyridoxal 5'-phosphate were present. The regain of activity obeyed second-order kinetics. In the absence of added dithiothreitol and pyridoxal 5'-phosphate, substantial formation of high-\(M_r\) aggregates occurred.

INTRODUCTION

Aspartate aminotransferase (\(L\)-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) occurs in two dimeric isoenzymes, one of which occurs in the mitochondrion and the other of which is confined to the cytosol. The two enzymes participate in the 'malate–aspartate' shuttle, which is involved in the transfer of reducing equivalents across the mitochondrial membrane (Newsholme & Start, 1973).

Amino acid sequences and X-ray-crystallographic structures have been derived for the isoenzyme from various sources, including pig heart and chicken heart (for a review see Christen & Metzler, 1985). These studies have shown that the various isoenzymes have a high degree of similarity. Thus, for instance, the amino acid sequences of the cytoplasmic and mitochondrial isoenzymes from pig heart are 48\% identical (Barra et al., 1980), and the tertiary structures of the cytoplasmic and mitochondrial isoenzymes from chicken heart are very similar (Borisov et al., 1985).

The mitochondrial isoenzyme is synthesized in the cytosol in a precursor form, and subsequently translocated across the mitochondrial membrane with proteolytic cleavage of the N-terminal targeting sequence. In the chicken enzyme this pre-sequence consists of 22 amino acids and follows the usual pattern in containing a number of positively charged, but not negatively charged, amino acids (Jaussi et al., 1985). Details of the folding and assembly processes involved in formation of the mature dimeric mitochondrial enzyme are obscure, though it has been shown that the enzyme is probably translocated in a monomeric form (O'Donovan et al., 1984), and that expression of the gene corresponding to the precursor form of the mitochondrial enzyme in Escherichia coli leads to the formation of aggregates of high \(M_r\), whereas expression of the gene corresponding to the mature form leads to the formation of active dimeric enzyme (Jaussi et al., 1987).

As part of our studies on the folding of translocated proteins (West & Price, 1988), we have studied the unfolding and refolding of the cytoplasmic isoenzyme of aspartate aminotransferase. Banks et al. (1968b), on the basis of sedimentation-velocity data, reported that the cytosolic enzyme from pig heart did not apparently unfold in 8 M-urea or 6 M-GdnHCl, since a dimeric structure was retained. On prolonged exposure to these agents, dissociation did occur. By contrast, Martinez-Carrion et al. (1970) and Bertland & Kaplan (1970) reported that the enzyme was unfolded in GdnHCl, as judged by loss of the near-u.v. c.d. spectrum and loss of activity respectively. The latter authors noted that partial regain of activity of the unfolded cytosolic apoenzyme from chicken muscle could be observed, provided that pyridoxal 5'-phosphate and 2-mercaptoethanol were present during refolding. The aim of the present report is to clarify some of the previous observations on the cytosolic isoenzyme and to provide a firm basis on which the unfolding and refolding behaviour of cytosolic and mitochondrial isoenzymes might be compared.

EXPERIMENTAL

The cytoplasmic isoenzyme of pig heart aspartate aminotransferase was initially purchased from Boehringer. However, as described in the Results section, a number of observations indicated that this preparation was unsatisfactory, and all subsequent work was performed on enzyme prepared by the procedure (Method 2) described by Banks et al. (1968a), but in which a final step of gel filtration on Sephacryl S-300 was included. The homogeneity of the preparation was assessed by SDS/polyacrylamide-gel electrophoresis on 12\%:polyacrylamide gels (Laemmli, 1970).

Concentrations of enzyme solution were determined spectrophotometrically at 280 nm by using a value of \(A_{280}^{\text{nm}}\) of 1.51 (Birchmeier et al., 1973).

Enzyme activity was assayed by a coupled assay procedure (Nisselbaum & Bodansky, 1966) with the following concentrations of substrates and coupling enzyme in 0.1 M-sodium phosphate buffer, pH 7.4, at 25°C: \(L\)-aspartate, 30 mm; 2-oxoglutarate, 5 mm; NADH, 0.09 mm; malate dehydrogenase, 5 \(\mu\)g/ml. In order to determine the kinetic parameters, concentrations of \(L\)-aspartate and 2-oxoglutarate were varied over the

Abbreviations used: GdnHCl, guanidinium chloride; \(Nbs_\text{p}\), 5,5'-dithiobis-(2-nitrobenzoic acid).
ranges 15 to 2.5 mM and 0.75 to 0.075 mM respectively. The data were then analysed by primary (double-reciprocal) plots and secondary plots in which the ordinate intercepts of the primary plots were plotted against the reciprocal of the concentration of the second substrate (Alberty, 1956; Price & Stevens, 1982).

Enzyme activity in the presence of GdnHCl was determined by a discontinuous assay procedure (Johnson & Price, 1987). A sample (0.05 ml) from a reaction mixture containing l-aspartate, 2-oxoglutarate and aspartate aminotransferase was taken at a known time after the start of the reaction and added to 0.95 ml of solution containing NADH and malate dehydrogenase (the concentrations of the substrates and coupling enzyme were the same as used for the continuous assay described above). The rapid decline in A490 on addition of the sample is due to oxaloacetate formed in the first (aspartate aminotransferase-catalysed) reaction. From the difference in these changes in A490 for two different sampling times, the rate of the first reaction can be determined. This discontinuous method ensures that the concentration of GdnHCl carried over to the malate dehydrogenase reaction is too small (<0.1 mM in the present experiments) to interfere with the activity of the coupling enzyme. Control experiments were performed to check that the rate of the aspartate aminotransferase-catalysed reaction was constant over the range of times studied and proportional to the amount of enzyme added.

Fluorescence studies were performed at 20 °C in a Perkin–Elmer MPF 3A fluorimeter. Spectra were recorded 15 min after addition of GdnHCl to samples; no further changes occurred after this time. The quenching of protein fluorescence by acrylamide and succinimide was performed as described by Eftink & Griron (1984), with the appropriate corrections described by these authors being made for dilution and 'inner filter' effects. Before use, acrylamide and succinimide were recrystallized from ethyl acetate and ethanol respectively. Analysis of the quenching data to determine the fraction of fluorophores accessible to the quenches was performed by a modified Stern–Volmer plot (Lehrer, 1971).

CD spectra were recorded at 25 °C in a Jobin–Yvon Dichrographe IV in cells of path length 0.1 mm (far u.v., 260–205 nm) or 10 mm (near u.v., 400–260 nm). The enzyme concentration in these experiments was 0.8 mg/ml. Spectra were recorded 15 min after the addition of GdnHCl.

GdnHCl (Aristar grade) was purchased from B.D.H. The concentrations of solutions of GdnHCl were checked by refractive-index measurements (Nozaki, 1972). Poly-(ethylene glycol) (M, approx. 10000) was purchased from Sigma. Enzyme samples were concentrated by dialysis against a 20% (w/v) solution of poly(ethylene glycol) in 0.1 M-sodium phosphate buffer, pH 7.4.

The refolding of aspartate aminotransferase was studied after the enzyme had been denatured by incubation for 15 min at 0 °C in GdnHCl dissolved in 0.1 M-sodium phosphate buffer, pH 7.4. Refolding was initiated by a 60-fold dilution into 0.1 M-sodium phosphate buffer, pH 7.4, at 20 °C.

RESULTS

Isolation of enzyme

Enzyme isolated by the method of Banks et al. (1968a), but including the additional gel-filtration step, had a specific activity of 154 μmol/min per mg under the assay conditions described in the Experimental section. This value can be compared with 120 μmol/min per mg for the commercial enzyme under these conditions and 138 μmol/min per mg reported by Banks et al. (1968a) under slightly different assay conditions.

The enzyme isolated in our laboratory was >95% homogeneous on SDS/polyacrylamide-gel electrophoresis as judged by staining with Coomassie Blue. The subunit Mr was estimated to be 45000 ± 2000, compared with the value of 46344 calculated from the amino acid sequence (Barra et al., 1980). The commercial enzyme showed significant (~10% of total staining) quantities of material with a subunit Mr 66000 and a number of minor bands in the Mr range 55000–60000, which were absent from the enzyme isolated in the laboratory.

The enzyme isolated in the laboratory possessed 2.0 thiol groups per subunit reactive towards Nbs2 (Habeeb, 1972) in the native enzyme, and 5.1 thiol groups per subunit in the presence of 6 M-GdnHCl or 0.1% SDS. These values are in good agreement with published data (Birchmeier et al., 1973; Arnone et al., 1977). By contrast, the commercial enzyme possessed <0.1 thiol group per subunit in the native enzyme and 2.9 thiol groups per subunit in the presence of 6 M-GdnHCl or 0.1% SDS; these values could not be increased significantly by prolonged (24 h) incubation with 1 mM-dithiothreitol followed by dialysis. It was therefore concluded that the commercial enzyme had undergone some irreversible
Unfolding and refolding of aspartate aminotransferase

Modification, and consequently enzyme isolated in our laboratory was used in all subsequent experiments reported in this paper.

Unfolding of enzyme in GdnHCl

Several measurements were made in order to assess the degree of unfolding of the enzyme caused by GdnHCl.

Enzyme activity. The enzyme was incubated in GdnHCl in 0.1 m-sodium phosphate buffer, pH 7.4, at 20 °C and then assayed in the presence of the same concentration of GdnHCl by using the discontinuous procedure described in the Experimental section. As shown in Fig. 1, there was a progressive loss of activity with increasing concentration of GdnHCl, with 50% activity lost at 0.7 M and <15% activity remaining at 2 M. These findings are consistent with the observation by Bertland & Kaplan (1970) that the cytoplasmic enzyme from chicken completely loses activity on incubation in 6 M-GdnHCl.

Fluorescence properties. When excited at 290 nm, aspartate aminotransferase exhibits a fluorescence emission maximum at 330 nm (Fig. 2a), characteristic of tryptophan side chains partially shielded from the aqueous solvent (Teipel & Koshland, 1971). On addition of GdnHCl, various changes occur in the fluorescence spectra. In the concentration range up to about 3 M, the fluorescence intensity decreases, with a shift in the emission maximum to 340 nm. As the concentration of GdnHCl is increased to 6 M, the intensity then increases and the emission maximum shifts to 350 nm (Fig. 2a), characteristic of tryptophan side chains exposed to the solvent (Teipel & Koshland, 1971). The changes in fluorescence at 350 nm are shown in Fig. 2b.

The increased exposure of tryptophan side chains on addition of GdnHCl is confirmed by measurement of the fraction accessible to quenching by succinimide. By the method of Lehrer (1971), this fraction (fₐ) was determined to be 0.52 for the native enzyme. As the concentration of GdnHCl was increased, the value of fₐ rose towards 1.0.

---

Fig. 2. Fluorescence properties of aspartate aminotransferase

Enzyme was incubated in 0.1 m-sodium phosphate buffer, pH 7.4, in the absence or presence of GdnHCl, at 20 °C. The excitation wavelength was 290 nm. (a) Emission spectra of enzyme (20 μg/ml). The continuous curves represent enzyme incubated in the absence of GdnHCl (i), and in the presence of 3 M- (ii) and 6 M- (iii) GdnHCl. The dashed curve represents enzyme refolded at a concentration of 5.4 μg/ml for 24 h in the presence of 0.1 mM-pyridoxal 5'-phosphate and 1 mM-dithiothreitol after unfolding in 6 M-GdnHCl. The refolded enzyme (which had regained 68% activity) was then subjected to a concentration and dialysis procedure as described in the text; the fluorescence spectrum was recorded at a concentration of 20 μg/ml. (b) The intensity of fluorescence at 350 nm at different [GdnHCl]. The enzyme concentration was 20 μg/ml. (c) Fraction of fluorophores accessible to quenching by succinimide. The enzyme concentration was 40 μg/ml, and emission was measured at 325 nm. Data were analysed by the method of Lehrer (1971).
Fig. 3. C.d. spectra of aspartate aminotransferase
Spectra of enzyme (0.8 mg/ml) were recorded in the absence of GdnHCl (---) and in the presence of 2 M- (- - - -), 4 M- (-----) and 6 M- (-----) GdnHCl. (a) Far-u.v. spectra; (b) near-u.v. spectra. The near-u.v. c.d. spectrum of the enzyme in the presence of 6 M-GdnHCl showed no signal.

Fig. 4. The reactive thiol groups of aspartate aminotransferase
Enzyme (0.2 mg/ml) was incubated in 0.1 M-sodium phosphate, pH 7.4, at 20 °C. The increase in \( A_{412} \) on reaction with Nbs₂ (250 \( \mu \)M) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

(Fig. 2c). Measurements were also made of \( f_a \) for acrylamide, which is known to be a very efficient quencher of tryptophan fluorescence (Eftink & Ghiron, 1984). In the native enzyme, \( f_a \) for acrylamide was found to be 1.0, and the value did not change appreciably from this value as the concentration of GdnHCl was increased (results not shown).

C.d. The far-u.v. c.d. spectrum of aspartate aminotransferase is shown in Fig. 3(a). The spectrum is similar to that reported by Martinez-Carrion et al. (1970). By using the reference values of \( \theta_{228} \) (Chen et al., 1974; Chang et al., 1978), the a-helix content can be calculated to be 49%. This agrees well with the value of 48% determined by X-ray crystallography of both cytoplasmic and mitochondrial forms of the chicken enzyme (Borisov et al., 1980, 1985).

On addition of 2 M-GdnHCl, there is relatively little change in the c.d. spectrum; larger changes are observed in 4 M-GdnHCl. In 6 M-GdnHCl there is apparently a complete loss of secondary structure, with \( \theta_{228} \) having a value \( \leq 3 \% \) of that of the native enzyme (Fig. 3a).

The near-u.v. spectrum for the native enzyme shows weak bands at 280 nm (positive), 300 nm (negative) and 365 nm (positive). The first two bands arise from tyrosine and tryptophan side chains (Adler et al., 1973), whereas the third is due to the pyridoxal 5'-phosphate cofactor. Addition of GdnHCl causes substantial changes in the spectra (Fig. 3b); the changes in the near u.v. (corresponding to tyrosine and tryptophan) occur at lower concentrations of GdnHCl than the changes in the far u.v. This is consistent with a general observation that the tertiary structure of proteins is more readily disrupted by GdnHCl than is the secondary structure (Creighton, 1978; Tsou, 1986). The decrease in the value of \( \theta_{228} \) at high concentration of GdnHCl is associated with the dissociation of the cofactor (see below).

Reactive thiol groups. The native enzyme was found to possess 2.0 thiol groups per subunit which reacted rapidly
Unfolding and refolding of aspartate aminotransferase

The unfolding and refolding of enzyme were performed as described in the text; the enzyme concentration during refolding was 2.3 µg/ml. The activity was assayed after 24 h refolding and expressed relative to a control sample from which GdnHCl had been omitted. Enzyme allowed to refold in the presence of 0.1 mM-pyridoxal 5'-phosphate plus 1 mM-dithiothreitol; enzyme allowed to refold in the absence of these ligands.

with Nbs2. Modification of these thiol groups led to little (< 10%) loss of activity of the enzyme, in agreement with previously reported data (Birchmeier et al., 1973). These thiol groups have been identified as the side chains of Cys-45 and Cys-82 (Arnone et al., 1977). As the concentration of GdnHCl was increased to 3 M, there was relatively little change in the number of reactive thiol groups. On further increase to 6 M-GdnHCl, the number of reactive thiol groups per subunit increases to 5.1, corresponding to the known content of cysteine (Fig. 4). The additional thiol groups exposed by unfolding of the enzyme at high concentrations of GdnHCl are Cys-191, Cys-252 and Cys-390 (Arnone et al., 1977).

Binding of cofactor. The absorption spectrum of aspartate aminotransferase shows a weak band at 360 nm corresponding to the bound pyridoxal 5'-phosphate cofactor ($A_{360}^{1\text{cm}} = 0.12$). This band did not change if the enzyme was dialysed at 4°C for 24 h against 100 mM-sodium phosphate, pH 7.4. In the presence of 3 M-GdnHCl, the band is shifted to 330 nm and slightly decreased ($A_{330}^{1\text{cm}} = 0.10$); however, there was no change on dialysis against buffer plus 3 M-GdnHCl, showing that the cofactor remained bound to the enzyme. In the presence of 6 M-GdnHCl, the spectrum shows a small peak at 390 nm ($A_{390}^{1\text{cm}} = 0.06$), and a peak of approximately equal intensity at 330 nm. These wave-lengths correspond to the maximum and shoulder respectively of the absorption spectrum of the pyridoxal 5'-phosphate in buffer or buffer plus 6 M-GdnHCl. The $A_{390}$ of the enzyme in the presence of 6 M-GdnHCl corresponds to approx. 0.8 molecule of free pyridoxal 5'-phosphate per enzyme subunit. On subsequent dialysis against buffer plus 6 M-GdnHCl, the peaks at 390 nm and 330 nm are effectively (> 90%) eliminated, confirming that the cofactor had dissociated from the enzyme. This conclusion is consistent with the changes in the near-u.v. c.d. spectrum noted above.

Refolding of enzyme after unfolding

Regain of enzyme activity. When the concentration of GdnHCl was lowered by 60-fold dilution of denatured enzyme into 0.1 M-potassium phosphate buffer, pH 7.4 at 20°C, recovery of enzyme activity occurred. The extent of regain of activity depended on the initial concentration of GdnHCl. Fig. 5 shows the data for regain of activity 24 h after dilution into buffer; clearly at concentrations of GdnHCl above 3 M there is a marked difference between samples allowed to refold in the presence of pyridoxal 5'-phosphate and dithiothreitol and samples allowed to refold in the absence of these compounds. Thus, under the conditions shown in Fig. 5, after incubation in 6 M-GdnHCl 65% activity was regained after 24 h in the presence of 0.1 M-pyridoxal 5'-phosphate plus 1 M-dithiothreitol, whereas only 2% activity was regained in the absence of these compounds.

Separate experiments showed that there was no significant increase in the regain of activity on increasing the pyridoxal 5'-phosphate concentration to 1 mM during refolding, and that either pyridoxal 5'-phosphate or dithiothreitol added separately led to much lower reactivation (~ 10% in each case).

Dependence of regain of activity on concentration of enzyme. The rate of regain of enzyme activity after refolding in 6 M-GdnHCl was studied as a function of enzyme concentration (pyridoxal 5'-phosphate and dithiothreitol were both added during refolding). The results (Fig. 6) show that both the rate and extent of reactivation increase with concentration of enzyme.

![Fig. 5. Extent of re-activation of aspartate aminotransferase after unfolding in GdnHCl](image)

![Fig. 6. Rate of re-activation of aspartate aminotransferase after refolding in 6 M-GdnHCl](image)
Table 1. Kinetic parameters for native and refolded aspartate aminotransferase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>V (µmol/min per mg)</th>
<th>$K_{A}$ (mM)</th>
<th>$K_{B}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>170 ± 10</td>
<td>3.0 ± 0.3</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Refolded</td>
<td>138 ± 14</td>
<td>3.0 ± 0.3</td>
<td>0.13 ± 0.015</td>
</tr>
</tbody>
</table>

The changes in the rate of re-activation with concentration of enzyme imply that an associative step is rate-determining in the formation of active enzyme. As shown in Fig. 6, the data can be fitted by a secondary-order progress course, with a rate constant $k = 5.4 \times 10^{3} \text{ M}^{-1} \cdot \text{s}^{-1}$.

Characterization of enzyme after refolding. When renatured in the presence of pyridoxal 5'-phosphate and dithiothreitol, aspartate aminotransferase regains substantial activity (Figs. 5 and 6). If the re-activated sample was concentrated by dialysis against poly(ethylene glycol) 10000 and then dialysed against 0.1 M-sodium phosphate buffer, pH 7.4, the fluorescence spectrum of the refolded enzyme resembled that of the native enzyme in terms of intensity and wavelength maximum (Fig. 2).

The kinetic parameters of the native and refolded enzyme (determined from primary and secondary plots) are shown in Table 1. The parallel lines in the primary plots show that both samples obey the characteristic 'enzyme substitution' mechanism. The data show that the Michaelis constants for the substrates are largely unchanged, whereas the value of $V$ for the refolded enzyme is somewhat lowered, consistent with the incomplete regain of activity on refolding.

Aggregation of enzyme during refolding. After unfolding in 6 M-GdnHCl, little activity is regained in the absence of pyridoxal 5'-phosphate and dithiothreitol (Fig. 5). This inactive material was concentrated by dialysis against poly(ethylene glycol) 10000 and then dialysed against 0.1 M-sodium phosphate buffer, pH 7.4, before being applied to a column (1.75 cm$^2 \times 12$ cm) of Sepharose 4B. The majority of the protein emerged at the void volume, but a significant amount was eluted at the position corresponding to the elution volume of native enzyme (Fig. 7). If enzyme was refolded in the presence of 0.1 mM-pyridoxal 5'-phosphate plus 1 mM-dithiothreitol and subjected to a similar concentration, dialysis and gel-filtration procedure, most of the protein emerged at the elution volume of native enzyme (Fig. 7), with only a small amount at the void volume. The enzyme activity was also eluted at the volume corresponding to that of native enzyme.

This experiment clearly shows that, in the absence of pyridoxal 5'-phosphate and dithiothreitol, unfolded aspartate aminotransferase refolds to yield a substantial amount of inactive aggregated material. The fluorescence emission spectrum of this material when excited at 290 nm showed a peak at 335 nm, significantly shifted from that of native enzyme or enzyme refolded in the presence of the ligands (330 nm in each case).

The following experiment was undertaken in order to check whether or not aggregated and/or other inactive material could still be induced to regain activity by addition of pyridoxal 5'-phosphate and dithiothreitol. After unfolding in 6 M-GdnHCl, refolding was initiated by 60-fold dilution; the final concentration of enzyme was 2 µg/ml. The solution was divided into four parts; to the first 0.1 mM-pyridoxal 5'-phosphate and 1 mM-dithiothreitol were added immediately. Re-activation proceeded as in Fig. 5, reaching a value of 60% after 24 h and 48 h relative to the non-denatured control sample. The second sample was incubated for 1 h at 20 °C (2% activity regained), and then pyridoxal 5'-phosphate and dithiothreitol were added. Re-activation occurred to a smaller extent (43% after 24 h and 48 h). The third sample was incubated at 20 °C for 24 h (3% activity regained), and then pyridoxal 5'-phosphate and dithiothreitol were added. In this case the extent of re-activation was much smaller (10% after a further 24 h). The fourth sample, to which no ligands were added, showed only 2% re-activation after 48 h. Thus it is clear...
from this experiment that, when refolding occurs in the absence of pyridoxal 5'-phosphate and dithiothreitol, there is a progressive decrease in the potential degree of re-activation of enzyme by subsequent addition of these ligands; this decrease is probably associated with the formation of aggregates.

**DISCUSSION**

The experiments described in this paper clearly show that cytosolic aspartate aminotransferase is unfolded by incubation in GdnHCl. The changes in enzyme activity occur at lower concentrations of GdnHCl than those in c.d., fluorescence and exposure of thiol groups, as has been found for a number of enzymes (Tsou, 1986). At low concentrations (< 3 M) of the denaturing agent, the changes in various parameters (exposure of tryptophan side chains to succinimide, secondary structure and reactive thiol groups) are relatively small; this can be correlated with the high degree of re-activation observed on dilution in both the absence and the presence of added ligands (Fig. 5). At higher concentrations of GdnHCl, however, unfolding is progressively greater, and the dependence of reactivation on added ligands is apparent, as previously noted by Bertland & Kaplan (1970). The requirement for pyridoxal 5'-phosphate can be explained by the observation that the cofactor dissociates from the enzyme at high concentrations of GdnHCl. Similarly, the requirement for dithiothreitol could be explained by the need to maintain the cysteine side chains exposed at high concentrations of GdnHCl (Fig. 4) in a reduced state.

The region of enzyme activity follows second-order kinetics (Fig. 6), with a rate constant (5.4 × 10^6 M^-1·s^-1) typical of that for association of subunits or groups of subunits during re-activation of denatured oligomeric enzymes (Jaenicke, 1987). The value is rather lower than the value of 10^7-10^9 M^-1·s^-1 expected for diffusion-controlled association of subunits (Koren & Hammes, 1976), implying a large steric restriction factor consistent with highly specific interactions between subunits at preformed contact sites (Jaenicke, 1987). The kinetics of reactivation are consistent with a mechanism in which association between subunits is required for the expression of catalytic activity. It is noteworthy that the X-ray crystallographic data show that each active site in the dimeric enzyme contains contributions from both subunits at the subunit interface (Borisov et al., 1985).

The decline in the degree of re-activation at low concentrations of enzyme (Fig. 6) can be ascribed to the long-term instability of intermediates in the refolding and assembly process (Jaenicke, 1987). Over the concentration range studied, formation of aggregates occurs to only a limited extent, provided that pyridoxal 5'-phosphate and dithiothreitol are present. In the absence of these ligands, aggregates represent the major product. The role of pyridoxal 5'-phosphate in the assembly process remains to be clarified; it may serve to stabilize a folded intermediate which has the correct binding site for subunit association to yield the dimeric enzyme. This type of effect has been suggested for a number of ligands during the refolding of proteins (Jaenicke, 1987).

We are unable to account for the observation reported by Banks et al. (1968b), who claimed that the enzyme did not apparently unfold in 6 M-GdnHCl except on prolonged (several weeks) incubation. It may be relevant that in some preliminary experiments the commercial enzyme was found to be more resistant to unfolding by GdnHCl than enzyme isolated in our laboratory. The processes which led to destruction of the reactive thiol groups may well have brought about this enhanced stability, and such effects may have been important in the sample examined by Banks et al. (1968b).

We thank the Science and Engineering Research Council for financial support, and Professor Roger Pain and Mr. Roman Hlodan for help in obtaining the c.d. spectra.

**REFERENCES**


Vol. 261


Received 12 January 1989/24 February 1989; accepted 1 March 1989