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The unfolding and attempted refolding of mitochondrial aspartate aminotransferase from pig heart

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The unfolding of the mitochondrial isoenzyme of aspartate aminotransferase from pig heart in solutions of guanidinium chloride (GdnHCl) has been studied. By a number of criteria (enzyme activity, protein fluorescence, c.d., thiol-group reactivity), the enzyme was judged to be almost completely unfolded in 2 M-GdnHCl. On dilution of the GdnHCl, no re-activation of the enzyme could be observed, whether or not pyridoxal 5'-phosphate and dithiothreitol were present. The behaviour of the mitochondrial isoenzyme is in marked contrast with that of the cytoplasmic isoenzyme [West & Price (1989) Biochem. J. **261**, 189–196], despite the similarities in the amino acid sequences and tertiary structures of the two isoenzymes. The implications of these findings for the process of folding and assembly of the mitochondrial isoenzyme *in vivo* are discussed.

INTRODUCTION

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) occurs in two dimeric isoenzyme forms, one of which occurs in the mitochondrion and the other of which is confined to the cytosol. The amino acid sequences and X-ray-crystallographic structures of the two isoenzymes from various sources have been determined (for a review, see Christen & Metzler, 1985). These studies have shown that the various isoenzymes have a marked 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 targetting sequence. In the chicken and pig enzymes the pre-sequences consist of 22 and 29 amino acids respectively and follow the normal pattern in containing a number of positively charged, but no negatively charged, amino acids (Jaussi *et al.*, 1985; Nishi *et al.*, 1989). The roles of various amino acids of the pre-sequence of the pig enzyme in the import and proteolytic cleavage processes have been explored (Nishi *et al.*, 1989). The enzyme is probably translocated in a monomeric form (O'Donovan *et al.*, 1984), so that assembly to the mature dimer occurs in the mitochondrion. Expression of the gene corresponding to the precursor of the chicken mitochondrial enzyme in *Escherichia coli* leads to formation of aggregates of high M_r, whereas expression of the gene corresponding to the mature enzyme leads to the formation of active dimeric enzyme (Jaussi *et al.*, 1987). However, a recent report shows that the gene corresponding to the precursor of the rat liver enzyme in *E. coli* leads to the formation of soluble active enzyme (Altieri *et al.*, 1989).

In a previous paper (West & Price, 1989) we showed that the cytoplasmic isoenzyme of aspartate aminotransferase from pig heart could be refolded in reasonable yield after denaturation in 6 M-GdnHCl, provided that pyridoxal 5'-phosphate and dithiothreitol were both present. In the absence of these compounds, substantial formation of aggregates of high M_r occurred. The reactivation reaction obeyed second-order kinetics.

The purpose of the present paper is to explore the unfolding and attempted refolding behaviour of the mitochondrial isoenzyme. Comparison with the behaviour of the cytoplasmic isoenzyme should shed light on steps involved in the formation of the mature mitochondrial isoenzyme. It should be noted that, in a preliminary experiment noted by Jaussi *et al.* (1987), the mitochondrial isoenzyme from chicken liver could not be refolded after denaturation, though no details were given.

EXPERIMENTAL

The mitochondrial isoenzyme of pig heart aspartate aminotransferase was isolated as described by Barra *et al.* (1976), but including an extra gel-filtration step on Sephacryl S-300. The enzyme was stored in 0.05 M-potassium phosphate buffer, pH 6.0, containing 0.1 mM-pyridoxal 5'-phosphate. After dialysis, the concentration of enzyme was determined spectrophotometrically at 280 nm by using a value of $A_{1\,cm}^{0.1\%}$ of 1.40 (Barra *et al.*, 1976). Enzyme activity was routinely assayed at 25 °C by using a coupled assay procedure involving malate dehydrogenase (Barra *et al.*, 1976). The final concentrations of the assay components in 0.05 M-potassium phosphate buffer, pH 6.0, were: L-aspartate, 3.3 mM; 2-oxoglutarate, 3.3 mM; NADH, 80 μM; malate dehydrogenase, 5 μg/ml. The specific activity of our preparation was 170 μmol/min per mg, a value identical with that reported by Barra *et al.* (1976). However, it was noted that, if the enzyme was stored in the absence of added

Abbreviations used: GdnHCl, guanidinium chloride; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid).

pyridoxal 5′-phosphate, the specific activity of the preparation was lower (100 μmol/min per mg); this value could be increased to 170 μmol/min per mg by incubation with cofactor. The results suggest that previous reports of lower specific activities of preparations of the mitochondrial isoenzyme (Wada & Morino, 1964; Michuda & Martinez-Carrion, 1969) may reflect partial loss of cofactor from the preparations.

The enzyme prepared in the laboratory was at least 95% homogeneous on SDS/polyacrylamide-gel electrophoresis in 12%-acrylamide gels (Laemmli, 1970), as judged by Coomassie Blue staining. The subunit M_r was determined to be 43000 ± 2000, a value consistent with the M_r calculated from the amino acid sequence, i.e. 44657 (Barra *et al.*, 1980).

Enzyme activity in the presence of GdnHCl was determined by the discontinuous assay procedure used in the previous studies on the cytoplasmic isoenzyme (West & Price, 1989). These assays were performed at 20 °C in 0.05 M-potassium phosphate buffer, pH 7.4, with the concentrations of L-aspartate and 2-oxoglutarate used in the earlier work (i.e. 30 mM and 5 mM respectively). 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 MPF3A fluorimeter. Spectra were recorded 15 min after addition of GdnHCl to samples. The quenching of protein fluorescence by acrylamide and succinimide was measured as described previously (West & Price, 1989).

C.d. spectra were recorded at 20 °C in a Jobin–Yvon Dichrographie IV in cells of path length 0.1 mm (far-u.v., 250–205 nm) or 10 mm (near-u.v., 400–260 nm). The enzyme concentrations in these experiments was 0.8 mg/ml. Spectra were recorded 15 min after 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).

The ability of aspartate aminotransferase to re-fold was studied by first incubating the enzyme for 15 min at 0 °C in GdnHCl dissolved in 0.05 M-potassium phosphate buffer, pH 7.4, followed by 60-fold dilution into 0.05 M-potassium phosphate buffer, pH 7.4, at 20 °C.

RESULTS

Unfolding of enzyme in GdnHCl

A number of measurements were made in order to assess the degree of unfolding of mitochondrial aspartate aminotransferase caused by GdnHCl.

Enzyme activity. The enzyme was incubated in GdnHCl in 0.05 M-potassium phosphate buffer, pH 7.4, at 20 °C for 15 min and then assayed in the presence of the same concentration of GdnHCl by the discontinuous assay 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 0.5 M and > 95% activity lost at 1.25 M. The comparative data for the cytoplasmic isoenzyme (also shown in Fig. 1) show that the mitochondrial isoenzyme is more sensitive to the denaturing agent.

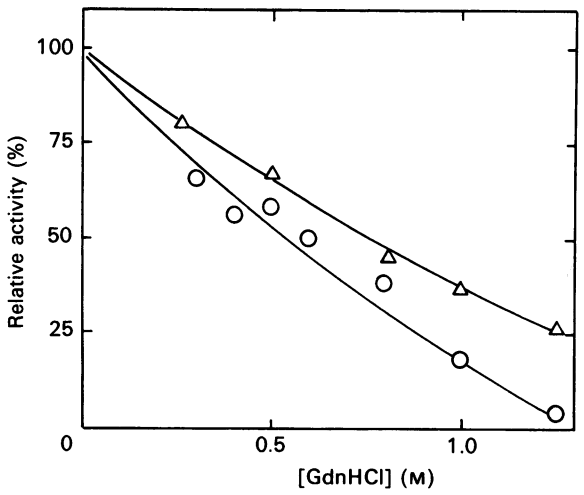

Fig. 1. Loss of activity of aspartate aminotransferases on incubation in GdnHCl: (○), mitochondrial isoenzyme; (△), cytoplasmic isoenzyme

The mitochondrial isoenzyme (40 μg/ml) was incubated at 20 °C for 15 min in 0.05 M potassium phosphate buffer, pH 7.4. A sample (8 μg) was taken for assay by the discontinuous procedure (West & Price, 1989). Activities are expressed relative to a control sample from which GdnHCl was omitted. The data for the analogous experiments on the cytoplasmic isoenzyme are taken from West & Price (1989).

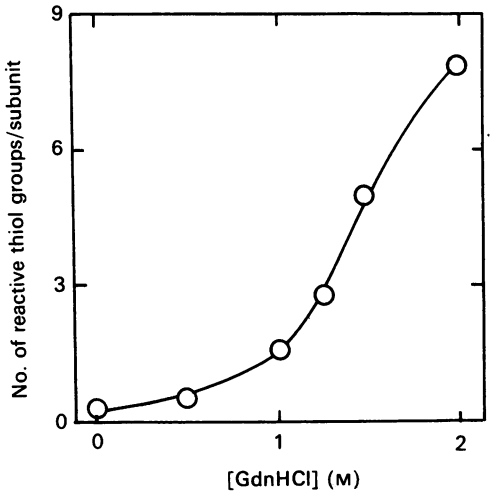

Fig. 2. Reactive thiol groups of mitochondrial aspartate aminotransferase

Enzyme (0.1 mg/ml) was incubated in 0.05 M-sodium phosphate buffer, pH 7.4, at 20 °C. The increase in A_{412} on reaction with Nbs_2 (250 μM) was used to calculate the number of reactive thiol groups. In each case the reaction was complete within 10 min.

Reactive thiol groups. In the absence of denaturing agents, the enzyme possessed virtually no thiol groups (< 0.2 group per subunit) which reacted with 250 μM-Nbs_2 over a 15 min period, in 0.05 M-sodium phosphate buffer, pH 7.4, at 20 °C. On addition of 0.1% SDS, a total of 7.1 thiol groups per subunit were found to be reactive towards Nbs_2 under these conditions; this value is consistent with the sequence data, which show 7

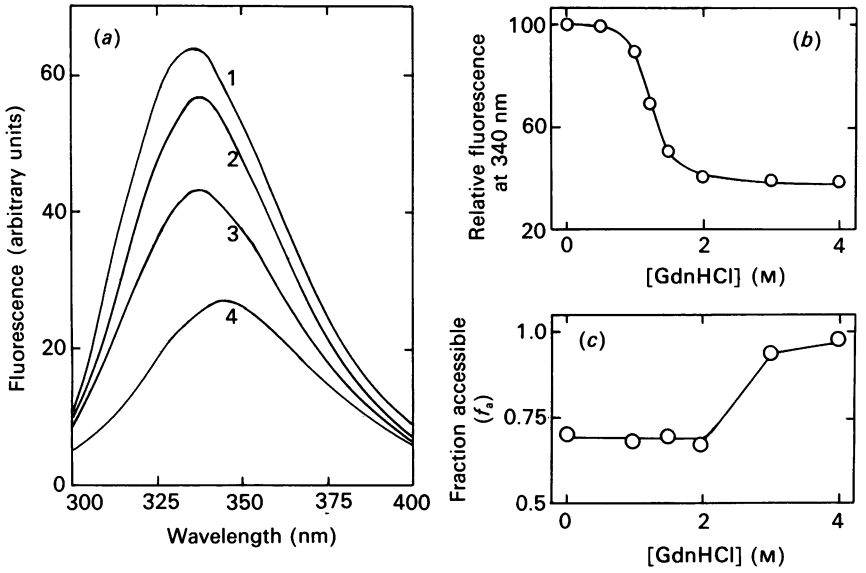

Fig. 3. Fluorescence properties of mitochondrial aspartate aminotransferase

Enzyme was incubated in 0.05 M-potassium phosphate buffer, pH 7.4, in the absence and presence of GdnHCl, at 20 °C. The excitation wavelength was 290 nm. (*a*) Emission spectra of enzyme (20 μg/ml). Curves 1, 2, 3, 4 represent enzyme in the absence of GdnHCl and in the presence of 1 M, 1.25 M- and 2 M-GdnHCl respectively. (*b*) The relative intensity of fluorescence at 340 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).

cysteines per subunit (Barra *et al.*, 1980). In the presence of increasing concentrations of GdnHCl, there is an increase in the number of reactive thiol groups (Fig. 2), with the major increase occurring between 1 M- and 2 M-GdnHCl, at which point all thiol groups have become exposed. These results can be contrasted with data for the cytoplasmic isoenzyme, where the increased exposure of thiol groups occurred in the GdnHCl concentration range 3–6 M (West & Price, 1989).

Fluorescence properties. When excited at 290 nm, mitochondrial aspartate aminotransferase exhibited a fluorescence maximum at 335 nm (Fig. 3*a*), characteristic of tryptophan side chains partially shielded from the aqueous solvent. On addition of GdnHCl there is a quenching of the fluorescence and a shift of the maximum (to 345 nm at 2 M-GdnHCl). The changes in fluorescence at 340 nm are shown in Fig. 3(*b*), and illustrate the marked structural changes occurring between 1 M- and 2 M-GdnHCl. On further addition of GdnHCl there is a small further red shift of the emission maximum (to 350 nm at 4 M- and 6 M-GdnHCl), but no significant change in fluorescence intensity at 340 nm.

The emission maximum for the mitochondrial isoenzyme is higher than that for the cytoplasmic isoenzyme (330 nm; West & Price, 1989), suggesting that the tryptophan side chains are more exposed to the solvent in the former isoenzyme. This conclusion is consistent with the results of fluorescence-quenching studies, where the fraction accessible to quenching by succinimide (f_a) is estimated as 0.70 for the mitochondrial isoenzyme, compared with 0.52 for the cytoplasmic isoenzyme (West & Price, 1989). In the presence of increasing concentrations of GdnHCl, the value of f_a for the mitochondrial isoenzyme increased towards 1.0 (Fig. 3*c*), though in these studies the major increase occurred between 2 M- and 3 M-GdnHCl. The value of f_a for acrylamide, which is known to be an efficient quencher of tryptophan fluorescence (Eftink & Ghiron, 1984), was estimated to be 1.0 in the absence of GdnHCl; this value did not change appreciably as the concentration of GdnHCl was increased to 4 M.

C.d. The far-u.v. c.d. spectrum of mitochondrial aspartate aminotransferase in the absence of GdnHCl is shown in Fig. 4(*a*), and is in reasonable agreement with that reported by Martinez-Carrion *et al.* (1970) under slightly different conditions. The spectrum shows minima at 208 nm and 220 nm, characteristic of a protein with a high content of α-helical structure (Chen *et al.*, 1974; Chang *et al.*, 1978). From the value of θ_{225}, the α-helix content can be calculated to be 65%, which is rather higher than the value (50%) determined by X-ray crystallography for the mitochondrial isoenzyme from chicken (Ford *et al.*, 1980). The reason for the discrepancy is not clear, but it may be relevant that the X-ray work shows that several of the helices are irregular, with interruptions to the hydrogen-bonding pattern.

The changes in the far-u.v. c.d. on addition of GdnHCl are shown in Fig. 4(*a*). In the presence of 1 M-GdnHCl, there is relatively little change in the c.d. spectrum, but substantial changes are noted between 1 M- and 2 M-GdnHCl; in the presence of 2 M-GdnHCl, virtually no secondary structure remains (Fig. 4*a*).

In the near-u.v., the c.d. spectrum (Fig. 4*b*) shows bands arising from tyrosine and tryptophan side chains of the protein (in the 260–300 nm region), and a band at 355 nm owing to the pyridoxal 5′-phosphate cofactor. There are pronounced changes in the near-u.v. spectrum on addition of GdnHCl, such that these bands are virtually absent in 1 M-GdnHCl. The increased sensitivity in the far-u.v. is consistent with general observations that

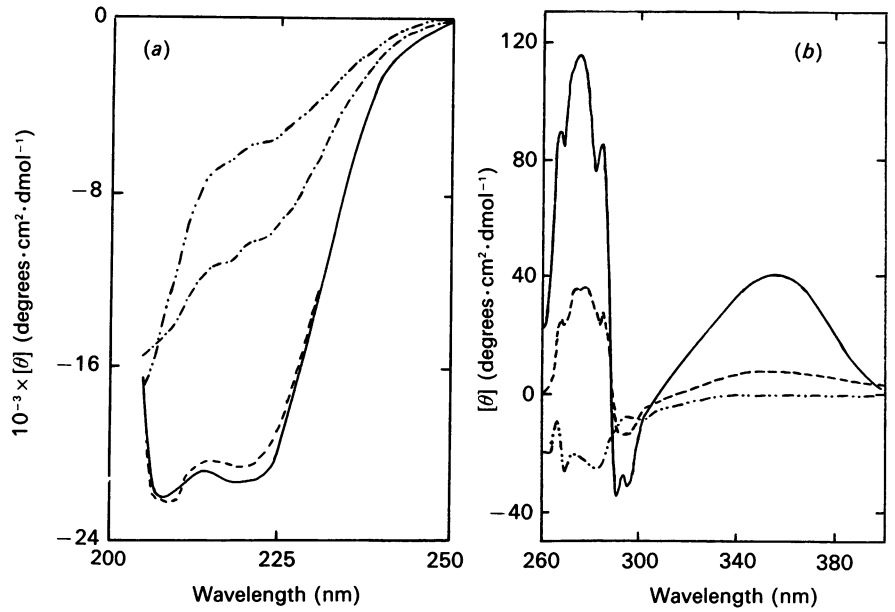

Fig. 4. C.d. spectra of mitochondrial aspartate aminotransferase

Spectra of enzyme (0.8 mg/ml) were recorded in the absence of GdnHCl (——) and in the presence of 1 M- (– – – –), 1.5 M- (–·–·–) and 2 M- (–··–··–) GdnHCl. (*a*) Far-u.v. spectra; (*b*) near-u.v. spectra.

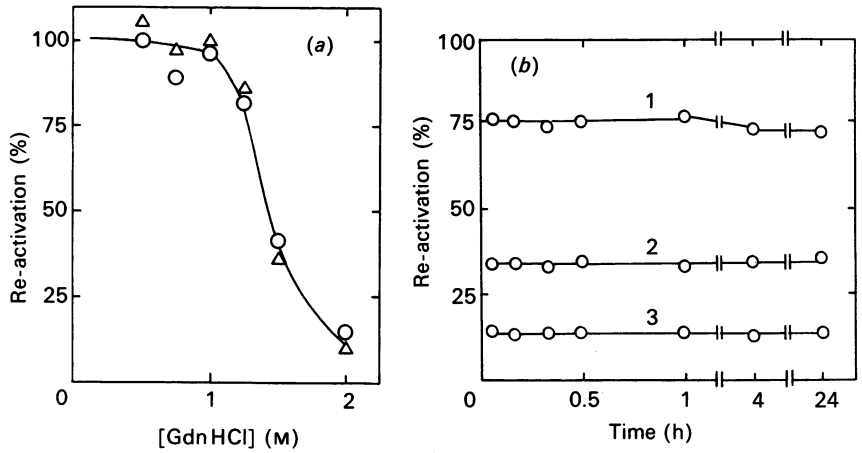

Fig. 5. Re-activation of mitochondrial aspartate aminotransferase after unfolding in GdnHCl

The unfolding and re-activation of enzyme were performed as described in the text; the enzyme concentration during refolding was 4 μg/ml. In each case, activity is expressed relative to a control sample from which GdnHCl had been omitted. (*a*) The activity was assayed after 24 h re-activation. (○) Enzyme allowed to refold in the presence of 0.1 mM-pyridoxal 5′-phosphate and 1 mM-dithiothreitol; (△) enzyme allowed to refold in the absence of these ligands. (*b*) Time-dependence of the extent of re-activation in the presence of 0.1 mM-pyridoxal 5′-phosphate and 1 mM-dithiothreitol. Curves 1, 2 and 3 refer to samples incubated in the presence of 1.25 M-, 1.5 M- and 1.75 M-GdnHCl respectively before re-activation.

the tertiary structure of proteins is more readily disrupted by GdnHCl than is the secondary structure (Creighton, 1978; Tsou, 1986). As shown below, the decrease in θ_{355} on addition of 2 M-GdnHCl is due to dissociation of the cofactor.

Binding of cofactor. The absorption spectrum of mitochondrial aspartate aminotransferase shows a weak band at 355 nm corresponding to bound pyridoxal 5′-phosphate ($A^{0.1\%}_{1\,cm} = 0.16$). This band did not change if the enzyme was dialysed at 4 °C for 24 h against 20 vol. of buffer (0.05 M-potassium phosphate, pH 7.4). In the presence of 2 M-GdnHCl, the spectrum showed bands at 330 nm ($A^{0.1\%}_{1\,cm} = 0.08$) and 390 nm ($A^{0.1\%}_{1\,cm} = 0.10$), which correspond to the bands in the absorption spectrum of pyridoxal 5′-phosphate under these conditions. On subsequent dialysis against buffer plus 2 M-GdnHCl, the bands at 330 nm and 390 nm were almost completely (> 90 %) eliminated, confirming that the cofactor had dissociated from the enzyme in the presence of 2 M-GdnHCl.

When the enzyme was incubated in the presence of 1 M-GdnHCl, there was a significant decrease in absorbance at 355 nm compared with native enzyme ($A^{0.1\%}_{1\,cm} =$

0.11); on dialysis this was decreased further (to 0.07). However, this result is difficult to interpret, because of the gradually increasing absorbance of the solution below 390 nm (probably owing to light-scattering, even though the solution was not visibly turbid). The data suggest that, in the presence of 1 M-GdnHCl, some dissociation of cofactor had occurred, consistent with the decline in the c.d. signal at 355 nm (Fig. 4*b*).

Attempted refolding of enzyme after denaturation

The ability of the mitochondrial isoenzyme to be re-activated after denaturation in GdnHCl was tested by 60-fold dilution, into 0.05 M-potassium phosphate buffer, pH 7.4, as described in the Experimental section. As shown in Fig. 5(*a*), full activity could be restored, provided that the initial concentration of GdnHCl was $\leqslant$ 1 M. At higher concentrations of GdnHCl, there was a pronounced loss of the ability of the enzyme to be re-activated, so that at 2 M-GdnHCl only $\approx$ 10% activity could be regained. This decline in re-activation was observed whether or not pyridoxal 5′-phosphate (0.1 mM) and dithiothreitol (1 mM) were present in buffer employed for the dilution step.

The data shown in Fig. 5(*a*) refer to samples assayed 24 h after dilution; however, as shown in Fig. 5(*b*), there was essentially no difference in the regain of activity over the period from 30 s to 24 h after dilution.

The behaviour of the mitochondrial enzyme contrasts markedly with that observed for the cytosolic isoenzyme (West & Price, 1989), in which the extent of re-activation of enzyme incubated at GdnHCl concentrations above 3 M depended on the presence of pyridoxal 5′-phosphate and dithiothreitol and was time dependent, obeying second-order kinetics.

DISCUSSION

The data reported in this paper show that the mitochondrial isoenzyme of aspartate aminotransferase is largely, if not completely, unfolded in 2 M-GdnHCl. In this respect the mitochondrial isoenzyme is significantly less stable than the cytoplasmic isoenzyme (West & Price, 1989), which, according to various criteria (protein fluorescence, c.d. and reactive thiol groups), retains substantial secondary and tertiary structure at this concentration of GdnHCl. The structural reasons for the different behaviour of the two isoenzymes are not clear, since the overall folding of the peptide chain is reported to be similar in the two cases (Borisov *et al.*, 1985). However, it may be significant that in one of the two regions of inter-subunit contact identified in the X-ray-crystallographic structure (Ford *et al.*, 1980) there is a lesser degree of identity of sequence identity than for the two isoenzymes as a whole (48%). In the contact region formed between the *N*-terminal arm of one subunit and the cofactor-binding domain of the other there is only 29% identity, compared with 49% identity in the other contact region. Thus it is possible that the strength of subunit interactions is significantly different in the two forms, with consequent effects on the stability of secondary and tertiary structure of each polypeptide chain.

For the mitochondrial isoenzyme, the major changes in enzyme structure occur at GdnHCl concentrations between 1 M and 2 M. The changes in enzyme activity (Fig. 1) occur at lower concentrations of GdnHCl, reflecting the general observation that enzyme activity is more critically dependent on structure than are properties such as fluorescence and exposure of amino acid side chains (Tsou, 1986).

The second major difference between the isoenzymes is that, whereas the cytoplasmic isoenzyme can refold after denaturation, provided that pyridoxal 5′-phosphate and dithiothreitol are present (West & Price, 1989), the mitochondrial isoenzyme cannot apparently do so. Thus the decline in the re-activation (Fig. 5*a*) matches the degree of unfolding monitored by reactive thiol groups (Fig. 2), fluorescence (Fig. 3) and c.d. (Fig. 4). Since there is no time-dependence of the regain of activity (Fig. 5*b*), it appears that it is only that fraction of enzyme molecules which retain native or native-like structure which can be re-activated within 30 s of dilution of the denaturing agent. Fully unfolded enzyme is apparently incapable of re-activation under these conditions.

The behaviour of the mitochondrial isoenzyme of aspartate aminotransferase is thus similar to that of other imported mitochondrial enzymes such as bovine liver glutamate dehydrogenase (West & Price, 1988) and pig heart citrate synthase (S. M. West & N. C. Price, unpublished work) in being unable to refold from isolated unfolded subunits. The mechanism by which the mature forms of these enzymes fold and assemble *in vivo* is thus a matter of some interest, since a number of studies, reviewed by Meyer (1988) and Bychkova *et al.* (1988), have shown that translocation of a protein across the mitochondrial and other membranes requires the protein to be in non-native, unfolded, state. It is probable that the folding and assembly of these imported mitochondrial proteins requires the involvement of specific binding proteins ('chaperonins') which have been implicated in the assembly of a number of imported proteins such as ribulose bisphosphate carboxylase/oxygenase (Hemmingsen *et al.*, 1988). In support of this idea, it has been recently shown (Cheng *et al.*, 1989) that one such chaperonin, namely the mitochondrial heat-shock protein hsp 60, is essential for the assembly of various oligomeric enzymes (such as F1 ATPase) imported into yeast mitochondria. The exact role played by such binding proteins in directing the folding and assembly processes of imported proteins remains a task for future work.

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