Dissociation, unfolding and refolding trials of pig kidney 3,4-dihydroxyphenylalanine (Dopa) decarboxylase

Paola DOMINICI,* Patrick S. MOORE† and Carla BORRI VOLTATTORNI†‡

*Università Cattolica del Sacro Cuore, Facoltà di Medicina e Chirurgia 'A.Gemelli', Istituto di Chimica Biologica, Largo P. Vito, 00168 Rome, Italy, and †Università degli Studi di Verona, Facoltà di Medicina e Chirurgia, Istituto di Chimica Biologica, Strada Le Grazie, 8, 37134 Verona, Italy

The effect of guanidinium chloride (GuCl) on enzyme activity, hydrodynamic volume, circular dichroism, and fluorescence of 3,4-dihydroxyphenylalanine (Dopa) decarboxylase from pig kidney (pkDDC) was studied under equilibrium conditions. Unfolding proceeds in at least three stages. The first transition, occurring between 0 and 1 M GuCl, gives rise to a dimeric inactive species which has lost pyridoxal 5'-phosphate (PLP), and has a high tendency to aggregate, but retains almost all of the native spectroscopic characteristics. The second equilibrium transition, between 1 and 2.2 M GuCl, involves dimer dissociation, with some loss of tertiary and secondary structure. Additionally, gross conformational changes at or near the PLP microenvironment were detected by fluorescence of NaBH₄-

reduced enzyme. The third step, presumably representing complete unfolding of pkDDC, appears to be complete at 4.5 M GuCl, as indicated by the lack of further substantial changes in any of the signals being studied. Attempts at refolding resulted in the findings that: (1) partial reactivation is observed only starting from enzyme denatured at concentrations below 1.5 M GuCl, and (2) starting from completely denatured protein, the refolding process is apparently reversible down to concentrations of approx. 2 M GuCl. Taken together, this would seem to indicate that the monomer-dimer transition is impaired under the experimental conditions tested. A plausible model is presented for the unfolding/refolding of pkDDC.

INTRODUCTION

The molecular mechanisms by which polypeptide chains fold into their biologically active three-dimensional structures have been widely investigated. For monomeric proteins, the unfolding/refolding transition under equilibrium conditions is generally a cooperative process that is often described by a two-state approximation, since frequently intermediates are insufficiently populated. Nonetheless, deviations from two-state behaviour have been observed even under equilibrium conditions. The protein folding problem is more complex when oligomeric proteins are considered. The acquisition of the quaternary structure of a given protein involves highly specific associations, usually vital for catalytic activity, which must be subtly concerted with proper folding of subunits. Both processes must be properly coordinated since the formation of the native quaternary structure requires the surfaces of the structured monomers to be preformed in the correct way, such that specific recognition is achieved (Jaenicke, 1978). As for the unfolding of monomeric proteins, the dissociation and unfolding of oligomers have been shown to be reversible in many cases. However, failure of many oligomeric proteins to regain activity when subjected to refolding when starting from denatured subunits has been documented (Jaenicke and Rudolph, 1986). There is good evidence that 'in vitro' refolding and reassociation of multimeric proteins generally proceeds via conformational states intermediate between those of the native and the fully denatured protein (Ghéris and Yon, 1982; Kim and Baldwin, 1982). The identification of these partially structured intermediates allows the correlation of catalytic function with single events in the multi-step process.

3,4-Dihydroxyphenylalanine (Dopa) decarboxylase (DDC;

EC 4.1.1.28) is an oligomeric protein, the unfolding/refolding characteristics of which have not been investigated. The enzyme catalyses the decarboxylation of L-Dopa and L-5-hydroxytryptophan to dopamine and 5-hydroxytryptamine respectively, which are the two major neurotransmitters of the mammalian nervous system. DDC from pig kidney (pkDDC) exists as a homodimer with a subunit molecular mass of 53 858 kDa (Borri Voltattorni et al., 1987). It is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and contains 1 mol of coenzyme per dimer, which is bound covalently to Lys-303 (Borri Voltattorni et al., 1979; Maras et al., 1991); the spectral properties of the coenzyme-bound apoenzyme and of the enzyme-substrate complexes have been elucidated (Borri Voltattorni et al., 1971, 1983; Fiori et al., 1975). The primary structure of pkDDC has been determined by Edman degradation, allowing for the localization of a number of structural features shown to be crucial for enzyme function (Maras et al., 1991; Dominici et al., 1991b). Crystals of pkDDC have been obtained recently (Malashkevich et al., 1992) and the X-ray structure is under investigation. Information regarding the correlation between its spatial structure and its catalytic activity has remained sparse.

The purpose of this study was to explore the denaturation of pkDDC in the apo- and holo-enzyme forms under equilibrium conditions using increasing concentrations of guanidinium chloride (GuCl) in an effort to identify the dissociation and denaturation steps involved in the unfolding pathway. The effect of bound coenzyme on this process, as well as the relationship between enzymic activity, dissociation and unfolding of pkDDC, was also determined. The non-coincidence of the equilibrium transition curves obtained by monitoring different signals implies that GuCl denaturation of pkDDC induces the appearance of

Abbreviations used: GuCl, guanidinium chloride; Dopa, 3,4-dihydroxyphenylalanine; pkDDC, Dopa decarboxylase from pig kidney; PLP, pyridoxal 5'-phosphate; DDT, dithiothreitol; PMP, pyridoxamine 5'-phosphate.

‡ To whom correspondence should be addressed.

successive molecular forms during the unfolding/refolding process. These intermediates were characterized and results of refolding attempts are also described.

EXPERIMENTAL

Materials

GuCl was obtained from Pierce (Sequenal grade). The concentrations of GuCl solutions were verified by refractive index measurements (Nozaki, 1972). All others reagents were of analytical grade. DDC was purified to homogeneity from pig kidney by the method of Borri Voltattorni et al. (1979). Apoenzyme was prepared by hydroxylamine treatment according to the method of Borri Voltattorni et al. (1971).

Protein concentration determination

Protein concentration was determined from the absorbance of the peptide backbone at 205 nm, as described by Scopes (1974). Utilizing this method, the apparent molar absorption coefficient at 280 nm was $1.30 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value is 0.5 % smaller than the theoretically determined molar absorption coefficient, $1.35 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$, which was calculated by using the individual absorption coefficients for tryptophan and tyrosine (Edelhoch, 1967). Holoenzyme concentration was also expressed in terms of bound coenzyme, determined by releasing the bound PLP into 0.1 M NaOH using a molar absorption coefficient of $6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for free PLP. Using the absorption coefficient determined by the method of Scopes and the absorption coefficient of PLP, among various enzyme preparations the ratio of mol of PLP/mol of enzyme homodimer consistently falls between 0.9 and 1.1.

Denaturation of pkDDC

Denaturation of pkDDC was performed in the following manner. Stock solutions of enzyme and 6 M GuCl, both dissolved in 50 mM potassium phosphate buffer, pH 7, containing 0.5 mM EDTA and 1 mM dithiothreitol (DTT) (buffer A), were mixed to give the desired concentration of protein and denaturant (see Figures for details). Each mixture was allowed to equilibrate by incubation at 25 °C for 30 min, which was a sufficient time to reach the limiting values for all enzyme properties listed below at each GuCl concentration used.

Enzyme activity

pkDDC activity was measured by the assay method of Sherald et al. (1973) according to the modification introduced by Charteris and John (1975).

C.d. spectroscopy

C.d. spectra were recorded on a Cary model 60 instrument at 20 °C using cuvettes of 0.1 cm path length. The protein concentration was typically 0.075–0.15 mg/ml for far-u.v. work and 1.5 mg/ml for the near-u.v.–visible c.d. experiments. Reported spectra are expressed as mean residue ellipticity, θ , and were calculated by using a value of 111 for the mean residue weight of the protein (Maras et al., 1991).

Fluorescence spectroscopy

Fluorescence measurements were carried out with a Kontron SFM 25 spectrofluorimeter at 25 °C. All solutions were filtered immediately before measurements were made. Protein fluorescence values were corrected by the subtraction of the apparent fluorescence of the solutions of GuCl. Changes in native protein fluorescence were monitored at 326 nm (excitation at 278 nm) using 5 nm bandwidths on both sides. Fluorescence of NaBH_4 -reduced enzyme was monitored at an emission wavelength of 385 nm (excitation at 325 nm).

Turbidity measurements

Turbidity was measured by fluorescence using entrance and exit monochromators set at 450 nm. The enzyme concentration was varied between 5 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$ in buffer A.

Gel-filtration chromatography

All experiments were conducted at 4 °C. A Superose 12 column (1 cm $\times$ 30 cm; Pharmacia) was calibrated with the following markers of known molecular mass in buffer A containing 300 mM NaCl: thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). A linear gradient of GuCl was formed by mixing 100 ml of buffer A containing 300 mM NaCl with 100 ml of buffer A containing 300 mM NaCl and 6 M GuCl (flow rate 0.1 ml/min) using an f.p.l.c. system. Successive 50 μl samples of native pkDDC (0.2 mg/ml) were injected after each 0.1 M increment of GuCl concentration. The enzyme remained on the column for periods of 120–140 min, depending on the GuCl concentration. The elution profile was monitored by recording the absorbance at 280 nm.

Refolding experiments

The refolding of pkDDC was attempted after the enzyme had been denatured by incubation for 30 min at 25 °C in 6 M GuCl dissolved in buffer A. The process was initiated either by the desired dilution into the appropriate buffer or by step-dialysis against a buffer containing the appropriate concentration of GuCl.

RESULTS

Enzyme activity

The holoenzyme was incubated in the presence of different concentrations of GuCl in buffer A at 25 °C, and then assayed (without the addition of PLP) in the presence of the same concentration of denaturant. As shown in Figure 1, there was a progressive loss of activity with increasing concentrations of GuCl, with 50 % of activity lost at 0.4 M GuCl and < 10 % of activity remaining at 1 M GuCl. Identical results were obtained with apoenzyme (results not shown). It should be noted that in this case the addition of PLP to the assay mixture is necessary to measure activity. After removal of GuCl, only a partial reactivation was observed, which depended on the initial concentration of GuCl. At 0.6 M GuCl, 50 % of the decarboxylase activity was recovered; above 1.5 M GuCl, the activity was irreversibly lost (Figure 1). Although the results presented in Figure 1 refer to samples assayed 24 h after dilution, it should be noted that there was essentially no difference in the degree of activity regained over the period from 1 to 24 h following dilution (results not shown). Attempts to regain enzymic activity

Figure 1 Inactivation and reactivation of holo-pkDDC as a function of GuCl concentration

For inactivation (○), the enzyme (8 $\mu\text{g}/\text{ml}$) was incubated in 50 mM potassium phosphate buffer containing 0.5 mM EDTA and 1 mM DTT in the presence of the indicated concentration of denaturant for 30 min at 25 °C and then an aliquot (2 μg) was assayed for activity in the same concentration of GuCl. For reactivation (●), identically treated samples were diluted to nearly zero GuCl concentration in the same buffer and then assayed for pkDDC activity.

starting from fully denatured pkDDC under a wide variety of experimental conditions proved to be unsuccessful (discussed later).

Fluorescence properties

The emission spectra of holo- and apo-DDC in the native or denatured states are shown in Figure 2. When excited at 278 nm, both of the native enzymic species display an emission maximum at 326 nm, which is characteristic of tryptophan side chain(s) buried in the interior of the protein and isolated from the aqueous solvent; however, the protein fluorescence of the holoenzyme has a quantum yield 40% lower than that of the apoenzyme. A similar phenomenon has been observed for aspartate aminotransferase from *Escherichia coli* (Herold and Leistler, 1992), in that the intrinsic fluorescence of holoDDC is quenched by bound coenzyme. For both of the enzymic species a red shift of the intrinsic fluorescence emission maximum, as well as a decrease in the intensity, was observed in the denatured state due to solvent exposure of buried tryptophan residues. The changes in fluorescence intensity at 326 nm, and in the emission maximum, as a function of GuCl concentration are reported in Figure 3. The overall transition occurs in three distinct phases. (1) Between 0 and 1 M GuCl the fluorescence intensity of apoDDC remains constant, while that of holoDDC increases, reaching the same value as that observed for apoDDC. No shift in the emission maximum was seen for either holo- or apo-DDC. The significant change observed in the holoenzyme may result from a loss of quenching due to the release of PLP. (2) From 1 M to an intermediate plateau value of 2 M GuCl the fluorescence intensity decreases in a cooperative manner, accompanied by a marked red shift from 326 nm to 341 nm: this transition appears to be dependent on protein concentration (results not shown), suggesting that the fluorescence signal could reflect both unfolding and dissociation of the dimeric enzyme. (3) The emission

Figure 2 Fluorescence emission spectra of apo- and holo-pkDDC in native and unfolded states

Excitation was at 278 nm. Buffer was as described in the legend of Figure 1, at 25 °C. Protein concentration was 5 $\mu\text{g}/\text{ml}$. At 0 M GuCl: (---) apoenzyme, (—) holoenzyme; at 6 M GuCl (--) the spectra of both the apo- and holo-enzymes were identical.

Figure 3 GuCl-dependent unfolding of apo- and holo-pkDDC measured by fluorescence emission at 326 nm

Excitation was at 278 nm. Relative fluorescence was normalized to the difference between 0 and 6 M GuCl. Buffer was as described in the legend of Figure 1, at 25 °C. Protein concentration was 5 $\mu\text{g}/\text{ml}$. Key to symbols: ●, apo; ○, holo. Inset: effect of increasing GuCl concentration on the emission maximum when holoenzyme was excited at 278 nm. An identical transition curve was obtained for apoenzyme.

intensity decreases further, reaching a minimum value at 4.5 M GuCl. The emission maximum displays a gradual red shift up to 4 M, followed by a sharp increase to 355 nm. These changes would seem to reflect the complete exposure of the tryptophan residues to the solvent.

Figure 4 GuCl-dependent unfolding of holo-pkDDC measured by c.d. spectroscopy

Buffer was as described for Figure 1, at 25 °C. Protein concentration was 0.13 mg/ml. Changes in ellipticity were measured at 222 nm at the indicated GuCl concentrations. Inset: C.d. spectra of holo-pkDDC in GuCl solutions of differing concentrations.

C.d. analysis

The far-u.v. c.d. spectrum of holoDDC, in the absence of GuCl, is shown in the inset of Figure 4. The spectrum displays minima at 208 nm and 222 nm, which is characteristic of a protein with a high content of α -helical structure. From the value of θ_{222} , the α -helix content is calculated to be 45%. When holoDDC was incubated in the presence of increasing concentrations of GuCl, there was a decline in the far-u.v. c.d. signals, reflecting the loss

of secondary structure (inset of Figure 4). Figure 4 shows that the mean residue ellipticity, θ_{222} , decreased as the concentration of GuCl was increased. The overall process appears to be biphasic and an intermediate state, which possesses about 50% of the native c.d. signal, appeared to be stable at about 2 M GuCl. The unfolding transition curve of apoDDC is identical to that of the holoenzyme (results not shown); thus, the presence of PLP does not appear to stabilize the secondary structure during the unfolding process.

Hydrodynamic measurements

Changes in the hydrodynamic volume of pkDDC during the unfolding process were investigated using size-exclusion chromatography through a gradient of increasing GuCl concentration. At 0 M GuCl pkDDC has a retention volume of 12.4 ml; this is consistent with an apparent molecular mass of 104 kDa, as expected for the homodimeric enzyme. As the GuCl concentration is increased the retention volume of the protein also increases, probably due to the dissociation of pkDDC into monomers. At 2 M GuCl the protein has a retention volume of 13.1 ml, corresponding to an apparent molecular mass of 56 kDa, which is in good agreement with the true subunit molecular mass of 53858 Da. It is possible that the subunits are in a globular conformation, although not as compact as in the dimeric form. It should be noted that a second peak elutes at 1.44 M and 1.75 M GuCl, the retention volume of which corresponds to a dimeric species, thus indicating that at these denaturant concentrations the dissociation into monomers is slow compared with the retention time on the column. Increasing the GuCl concentration beyond 2.2 M led to a gradual decrease of elution volume, corresponding to the complete unfolding of the monomeric protein (Figures 5a and 5b).

Binding of coenzyme

The absorption spectrum of holoenzyme shows peaks at 335 nm and at 420 nm (Borri Voltattorni et al., 1971, 1979), which are associated with an asymmetrically bound coenzyme, as shown by

Figure 5 Dissociation and unfolding of holo-pkDDC as monitored by GuCl-gradient gel-filtration chromatography

Experimental conditions were as described in the Experimental section. (a) Elution profiles of pkDDC at the indicated concentrations of GuCl. (b) Change of elution volume as a function of GuCl concentration (●). Open circles represent the elution volume of the second peak at 1.44 and 1.75 M GuCl as described in the text (see also a).

Figure 6 Visible absorption (a) and dichroic (b) spectra of holo-DDC in the presence of increasing GuCl concentrations

Buffer was as described for Figure 1, at 20 °C. Protein concentration was 0.53 mg/ml in (a) and 1.5 mg/ml in (b). For both (a) and (b), 0 M GuCl (curve A), 0.5 M GuCl (curve B) and 1 M GuCl (curve C) are shown.

Figure 7 Fluorescence of NaBH_4 -reduced holo-pkDDC as a function of GuCl concentration when excited at 325 nm

Changes in emission intensity were measured at 385 nm for (○) pkDDC and (●) free PMP. Buffer was as described for Figure 1, at 25 °C. Protein concentration was 5 $\mu\text{g}/\text{ml}$. Inset: Effect of GuCl on the emission maximum of reduced holo-pkDDC.

the visible c.d. spectra (Borri Voltattorni et al., 1979). The presence of increasing concentrations of GuCl causes the gradual decrease of these absorbing and dichroic bands and the concomitant appearance, due to free PLP, of a 388-nm-absorbing band (Figures 6a and 6b). Above 1 M GuCl no further changes were observed, thus indicating that the coenzyme has fully dissociated from the enzyme in the presence of 1 M GuCl.

The fluorescence emission intensity of NaBH_4 -reduced holo-DDC in the presence of increasing concentrations of denaturant was measured to provide information on the conformational changes that GuCl induces on the coenzyme-binding

region. In fact, upon reduction of the holoenzyme with NaBH_4 , the resulting coenzymic form is bound to the protein through a single covalent bond and is therefore free to rotate easily. Thus this probe, through changes in its fluorescence, reports even minute conformational changes occurring in this site. It is not possible at present to distinguish whether the single molecule of coenzyme per dimer is bound asymmetrically or is shared between the two subunits. Nonetheless, these fluorescence signals reflect changes in the PLP microenvironment regardless of the coenzyme location. When excited at 325 nm, NaBH_4 -reduced enzyme displays an emission spectrum centred at 385 nm. In the presence of increasing concentrations of GuCl, marked changes occur in the range up to 2 M. The fluorescence intensity increases dramatically, reaching a maximum at 1 M GuCl followed by a decline reaching a value coincident with that of the free chromophore (at the same concentration as the reduced coenzyme) at 2 M (Figure 7). Additionally, a gradual shift in the emission maximum from 385 nm to 398 nm occurs over the range 0–2 M, after which no further changes are observed, either in the emission maximum or intensity (Figure 7 and inset). These results would seem to indicate that between 0 and 1 M GuCl local structural alterations of the coenzyme microenvironment take place, and that at 2 M GuCl the local environment of the PLP-binding site is completely exposed to the solvent.

We also performed measurements of the intrinsic fluorescence of the reduced holoenzyme. This enzymic species fluoresces not only at 326 nm, but also at 390 nm (results not shown). This PLP emission is probably due to the occurrence of energy transfer from tryptophan residue(s) to the reduced coenzyme. A similar process has been observed already for *N*-(bromoacetyl)pyridoxamine 5'-phosphate-modified apoenzyme (Dominici et al., 1991a). The intrinsic emission fluorescence spectra of reduced holo-DDC in GuCl solutions up to 6 M are characterized by changes (red shift of the 326 nm emission and a decrease in the emission intensity) identical to those observed for the apoenzyme. Thus, reduction of the internal aldimine does not seem to alter the stability of the enzyme, or at least does not change the profile of tertiary structure loss upon progressive addition of denaturant. In addition, the 385 nm emission begins to decrease at 1 M GuCl and fully disappears at 2 M, thus indicating that in this range the adequate requirements of distance and orientation between donor

and acceptor fluorophores, which give rise to the energy-transfer process in the reduced holoenzyme, are not met.

Turbidity measurements during unfolding

Aggregation is considered a general feature of proteins in solution which can be observed also under moderate or even strong denaturing conditions. Evaluation of aggregate formation by turbidity measurements during the unfolding process of pkDDC indicated that the intensity of light diffracted by pkDDC solutions reaches a maximum at about 1 M GuCl (in the range 0.5–4 M). While this phenomenon was not observed at low (e.g. 5 µg/ml) protein concentrations, it becomes marked at 50 µg/ml and increases considerably at higher concentrations (results not shown).

Attempted refolding of enzyme after denaturation

As noted previously, partial enzymic activity can be restored after treatment with denaturant providing that the initial GuCl concentration is < 1.5 M (Figure 1). At higher concentrations of denaturant, there is an irreversible loss of the ability of the enzyme to be reactivated. Attempts to reactivate pkDDC under conditions recommended by Jaenicke and Rudolph (1989) for oligomeric proteins proved to be unsuccessful. No reactivation was observed starting from material denatured at GuCl concentrations of 1.5 M or higher, either by quick dilution or step dialysis. Similarly, neither a low (1–5 µg/ml) nor a high (50–100 µg/ml) final concentration of pkDDC after dilution or dialysis had any effect on the degree of activity regained. With the use of high protein concentrations aggregates were easily seen when the concentration of GuCl was less than 2 M. These aggregates were attributed to non-specific hydrophobic interactions rather than interchain disulphide bond formation, based on the observation that after 'refolded' samples were subjected to SDS/PAGE, in the absence or presence of 2-mercaptoethanol, aggregates were not seen. It is likely that incorrect intermolecular reactions occur between sticky exposed hydrophobic stretches, resulting in aggregation rather than allowing the correct hydrophobic core of the protein to re-form. We therefore focused our further efforts using a low final concentration of protein. The addition of the cofactor PLP, 2-mercaptoethanol, non-ionic detergent, the use of different pH levels in the inactivation buffer, or a variation of the time allowed for reactivation all resulted in failure to restore pkDDC activity to the unfolded polypeptide. The aforementioned conditions yielded fluorescence spectra quite dissimilar to that of native protein. The addition of the 'structure making' compound sodium sulphate to buffer A also failed to restore any detectable activity. However, when fully denatured samples were brought, by quick dilution (100-fold) in the presence of 0.9 M sodium sulphate, to a GuCl concentration that would be expected to support native enzyme, a fluorescence spectrum almost coincident with that of the native enzyme was observed. This species, termed X, exhibits a 1 nm red shift in the emission maximum and a 15% increase in emission intensity with respect to the native enzyme, while its hydrodynamic volume is between that of the native enzyme and the fully unfolded form. The fact that X is able to interact with coenzyme was demonstrated by the observation that it exhibits a fluorescence maximum at 390 nm (excitation 325 nm) after reduction with NaBH₄ in the presence of exogenous PLP and dialysis. Nonetheless, the reduced species does not display energy transfer (results not shown). Unfortunately, due to the low concentration of protein obtained after dilution, c.d. measurements could not be carried out. Characterization of the unfolding transition of X by GuCl, as

monitored by intrinsic fluorescence emission, showed that with an increasing concentration of denaturant there is a decrease of the emission intensity accompanied by a red shift. Unlike that of the native enzyme, the denaturation transition is symmetrical and sigmoidal with a pre-transition baseline from 0 to 1 M GuCl and a post-transition baseline from 4 to 6 M (results not shown). Therefore, denaturation of this species and the native enzyme differ in the 1.5–4 M GuCl concentration range.

To investigate the reversibility of the refolding process further, fully denatured samples were subjected to step-dilution in the presence of sodium sulphate and changes in fluorescence were monitored. In the range of 6–2 M GuCl the values of emission intensity and the shift of the emission maximum from 355 to 341 nm parallel those of the unfolding pathway. In the range below 2 M there was no further change in the emission maximum. Although the values of the emission intensity varied greatly from experiment to experiment, they were, however, always consistently lower than those of the unfolding pattern. These results support the concept that the refolding process is fully reversible only in the range of 2–6 M GuCl, at least under the conditions tested.

DISCUSSION

Several experimental properties which reflect different aspects of the structural integrity of pkDDC were monitored using different signals through the course of equilibrium denaturation. Non-coincident equilibrium transition curves were obtained, thus suggesting that a two-state model is inadequate to describe the overall unfolding process of the enzyme and that the unfolding of pkDDC is a complex process involving intermediates that are detectable at equilibrium.

The unfolding process itself can be divided into at least three general steps. The first step is associated with the loss of pkDDC activity. In the GuCl concentration range of 0–1 M, where the enzyme has been unfolded to a very slight extent, pkDDC activity is largely, if not completely, lost. This result reflects the general observation that active sites are usually confined to a limited region of the enzyme molecule, which is more flexible and more easily affected by denaturants than the rest of the protein. As revealed by absorption and c.d. spectra of holoDDC in the absence and in the presence of various GuCl concentrations, PLP appears to be released in this concentration range. The supposition that the protein loses the coenzyme is supported by the observed changes in the intrinsic fluorescence emission intensity of holo-DDC in this GuCl concentration range. Additionally, phosphopyridoxyl-lysine was used as a probe in the NaBH₄-reduced holoenzyme to follow local conformational changes at the active site during the denaturation process. When excited at 325 nm the first fluorescent transition is coincident with the loss of activity, strongly suggesting that a slight disturbance of the correct spatial geometry of the functional groups in the PLP microenvironment has occurred.

The results of gel filtration through a GuCl gradient indicate that pkDDC remains in its dimeric form over the 0–1 M denaturant concentration range. Thus, the first step in pkDDC denaturation, in which enzymic activity is completely lost, does not involve its dissociation into subunits, but rather loss of coenzyme. The observation that this inactivation transition is independent of protein concentration further supports this hypothesis (results not shown). For many oligomeric proteins the dissociation into subunits upon exposure to denaturant is the first step in the unfolding process (Parr and Hammes, 1975; Jaenicke et al., 1981; Yao et al., 1984; Herold and Kirschner, 1990; Kelly and Price, 1991). This event usually results in the loss

of enzymic activity and suggests, at least in these systems, that the intersubunit interactions are relatively weak compared with the interactions responsible for the formation of the secondary and tertiary structure of the individual subunits. Nonetheless, inactivation of multimeric enzymes before subunit dissociation and unfolding has been documented (Rinas et al., 1990; Liang et al., 1990; Nozais et al., 1992). In pkDDC, the inactivation transition gives rise to a dimeric but inactive species, D', which retains almost all of the native fluorescence and c.d. signals. The species does not, however, bind PLP. It should be noted that pkDDC loses PLP at a different GuCl concentration from that observed in other PLP-dependent enzymes. In aspartate aminotransferase from *E. coli*, PLP dissociates during the transition from structured monomers to a compact intermediate monomeric conformation, which takes place at concentrations between 0.6 and 0.9 M GuCl (Herold and Leistler, 1992). In cytosolic aspartate aminotransferase from pig heart, the complete unfolding is accompanied by coenzyme dissociation at about 6 M GuCl (West and Price, 1989). In contrast, mitochondrial aspartate aminotransferase is completely unfolded at 2 M GuCl, losing PLP at the same concentration (West and Price, 1990). Most likely, the loss of the active-site integrity which characterizes D' results from a conformational change which exposes previously buried hydrophobic residues to solvent, thus favouring the formation of aggregates. Similar aggregation of denaturation intermediates has been observed for a wide variety of proteins which are quite often oligomeric (Parr and Hammes, 1975; Jaenicke et al., 1981; Zettlemessl et al., 1984; Rinas et al., 1990; Liang et al., 1990; Herold and Kirschner, 1990; Plomer and Gafni, 1992). This phenomenon could be responsible for the partial reversibility of this transition.

The second step of the unfolding process represents the dissociation of the dimeric enzyme into its subunits ($D' \rightarrow 2M$) and occurs at levels of GuCl between 1 and 2.2 M. This transition alters not only the quaternary structure but also the secondary structure, as indicated by changes in ellipticity, and the tertiary structure, as indicated by changes in intrinsic fluorescence. This phase is also characterized by: (i) the complete exposure to the solvent of the reduced PLP, as revealed by its emission intensity, which is almost identical to that of free PMP, and (ii) the disappearance of the energy-transfer process between tryptophan residue(s) and the reduced coenzyme. These latter observations indicate that, although the enzyme molecule is not yet in the completely unfolded form, in this concentration range gross conformational changes have occurred at or near the PLP microenvironment. This process also affects the distance and the orientation of the donor-acceptor pair. Judging from the aforementioned characteristics of this transition, it is possible that this step gives rise to a 'structured' monomer.

The third step, presumably representing the complete unfolding of pkDDC subunit, appears to be complete at about 4.5 M GuCl, as indicated by the lack of further substantial changes in any of the parameters followed.

Starting from the unfolded form, pkDDC was able to regain on step-dilution or dialysis its fluorescence features only down to about 2 M GuCl. This suggests that the bimolecular reaction corresponding to the monomer-dimer transition ($2M \rightarrow D'$) is impaired, at least under the experimental conditions tested. The inability of the enzyme to reassociate from partially folded subunits could be due to the formation of wrong conformers and/or to aggregation during the refolding process, competing with the reformation of the native structure. Renaturation of pkDDC by fast dilution of completely unfolded enzyme to conditions favouring the native state yielded a 'refolded' form X, which is inactive and binds PLP, but does not display energy

transfer when reduced with NaBH_4 . It was not possible to distinguish whether the species is in the monomeric or the dimeric state from measurements of the hydrodynamic volume. Furthermore, since attempts to cross-link the native enzyme have been unsuccessful, this technique cannot be used to resolve this question. Characterization of the secondary structure is hampered by the inability to take c.d. measurements at low protein concentrations. However, fluorescence measurements showed only a slight red shift and a modest increase in intensity with respect to the native enzyme, suggesting that the tertiary structure has largely been restored. It is likely that when the concentration of GuCl is lowered suddenly, the enzyme is prevented from acquiring its proper native structure by kinetic-energy barriers. Thus the species X, which has the properties of neither the denatured nor the native enzyme, could represent a kinetically trapped conformer that limits refolding. Furthermore, it should be noted that the symmetrical shape of the denaturation transition of the species X is consistent with a two-state denaturation mechanism involving only X and the unfolded monomer.

Based on these studies, a simplified model for the unfolding/refolding of pkDDC can be proposed:

where D and D' indicate 'folded dimeric forms' of the enzyme which are the fully active and partially reversible inactivated states respectively; M represents the 'structured' monomer; U, the unfolded monomer; and X the inactive abortive state. In the above scheme, continuous and broken arrows indicate reversible and irreversible transitions respectively. Such a model is the simplest plausible mechanism which would account for most of the data obtained up to now. Whether or not this scheme completely describes the denaturation of pkDDC is currently being investigated by kinetic studies which should provide a more complete resolution between potential refolding and reassociation steps.

This research was supported by grants from the Italian MURST. We thank Dr. Paola Filipponi for performing the c.d. measurements.

REFERENCES

- Borri Voltattorni, C., Minelli, A. and Turano, C. (1971) *FEBS Lett.* **17**, 231–235
- Borri Voltattorni, C., Minelli, A., Vecchini, P., Fiori, A. and Turano, C. (1979) *Eur. J. Biochem.* **93**, 181–188
- Borri Voltattorni, C., Minelli, A. and Dominici, P. (1983) *Biochemistry* **22**, 2249–2254
- Borri Voltattorni, C., Giartosio, A. and Turano, C. (1987) *Methods Enzymol.* **142**, 179–187
- Charteris, A. and John, R. A. (1975) *Anal. Biochem.* **66**, 365–371
- Dominici, P., Maras, B., Mei, G. and Borri Voltattorni, C. (1991a) *Eur. J. Biochem.* **201**, 393–397
- Dominici, P., Maras, B., Barra, D., Bossa, F. and Borri Voltattorni, C. (1991b) in *Enzymes* Dependent on Pyridoxal Phosphate and Other Carbonyl Compounds as Cofactors (Fukui, T., Kagamiyama, H., Soda, K. and Wada, H., eds.), pp. 83–85, Pergamon Press, Oxford
- Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
- Fiori, A., Turano, C., Borri Voltattorni, C., Minelli, A. and Turano, C. (1975) *FEBS Lett.* **54**, 122–125
- Ghéllis, C. and Yon, J. M. (1982) in *Protein Folding* (Horecker, B., ed.), pp. 289–296, Academic Press, Orlando, FL
- Herold, M. and Kirschner, K. (1990) *Biochemistry* **29**, 1907–1913
- Herold, M. and Leistler, B. (1992) *FEBS Lett.* **308**, 26–29
- Jaenicke, R. (1978) *Naturwissenschaften* **65**, 569–577
- Jaenicke, R. and Rudolph, R. (1986) *Methods Enzymol.* **131**, 218–250
- Jaenicke, R. and Rudolph, R. (1989) in *Protein Structure – A Practical Approach* (Creighton, T. E., ed.), pp. 191–233, IRL Press, Oxford
- Jaenicke, R., Vogel, W. and Rudolph, R. (1981) *Eur. J. Biochem.* **114**, 525–531
- Kelly, S. M. and Price, N. C. (1991) *Biochem. J.* **275**, 745–749

- Kim, P. S. and Baldwin, R. L. (1982) *Annu. Rev. Biochem.* **51**, 459–489
- Liang, S., Lin, Y., Zhou, J., Tsou, C., Wu, P. and Zhou, Z. (1990) *Biochim. Biophys. Acta* **1038**, 240–246
- Malashkevich, V. N., Fillipponi, P., Sauder, U., Dominici, P., Jansonius, J. N. and Borri Voltattorni, C. (1992) *J. Mol. Biol.* **224**, 1167–1170
- Maras, P., Dominici, P., Barra, D., Bossa, F. and Borri Voltattorni, C. (1991) *Eur. J. Biochem.* **201**, 385–391
- Nozais, M., Bèchet, J.-J. and Houadjieto, M. (1992) *Biochemistry* **31**, 1210–1215
- Nozaki, Y. (1972) *Methods Enzymol.* **27**, 43–50
- Parr, G. R. and Hammes, G. G. (1975) *Biochemistry* **14**, 1600–1608
- Plomer, J. J. and Gafni, A. (1992) *Biochim. Biophys. Acta* **1122**, 234–242
- Rinas, U., Risse, B., Jaenicke, R., Abel, K.-J. and Zettlmeissl, G. (1990) *Biol. Chem. Hoppe-Seyler* **371**, 49–56
- Scopes, R. K. (1974) *Anal. Biochem.* **59**, 277–282
- Sherald, A. F., Sparrow, J. C. and Wright, T. R. F. (1973) *Anal. Biochem.* **56**, 300–305
- West, M. and Price, N. C. (1989) *Biochem. J.* **261**, 189–196
- West, M. and Price, N. C. (1990) *Biochem. J.* **265**, 45–50
- Yao, Q.-Z., Tian, M. and Tsou, C.-L. (1984) *Biochemistry* **23**, 2740–2744
- Zettlmeissl, G., Teschner, W., Rudolph, R., Jaenicke, R. and Gade, G. (1984) *Eur. J. Biochem.* **143**, 401–407