Eur. J. Biochem. *255*, 439−445 (1998)

Stability of *Escherichia coli* phospho*enol*pyruvate carboxykinase against urea-induced unfolding and ligand effects

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(Received 3 February 1998) − EJB 98 0160/3

The urea-induced unfolding at pH 7.5 of *Escherichia coli* phospho*enol*pyruvate (*P*-pyruvate) carboxykinase was studied by monitoring the enzyme activity, intrinsic protein fluorescence, circular dichroism spectra, and 1-anilino-8-naphthalenesulfonate binding. These studies were performed in the absence and presence of substrates and ligands. ATP or *P*-pyruvate plus $MnCl_2$, or of the combined presence of ATP plus $MnCl_2$ and oxalate, conferred great protection against urea-induced denaturation. The unfolding process showed the presence of at least one stable intermediate which is notably shifted to higher urea concentrations in the presence of substrates. This intermediate protein structure was inactive, contained less tertiary structure than the native protein and retained most of the original secondary structure. Hydrophobic surfaces were more exposed in the intermediate than in the native or unfolded species. Refolding experiments indicated that the secondary structure was completely recovered. Total recovery of tertiary structure and activity was obtained only from samples denatured at urea concentrations lower than those where the intermediate accumulates.

Keywords: phospho*enol*pyruvate carboxykinase; urea unfolding; unfolding intermediate; ligand effects on unfolding.

Escherichia coli phospho*enol*pyruvate carboxykinase catalyzes the ATP-dependent decarboxylation and phosphorylation of oxaloacetate to phospho*enol*pyruvate (*P*-pyruvate), ADP, and CO_2 in the presence of a bivalent metal ion, corresponding to one of the first steps in the biosynthesis of glucose from three and four carbon precursors [1].

This enzyme is a monomer of 540 residues with a molecular mass of 59 583 Da [2]. From the X-ray crystal structure, it has been deduced that the polypeptide consists of a 275-residue N-terminal domain and a 265-residue C-terminal or mononucleotide-binding domain, with the active site located within a cleft between the two domains [2]. The X-ray diffraction data indicates 27% α helix, comparable with the 24% content estimated from CD spectroscopy [3]. Kinetic studies of this enzyme and of the homologous *Saccharomyces cerevisiae P*-pyruvate carboxykinase [4] suggested the existence of two metal-ion-binding sites, one binding a cation-nucleotide complex and the other binding a free divalent cation [5, 6]. Work carried out in GTP-dependent *P*-pyruvate carboxykinases indicate that Mn^{2+} is the most effective cation for binding and activation at the free divalent cation-binding site, while Mn^{2+}, Mg^{2+} or other cations can form the metal-nucleotide complex which serves as the substrate for the reaction [7−9]. A structure for the quaternary complex between the enzyme, oxaloacetate, Mn^{2+} and MgGTP has been proposed, where protein-bound Mn^{2+} bridges oxaloacetate and the γ-phosphoryl group of MgGTP in the enzyme active site [10].

In *E. coli P*-pyruvate carboxykinase, the binding of Mn^{2+} or MgATP originates alterations in the intrinsic fluorescence, thus implying conformational changes in the protein [11]; however, the lack of alterations in the CD and Fourier transform infrared spectra indicate that they do not affect the enzyme secondary structure [3]. Recently, Tari et al. [12] have crystallised a complex between the *E. coli* enzyme, ATP, Mg^{2+} and oxalate (a structural analog of *enol*pyruvate, a proposed reaction intermediate [10, 13]) showing that a 20° rotation movement of the domains occurs upon binding of the ATP-Mg^{2+}-oxalate complex. Evidence of ligand-induced conformational changes have been also reported for chicken and rat liver *P*-pyruvate carboxykinases. For the chicken liver enzyme, it has been suggested [14, 15] that the binding of IDP and HCO_3^- in the presence of Mn^{2+} produces a protein conformational change which alters the metal environment, while Chen et al. [16] reported rapid structural changes upon MgGTP binding. The binding of Mn^{2+} or the magnesium complexes of guanosine and inosine nucleotides to the rat liver enzyme produces conformational changes that are easily detected by intrinsic fluorescence quenching [11].

In spite of the information available on the structure and ligand-induced conformational changes in *P*-pyruvate carboxykinases, the conformational changes during the unfolding and refolding of this enzymes have not been explored. At this respect, folding/unfolding studies can give valuable clues on the

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Abbreviations. ANS, 1-anilino-8-naphthalenesulfonate; GdnHCl, guanidinium hydrochloride; *P*-pyruvate, phospho*enol*pyruvate.

Enzymes. Malate dehydrogenase, L-malate:NAD^+ oxidoreductase (EC 1.1.1.37); octopine dehydrogenase, 17β-oestradiol-UDP glucuronyltransferase (EC 2.4.1.59); phospho*enol*pyruvate carboxykinase, ATP/GTP:oxaloacetate carboxylyase (transphosphorylating) (EC 4.1.1.48/32); pyruvate kinase, ATP:pyruvate 2-*O*-phosphotransferase (EC 2.7.1.40); rhodanese, thiosulphate:cyanide sulphurtransferase (EC 2.8.1.1).

conformational stability of proteins. In this work, we have studied the effect of urea on the equilibrium unfolding of *E. coli* *P*-pyruvate carboxykinase, an enzyme from the ATP-dependent class of *P*-pyruvate carboxykinases that presently is the only *P*-pyruvate carboxykinase for which a three-dimension structure is available [2]. Particular relevance was given to the unfolding mechanism in the absence and presence of substrates and metal ions.

EXPERIMENTAL PROCEDURES

Materials. Urea (electrophoresis grade), GroEL/GroES mixture (Chaperonin 60/Chaperonin 10, 1:1 mixture), *P*-pyruvate, ATP, $MnCl_2$, $MgCl_2$ and N-Hepes, were from Sigma Chemical Co. Sodium oxalate was from Merck. Concentrated stock solutions of urea were prepared in 50 mM Hepes, pH 7.5, immediately before use. 1-anilino-8-naphthalenesulfonate (ANS) was from Molecular Probes, and its concentration was determined from $\varepsilon = 6800\ M^{-1}\ cm^{-1}$ at 370 nm. *E. coli* *P*-pyruvate carboxykinase was purified as described [17]. The enzyme concentration was determined using an absorption coefficient of $67400\ M^{-1}\ cm^{-1}$ at 280 nm [2, 5].

***P*-pyruvate carboxykinase assay.** The enzyme was assayed at 30 °C as described [18] using a continuous assay which couples *P*-pyruvate carboxykinase to malate dehydrogenase, except that 100 mM sodium bicarbonate was used. In order to avoid enzyme refolding during the activity determinations, urea was included in the assay medium at the appropriate concentrations. It was determined that the auxiliary enzyme remained active during the assay times under these conditions.

Spectroscopic measurements. Most spectroscopic experiments were done with a protein concentration of 0.1 mg/ml. Fluorescence measurements were made at 20 °C in a Spex 1681 Spectrolog fluorimeter with 1.25 nm slits for excitation and emission. The excitation wavelength to follow the tryptophan fluorescence was 290 nm. The binding of ANS to *E. coli* *P*-pyruvate carboxykinase was monitored by the shift of the dye λ_{max} emission; the excitation was at 390 nm. CD spectroscopy was carried out at 20 °C in a Jasco J-720 spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. The spectra were acquired in the 200−260-nm wavelength region using a cell of pathlength 0.1 mm or 0.2 mm. Each spectrum was the average of at least ten scans, with a time resolution of 2 s.

Sample preparation. For denaturation experiments, protein samples were incubated in 50 mM Hepes, pH 7.5, for at least 5 h, at 16 °C, at each urea concentration. No changes were apparent at longer incubation times (up to 24 h). To compare the transitions detected by the different methods, each unfolding curve was expressed relative to the folded form, Y_F, using the following relationship:

$$Y_F = \frac{P_{obs} - P_U}{P_N - P_U} \times 100 \qquad (1)$$

where P_{obs} is the value of the parameter being monitored at a given denaturating condition and P_N and P_U are the values for the native and unfolded state, respectively. Data points are the average of at least two unfolding experiments.

Renaturation experiments. The protein samples were incubated for 5 h at 20 °C at different urea concentrations. After incubation, the samples were diluted 6−8-fold in the buffer and left to stand for 1 h at room temperature before the spectroscopic or activity measurements were done. This time period was sufficient for refolding to occur. Incubation for longer periods did not vary the recovery of the measured property. For the experiments in the presence of substrates, the dilution buffer contained appropriate concentrations of the different ligands.

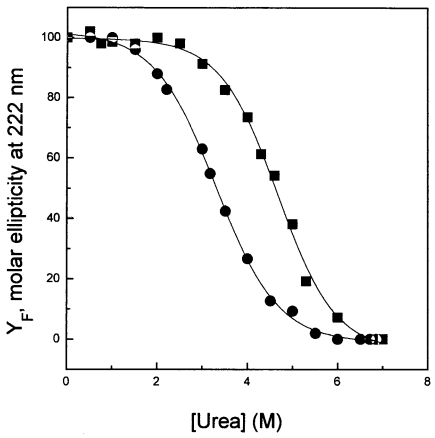

Fig. 1. Ellipticity at 222 nm of *E. coli* *P*-pyruvate carboxykinase equilibrated in urea. The enzyme (0.2 mg/ml), was equilibrated for 10 h with different urea concentrations and the CD spectra were recorded. Shown are the ellipticity changes calculated according to equation 1 in the absence (●) or presence (■) of 0.2 mM ATP plus 0.4 mM $MnCl_2$. Each point represents the average of at least three different measurements. Standard deviations were less than 5%.

Ultracentrifugation experiments. Measurements were carried out in 30 mM Hepes, pH 7.5, with the indicated urea concentrations. All experiments were performed in a Beckman XLA-Analytical Ultracentrifuge at 15 °C, employing sample volumes of 80 µl in two-channel and six-channel cells, in a titanium rotor at 8000, 12000, 14000, 18000 and 20000 rpm. Radial scans of absorbance at 275, 245 and 230 nm were taken at 2 h intervals after 10 h centrifugation, when equilibrium was achieved (successive scans gave no significant difference). Baselines were determined after 5 h at 42000 rpm and return to the speed of the experiment, and subtracted when necessary. Partial specific volume values used [19] were 0.735 in buffer at 15 °C, and 0.721 in 8 M urea [20]. Values at intermediate urea concentrations were calculated by direct interpolation. Maximum deviation from the real value was estimated to be 0.4%, which implies an error of 1.3% in the molecular-mass determination. Urea concentrations were determined refractometrically. Density values used were the tabulated values for urea at the desired concentration at 20 °C [21]. Differences in tabulated values at 4−20 °C were around 0.2%. Molecular masses were calculated using the Eqassoc program [22].

RESULTS

Unfolding detected by CD spectroscopy. The far-ultraviolet CD spectrum of the *E. coli* *P*-pyruvate carboxykinase has been previously described, and the secondary structure content has been estimated [3]. The unfolding was followed by the decrease of the CD ellipticity at 222 nm as a function of increasing urea concentrations. The urea-induced unfolding showed a monophasic transition with midpoint at 3.3 M urea (Fig. 1). At 5 M urea, a continuous spectrum was observed (not shown), indicating an almost complete loss of secondary structure. In the presence of saturating amounts of ATP plus $MnCl_2$, the midpoint was shifted to 4.8 M urea (Fig. 1).

Unfolding detected by fluorescence. *E. coli* *P*-pyruvate carboxykinase has nine Trp, six in the N-terminal and three in the C-terminal domains of the protein [2]. The characteristics of the fluorescence spectrum are therefore due to residues located over the whole protein molecule. When excited at 290 nm, the en-

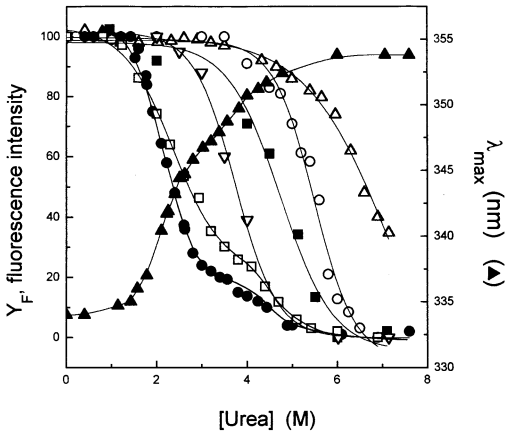

Fig. 2. Characteristics of the intrinsic fluorescence of *E. coli P*-pyruvate carboxykinase as a function of urea concentration. The enzyme (0.1 mg/ml), was equilibrated for 10 h at 15 °C with different urea concentrations. Shown are the fluorescence intensity at 334 nm (●) and the emission maximum wavelength (▲). The enzyme was also incubated at the indicated urea concentrations in the presence of 0.2 mM ATP plus 0.4 mM $MgCl_2$ (□), 2 mM *P*-pyruvate (▽), 0.2 mM ATP plus 0.4 mM $MnCl_2$ (■), 0.2 mM ATP plus 0.2 mM oxalate and 0.2 mM $MnCl_2$ (○), or 2 mM *P*-pyruvate plus 2 mM $MnCl_2$ (△). These fluorescence intensity data are expressed as relative changes, calculated according to Eqn 1.

Table 1. Concentration of urea required for 50% unfolding of *E. coli P*-pyruvate carboxykinase in the presence of ligands, as calculated from the change in fluorescence emission at 334 nm. Experiments were carried out as described in Fig. 2. The enzyme concentration was 0.1 mg/ml, and the concentrations of added ligands (mM) were: $MgCl_2$, 0.4; $NaHCO_3$, 25; ATP, 0.2; sodium oxalate, 0.2; *P*-pyruvate, 2. $MnCl_2$ was 0.2 mM in all cases but 2 mM when $NaHCO_3$ or *P*-pyruvate were tested. Errors represent the standard deviation of two or more different unfolding experiments

Ligand	Urea concentration
	M
None	2.3 ± 0.1
$MgCl_2$	2.3 ± 0.1
$MnCl_2$	2.3 ± 0.1
$NaHCO_3$	3.0 ± 0.1
$NaHCO_3$ + $MnCl_2$	4.1 ± 0.2
ATP	3.0 ± 0.2
ATP + $MgCl_2$	2.6 ± 0.1
ATP + $MnCl_2$	4.6 ± 0.1
Oxalate + $MnCl_2$	3.6 ± 0.1
Oxalate + ATP + $MnCl_2$	5.4 ± 0.1
Oxalate + ATP + $MgCl_2$	3.1 ± 0.1
P-pyruvate	3.7 ± 0.2
P-pyruvate + $MnCl_2$	6.7 ± 0.2

zyme shows a fluorescence maximum at 334 nm, characteristic of Trp sidechains partially shielded from the aqueous solvent [11]. Addition of increasing concentrations of urea caused a progressive quenching of the fluorescence intensity and a red shift in the emission maximum. At 6 M urea, the λ_{max} was 352 nm, the value found for fully exposed Trp, indicating that the unfolded state does not contain residual structures. The change in emission λ_{max} with the urea addition showed (Fig. 2) a monophasic transition with a midpoint of 2.7 M urea. The fluorescence intensity is markedly quenched by urea addition; it decreased approximately 50% of the initial value at 6 M urea. The data, normalized with respect to the fluorescence of the native protein, are shown (Fig. 2). These results show that fluorescence intensity changes associated with the denaturant concentration present a different pattern to that monitored by the shift in λ_{max}; the overall process detected is biphasic with a first transition that presents a fairly sharp change and reaches approximately 75% of the initial value. This transition is sigmoidal in nature and is centered at 2.1 M urea. A second broad transition starts at 2.8 M urea, reaching a constant value at 6 M urea. At 2.8 M urea, λ_{max} has experienced a 50% change, and 75% secondary structure remains. This biphasic behaviour reveals the presence of an intermediate in the unfolding process, where the protein has lost much of its tertiary structure.

Mn^{2+} ion does not affect the unfolding pattern detected by fluorescence intensity. Substrates in the absence of metal ions or in the presence of Mg^{2+} caused minor protection effects, and the unfolding curves remained biphasic. However, substrates in the presence of Mn^{2+} elicited a strong protection of unfolding by urea (Fig. 2), and the curves became monophasic. Urea concentrations required for 50% unfolding of the enzyme in the presence of several ligands are shown (Table 1). At 5 M urea in the presence of *P*-pyruvate plus $MnCl_2$, 95% of the tertiary structure remains, whilst in the absence of substrates it is completely lost.

ANS binding. To gain further insight on the intermediate unfolding stage, the binding of ANS to *E. coli P*-pyruvate carboxykinase was studied as a function of the urea concentration, both in the free and substrate-bound enzyme. It is well established

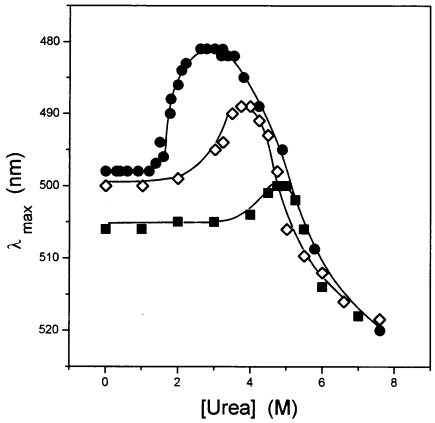

Fig. 3. ANS binding as a function of urea concentration. Enzyme samples (0.1 mg/ml) were incubated with different urea concentrations for 10 h in the absence of substrates (●), in the presence of 0.2 mM ATP plus 0.4 mM $MnCl_2$ (■), or in the presence of 25 mM $NaHCO_3$ plus 2 mM $MnCl_2$ (◇). Then, the solution was made 60 μM in ANS, and after 30 min the fluorescence spectra of ANS (λ_{exc} = 390 nm) was recorded. Wavelength at the maximum emission has an error of ± 1.5 nm.

that ANS binds with high affinity to nonpolar sites of proteins in the folded state and to hydrophobic intermediates, while it interacts very poorly with fully unfolded proteins [23]. ANS fluorescence intensity is markedly increased in nonpolar environments where a blue shift in the emission maximum occurs. In aqueous solution, ANS presents a maximum at 520 nm that decreases approximately 25 nm in the presence of *E. coli P*-pyruvate carboxykinase, indicating affinity of the protein for ANS. In the absence of substrates, λ_{max} was constant until 1.5 M urea, then it shifted to lower wavelengths reaching a minimum at 2.8 M urea (Fig. 3). These changes were also accompanied by an increase in the fluorescence intensity of ANS. Further urea additions resulted in a red shift on the spectral position and a decrease in the fluorescence intensity. At urea concentration of ≈7 M, λ_{max} reached 520 nm, the value obtained in aqueous me-

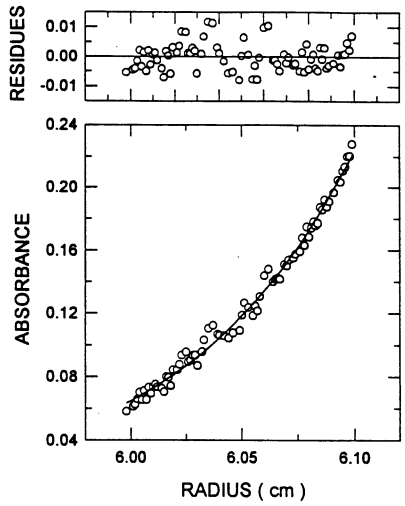

Fig. 4. Sedimentation equilibrium of *E. coli P*-pyruvate carboxykinase in urea solutions. The equilibrium sedimentation profile was carried out at 0.1 mg/ml in 30 mM Hepes, pH 7.5, and 3.0 M urea at 18000 rpm and 15°C. The continuous line represents the best fit to the data according to the program Eqassoc. Upper panel shows the residuals.

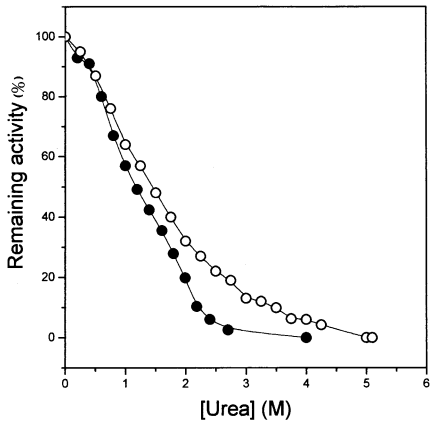

Fig. 5. Enzyme activity of *E. coli P*-pyruvate carboxykinase as a function of the urea concentration. The enzyme (0.1 mg/ml) was incubated 10 h at 16°C at the indicated urea concentrations (●). The activity of the enzyme measured without previous incubation in urea is also shown (○). The remaining enzyme activity was determined in a media containing the indicated urea concentrations. Each point represents duplicate determinations of activity. Changes in activity are expressed relative to the activity in the absence of urea. Further details in Experimental Procedures.

dia, showing that the denatured state of the protein does not bind ANS. These changes coincide with those found from the decrease of the Trp fluorescence intensity (Fig. 2). In contrast, both the blue shift and the increase in fluorescence intensity of ANS observed in the urea range 1.5−3 M suggest more exposed hydrophobic surface areas than the native state, and point to the existence of a rather non-polar stable intermediate in the denaturation process of *E. coli P*-pyruvate carboxykinase. ANS binds to the enzyme in the presence of substrates (Fig. 3) and it is clear from these ANS-binding experiments that the formation of an intermediate in the presence of urea, shifts to higher denaturant concentrations. The presence of an hydrophobic intermediate in the unfolding pathway was also detected when the enzyme was denatured in the presence of guanidinium hydrochloride (Gdn/HCl). This intermediate appears in the range 0.5−1.5 M Gdn/HCl (data not shown). The denaturant concentrations are lower than those obtained with urea, as expected from the stronger denaturant effect of Gdn/HCl.

Aggregation analyses by sedimentation equilibrium. The molecular mass of *E. coli P*-pyruvate carboxykinase was studied by sedimentation equilibrium analysis at different urea concentrations. The enzyme was sediment to equilibrium at 0.1−1 mg/ml and at four different speeds to measure the degree of self-association. For the native enzyme, the molecular mass was independent of the protein concentration and velocity of the experiment, and the mass average was 58.2 ± 3.2 kDa. At 0.1 mg/ml and at 3.0 M urea, the mass (59.5 kDa) was also velocity independent and the equilibrium sedimentation gradient could be described by a simple sedimenting species (Fig. 4). However, at 3.5 M urea, there was small aggregation speed dependency (68.5 kDa at 14000 rpm and 63.2 kDa at 18000 rpm). At 1 mg/ml and 3.0 M and 3.5 M urea, there was significant loss of material (about 50%) and the molecular mass showed a strong speed-dependency: at 3.0 M urea it shifted from 59.0 kDa at 14000 rpm to 55.5 kDa at 18000 rpm, while at 3.5 M urea it was 74 kDa at 14000 rpm, but 64 kDa at 18000 rpm. The loss in material and the velocity-dependency of the molecular mass imply aggregate formation at 1 mg/ml. The average molecular mass returned to 60 kDa in 8 M urea.

Enzyme activity. Activity measurements of the enzyme incubated to equilibrium in urea solutions, then assayed in media containing the same denaturant concentration as that during the incubation (Fig. 5), showed that the enzyme activity was affected by low urea concentrations. Thus, the protein lost 50% of its activity in 1.5 M urea. At this concentration, there is no detectable changes in either the secondary structure or the intrinsic fluorescence (Figs 1 and 2). It was also found that the inactivation curve changed only to a small extent when the enzyme was assayed without previous incubation in urea. Major time dependence was observed at concentrations higher than 1.5 M denaturant (Fig. 5). In the presence of substrates (Mn^{2+} plus either ATP or *P*-pyruvate), the curve of activity loss after equilibrium as a function of the urea concentration superimposed that obtained at zero incubation time (not shown).

Refolding of *E. coli P*-pyruvate carboxykinase. The enzyme activity could be completely recovered upon sixfold dilution of samples denatured in urea concentrations up to almost 2 M. Denaturation with higher urea concentrations gave minor enzyme activity restoration. No significant improvement was detected when a commercial chaperonin mixture (1:1 GroEL/GroES) was added at 0.4 or 0.8 mg/ml, to a 50-fold dilution of *P*-pyruvate carboxykinase denatured with 4 M urea for 4 h under the conditions described by Zhi et al. [24] (2 mM ATP, 10 mM KCl, 10 mM $MgCl_2$). When the unfolding process was carried out in the presence of substrates, notable effects on the renaturation were seen. Thus, from an enzyme sample incubated with 5 M urea in the presence of ATP and $MnCl_2$, a condition where the enzyme was completely inactive and 65% of the tertiary structure remained, 100% activity recovery was detected after dilution. At 6 M urea, 50% activity was recovered. Similar experiments in the presence of *P*-pyruvate plus $MnCl_2$ indicated 100% recovery from samples denatured up to 6 M urea.

The refolding of *E. coli P*-pyruvate carboxykinase was also analysed with respect to the fluorescence spectrum of the protein. When sixfold diluted in 50 mM Hepes, pH 7.5, enzyme samples denatured at different urea concentrations showed a shift in λ_{max} that was almost completely reversed. However, less refolding was observed when followed by the fluorescence in-

tensity, and it was dependent on the original urea concentration. When the enzyme was incubated at urea concentrations lower than 3.5 M, the recovery was total, however it decreased at higher concentrations, reaching 60% at 7.5 M urea denaturant. Samples denatured in the presence of ATP plus $MnCl_2$ provided 100% recovery from 5 M urea, a value that decreased to 60% at 6 M urea. In the presence of *P*-pyruvate plus $MnCl_2$ the original fluorescence intensity was reached, even in 6.8 M urea.

Recovery of the native secondary structure was monitored through the change in the CD spectrum of the protein. Samples denatured in urea concentrations of 2.5−6 M regained the original CD spectrum almost completely. These results show that the loss in secondary structure in the urea-induced unfolding is completely reversible.

Additionally, ANS was added to the diluted samples. When the dye was added to protein denatured at urea concentrations higher than those where the intermediate is evidenced, the λ_{max} of ANS emission was blue-shifted relative to that of the native protein. This fact holds for experiments carried out in the absence or presence of substrates, thus suggesting that the intermediate accumulates.

DISCUSSION

The noncoincidence of the transition curves obtained by monitoring intrinsic emission intensity, fluorescence wavelength maximum, and ellipticity at 222 nm clearly indicate that the unfolding of *E. coli* PEP carboxykinase by urea is not a two-state transition. Furthermore, the transition observed from emission intensity and the experiments in the presence of ANS indicate the existence of an intermediate protein conformation in the unfolding process, which was also observed with other chemical denaturants such as Gdn/HCl. In the presence of ATP plus Mg^{2+}, the quenching by urea of the intrinsic fluorescence remains biphasic, but it becomes monophasic in the presence of ligands that cause high protection effects. However, the existence of an intermediate in the unfolding pathway in the presence of ligands is clearly seen through the ANS-binding experiments (Fig. 3). The existence of these hydrophobic structures at high urea concentrations implies that the substrates are still bound to the protein and contribute to its stability against urea denaturation. The urea concentrations at which the intermediate is seen are related to the protective effects of the ligands. These observations indicate that, in the presence of substrates, the unfolding pathway also involves an hydrophobic intermediate, even in those cases where a strong protective effect takes place. For red kidney bean acid phosphatase, it has been reported that phosphate binding does not shift the Gdn/HCl concentrations where a similar intermediate appears [25], although a clear stabilizing effect of the ligand was observed.

E. coli P-pyruvate carboxykinase lost its activity at urea concentrations much lower than those where changes in the overall protein structure were detected. This observation may be due to a location of the active site in a fragile and flexible region of the protein, or it may reflect a reversible inhibition effect by urea. Indeed, we found a very small dependence of inactivation at low urea concentrations. However, for reversible inhibitors, it is expected that the inactivation rate will be higher than that which was observed in our experiments. Activity loss at very low denaturant concentrations has been observed in many enzymes, and for those proteins it has been suggested that the active site is located in limited and flexible molecular regions [26−30]. Then, it seems unlikely that the activity loss at low urea concentrations be due only to an inhibitor effect. Hence, it is possible that the loss in local structures in the binding site, located in a deep cleft between the two domains in the bacterial carboxykinase [2], play a role in the urea effect.

Our experiments indicate that total reactivation of *E. coli P*-pyruvate carboxykinase was only possible from enzyme incubated with urea at concentrations lower than those where the unfolding intermediate is accumulated. Renaturation experiments carried out with enzyme equilibrated with higher urea concentrations showed that the native enzyme was not recovered, but a protein species with characteristics similar to that of the unfolding intermediate originated. Similar features were observed when the enzyme was incubated in the presence of substrates. The highly exposed hydrophobic surface of equilibrium folding intermediates can often lead to the irreversible aggregation of the protein through non-specific intermolecular interactions [31−33]. The equilibrium centrifugation experiments showed that, at 0.1 mg/ml, the protein concentration where the experiments here described were carried out, very small (<10%) aggregate formation was detected at urea concentrations where the intermediate is formed. This discards aggregation as a main reason for the lack of complete recovery of full native enzyme structure at low protein concentrations. The uncompleted refolding could be related to the domain structure of the protein [2]. It is possible that once the two protein domains are formed, wrong pairing reactions may occur, giving rise to uncompleted reconstitution. Similar considerations have been proposed to explain the uncompleted reconstitution of denatured octopine dehydrogenase [34] and the partial recovery of the native structure from denatured rhodenase [35] and tubulin [36].

Of particular relevance are the protection effects against urea-induced unfolding exerted by ATP and *P*-pyruvate in the presence of Mn^{2+}, and by the combined presence of oxalate, ATP and Mn^{2+}. Previous data from this laboratory [11] showed that Mn^{2+} or ATPMg binding to *E. coli P*-pyruvate carboxykinase induce conformational changes that can be sensed through alterations in the protein intrinsic fluorescence. Our findings indicate that no protection is exerted by $MnCl_2$, while small effects are produced by ATP, either alone or in the presence of $MgCl_2$. These effects were not improved by the simultaneous presence of ATP, oxalate, and $MgCl_2$, a condition where a 20° hinge-like rotation of the N-terminal and C-terminal domains which closes the active-site cleft occurs [12]. Then, the conformational changes described above do not lead to protein stabilization against urea-induced denaturation. In contrast, notable protection effects were exerted by the combined presence of Mn^{2+} plus either ATP, ATP + oxalate or *P*-pyruvate (see Table 1). These remarkable stabilization effects indicate that the specific interaction of Mn^{2+} with the substrates or with ATP plus oxalate (an analog of *enol*pyruvate, a proposed reaction intermediate for both *P*-pyruvate carboxykinase and pyruvate kinase [10, 13]), give rise to protein forms where new interactions that are not easily disrupted by urea are formed. Considering that urea is known not to affect electrostatic interactions [37, 38], the possibility exists that such interactions might be important for stabilizing the conformation adopted by *E. coli P*-pyruvate carboxykinase in the presence of these ligands.

The protective effects against urea-denaturation exerted by the combined presence of Mn^{2+} ions plus ATP, *P*-pyruvate, or ATP + oxalate suggest a specific role for *E. coli P*-pyruvate carboxykinase-bound Mn^{2+}. In GTP-dependent *P*-pyruvate carboxykinases, divalent cations (Mg^{2+} or Mn^{2+}) can form the active bidentate metal-nucleotide complex, while Mn^{2+} is the species that binds to and activates the enzyme [14]. In this way, protein-bound Mn^{2+} ion is a ligand for MgGTP and oxaloacetate, thus playing a critical role in facilitating phosphoryl transfer [10, 15, 39]. The existence of a metal-binding site specific for Mn^{2+} (but not for Mg^{2+}) in the ATP-dependent *P*-pyruvate car-

boxykinases has also been documented before [5, 11, 18]. Our results suggest that interactions between protein-bound Mn^{2+} and the substrates also take place in *E. coli P*-pyruvate carboxykinase; thus, it is possible that the model proposed for GTP-dependent *P*-pyruvate carboxykinases [39] may be applicable to the ATP-dependent carboxykinases.

In conclusion, we have shown that the equilibrium urea-induced unfolding of *E. coli P*-pyruvate carboxykinase occurs by a two-step mechanism involving the presence of a stable inactive intermediate at 2.5 M urea that retains most of the secondary structure and a significant amount of the tertiary structure of the native protein. Renaturation to the active folded form was possible only when refolding was done from enzyme in urea concentrations less than 2 M. Folding of the carboxykinase from its unfolded state in 6−8 M urea could be achieved only up to a partly folded state that resembles the intermediate seen during unfolding. Substrates in the presence of Mn^{2+} greatly protected the native protein against denaturant-induced unfolding, with an increase of the urea concentration at which the intermediate appears. The role of Mn^{2+} in mediating the protective effects of substrates and ligands in unfolding points to interactions between the metal ion, the ligands, and the protein that are reminiscent of those described for GTP-dependent PEP carboxykinases [39].

This work was supported by FONDECYT 1970670, DGES PB950116, DICYT 0299441CU, and CSIC-USACh.

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