Folding pathway of the pyridoxal 5'-phosphate C-S lyase MalY from *Escherichia coli*

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MalY from *Escherichia coli* is a bifunctional dimeric PLP (pyridoxal 5'-phosphate) enzyme acting as a β -cystathionase and as a repressor of the maltose system. The spectroscopic and molecular properties of the holoenzyme, in the untreated and NaBH₄-treated forms, and of the apoenzyme have been elucidated. A systematic study of the urea-induced unfolding of MalY has been monitored by gel filtration, cross-linking, ANS (8-anilino-1-naphthalenesulphonic acid) binding and by visible, near- and far-UV CD, fluorescence and NMR spectroscopies under equilibrium conditions. Unfolding proceeds in at least three stages. The first transition, occurring between 0 and 1 M urea, gives rise to a partially active dimeric species that binds PLP. The second equilibrium transition involving dimer dissociation, release of PLP and loss of lyase activity leads to the formation of a monomeric equilibrium intermediate. It is a partially unfolded molecule that retains most of the native-state secondary structure,

binds significant amounts of ANS (a probe for exposed hydrophobic surfaces) and tends to self-associate. The self-associated aggregates predominate at urea concentrations of 2–4 M for holoMalY. The third step represents the complete unfolding of the enzyme. These results when compared with the urea-induced unfolding profiles of apoMalY and NaBH₄-reduced holoenzyme suggest that the coenzyme group attached to the active-site lysine residue increases the stability of the dimeric enzyme. Both holo- and apo-MalY could be successfully refolded into the active enzyme with an 85% yield. Further refolding studies suggest that large misfolded soluble aggregates that cannot be refolded could be responsible for the incomplete re-activation.

Key words: aggregates, *Escherichia coli*, folding, MalY, pyridoxal 5'-phosphate, urea.

INTRODUCTION

MalY from *Escherichia coli* is a member of the α -family and one of the fold type I PLP (pyridoxal 5'-phosphate) enzymes. MalY is an interesting bifunctional enzyme showing cystathionase activity [1] and it is a negative effector of MalT, a transcriptional activator of maltose regulon [2]. The spatial structure of MalY has been determined by X-ray crystallography [3]. The enzyme is a homodimer with 399 amino acids per subunit and has a large subunit interface. Each subunit of the MalY dimer contains a large PLP-binding domain and a small one similar to aminotransferases. However, some unusual structural features distinguish MalY from the other known fold type I PLP enzymes. In particular, the MalY active site is much larger and more open and the PLP cofactor is partly solvent-exposed compared with other type I PLP enzymes. This more-open active site could be a functional requirement for catalysing the physiological substrate of MalY, which is still unknown. Moreover, two regions in the PLP domain (residues 79–89 and 207–224) seem to be unique for MalY and possibly represent a potential protein–protein interaction site involved in its repressor function [3]. Enzymatic activity of MalY is not required for repression; a mutant lacking the cystathionase activity still shows repressor activity [1]. On the other hand, mutants can be isolated that exhibit normal cystathionase activity but are defective in their repressor activity [3]. Binding studies between MalY and MalT have indicated that the MalY–MalT complex contains one MalY dimer and two MalT protomers [4]. Since no information has been available on the structure–function

relationship of MalY, studies are in progress to identify the chemical nature of the physiological substrate, which is probably linked to sugar metabolism.

In the course of these investigations, it was found that, although the holoenzyme in either the unreduced or the reduced form is a dimer, the apoenzyme is in part dissociated into monomers. This finding together with the unusual mode of binding of PLP in MalY prompted us to collect extensive urea-induced unfolding equilibrium data of holoMalY, NaBH₄-reduced holoMalY and apoMalY with the aim of probing how MalY's looser mode of PLP binding affects its unfolding and comparing the interplay of MalY folding and coenzyme interaction with those of other PLP enzymes examined so far [5–13]. Global analysis of the results reveals (i) a multistate unfolding process involving, in both apo and holo forms, the formation of a monomeric intermediate that is enzymatically inactive, which is prone to aggregation, and (ii) the influence of PLP and of its mode of binding to MalY on the dissociation and the unfolding steps. The present study is the first to examine the unfolding pathway of a C-S lyase and to use high-field NMR (800 MHz) to study the unfolding of a fold type I PLP enzyme. In addition, we report that completely denatured MalY, regardless of the presence or absence of a cofactor, could be refolded to its native state with a yield of 85%, thus suggesting that the coenzyme is not mandatory in the refolding pathway, although it is essential for achieving the catalytically active dimeric state. Further refolding experiments provide evidence showing that the reduced recovery of native enzyme features is due to the formation of misfolded aggregates during refolding.

Abbreviations used: ANS, 8-anilino-1-naphthalenesulphonic acid; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; PLP, pyridoxal 5'-phosphate.

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EXPERIMENTAL

Chemicals

PLP, urea, L-cystine, β -chloro-L-alanine, pyruvate, NADH, lactate dehydrogenase, dithiothreitol and glutaraldehyde, as well as SigmaMarker™ High Range (molecular mass 36–205 kDa) SDS/PAGE molecular-mass standards were obtained from Sigma. ANS (8-anilino-1-naphthalenesulphonic acid) was purchased from Molecular Probes. 1,3-Bis[tris(hydroxymethyl)methylamino]propane (referred to as Bis-Tris propane) was obtained from Fluka (Madrid, Spain). All other chemicals were of the highest purity available.

Expression and purification of MalY

MalY cDNA and *E. coli* EZ5 strain cells were kindly provided by Professor W. Boos (University of Konstanz, Konstanz, Germany). The conditions used for the expression and purification of MalY in *E. coli* were as described previously [1]. The enzyme concentration was determined using molar absorption coefficient ϵ_M $18.80 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm [4]. PLP content of holoMalY was determined by releasing the coenzyme in 0.1 M NaOH and by using ϵ_M $6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 388 nm [14].

Assay of enzymatic activity

During the purification of MalY, pyruvate formation from L-cystine or β -chloro-L-alanine was determined with the NADH-dependent lactate dehydrogenase [15]. In the experiments designed to determine the effect of various concentrations of urea on lyase activity, pyruvate production was measured by an assay based on measuring the dinitrophenylhydrazine derivative of pyruvate by HPLC as reported previously [16].

Preparation and reconstitution of apoenzyme

The coenzyme PLP was removed as follows: to a solution containing approx. 5 mg of holoenzyme in 10 ml of 0.5 M potassium phosphate buffer (0.5 M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 6.9), hydroxylamine was added to a final concentration of 50 mM. After 1 h at 25 °C, the enzymatic solution was loaded on to a desalting column equilibrated with the same buffer. The eluted protein was concentrated and washed in Amicon Ultra-15 devices (Millipore, Eschborn, Germany) concentrators using 20 mM Bis-Tris propane (pH 8). To reconstitute the apoenzyme to holoenzyme, PLP was added to a final concentration of 20 μM to the apoenzyme and, after 30 min of incubation at 25 °C, excess of coenzyme was removed by filtration on PD10 column. The apparent equilibrium constant for dissociation of PLP from MalY, K_d , was determined by measuring enzymatic activity of the apoenzyme (15 nM) in the presence of PLP at a concentration ranging from 0.5 to 2000 nM.

Urea denaturation

Denaturation of MalY was performed in the following manner. Stock solutions of MalY and 10 M urea (freshly prepared), dissolved in 20 mM Bis-Tris propane (pH 8) respectively, were mixed to give the desired concentration of enzyme and denaturant. Each mixture was allowed to equilibrate at 25 °C for 12 h, which was a sufficient time to reach the limiting values for all enzyme properties listed below at each urea concentration used. The actual urea concentration was checked using refractive index data [17]. Urea-induced unfolding of MalY was probed at protein concentrations above the K_d for PLP binding to apoenzyme.

Fluorescence measurements

All fluorescence measurements were performed by using a 1 ml cell in a Jasco FP-750 spectrofluorimeter equipped with a thermostatically controlled cell holder. Tryptophan emission spectra were recorded from 300 to 550 nm using excitation at 280 nm. Holoenzyme-reduced emission spectra were recorded from 350 to 550 nm using an excitation wavelength of 325 nm. Steady-state fluorescence measurements were performed at 25 °C at 0.01–1 μM protein concentration. All the spectra were corrected by subtracting the emission spectrum of the buffer.

ANS binding experiments were carried out as follows: aliquots of protein at a final concentration of 1 μM were equilibrated at the desired urea concentration overnight at 25 °C. They were then mixed with a concentrated solution of ANS dissolved in 20 mM Bis-Tris propane (pH 8) and incubated for 1 h. The excitation wavelength was 365 nm, and the emission was recorded from 400 to 560 nm. The final ANS concentration was 10 μM . The values were normalized by subtracting the baseline recorded for the probe alone under identical conditions.

All fluorescence experiments were performed using 5 nm excitation and emission bandwidths.

CD measurements

CD measurements were made with a Jasco J-710 spectropolarimeter. The CD spectra were recorded at enzyme concentrations of 1 μM with a 1 mm cell and 0.13–6 μM with a 1 cm cell at 25 °C for far- and near-UV measurements respectively. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration as the denaturant under similar conditions. The secondary-structure content of MalY was determined by the neural net deconvolution procedure of Böhm et al. [18] using a set of 33 proteins.

NMR spectroscopy

One-dimensional ^1H NMR spectra of MalY samples dissolved in 0, 1.1, 2.5, 3.0, 4.0, 4.2 and 8.0 M urea were acquired using a Bruker 800 MHz Advance US2 spectrometer at 25.0 °C. Typically, MalY (final concentration, 10 mg/ml) was dissolved in $^2\text{H}_2\text{O}$ with phosphate (50 mM) or Tris/HCl buffer (10 mM), 50 μM DSS (sodium 4,4-dimethyl-4-silapentane-1-sulphonate) as the internal chemical shift reference, and a concentrated urea solution (final pH^* 7.0–7.3; where pH^* stands for pH meter reading in $^2\text{H}_2\text{O}$ uncorrected for the deuterium isotope effect). The urea stock solution had been deuterated by repeated exchange/freeze-drying in $^2\text{H}_2\text{O}$, and its concentration was determined from the refractive index [17]. After dissolving the sample, a series of spectra (256–1024 transients/spectrum) was acquired over a time period of 14–16 h. Presaturation was used to suppress the residual $^1\text{H}_2\text{O}$ signal. The data were Fourier-transformed and analysed using standard Bruker software.

Cross-linking experiments using glutaraldehyde

To native or urea-treated (12 h at 25 °C) MalY (1 μM), an aliquot of 25 % (w/v) glutaraldehyde was added to give a final glutaraldehyde concentration of 1 %. These samples were incubated at 25 °C for 5 min followed by quenching the cross-linking reaction by the addition of 200 mM sodium borohydride. After 20 min of incubation, 3 μl of aq. 10 % (v/v) sodium deoxycholate was added. The pH of the reaction mixture was lowered to 2–2.5 by the addition of orthophosphoric acid (85 %, w/v), which resulted in the precipitation of the cross-linked protein. After centrifugation at 5500 g for 10 min, the obtained precipitate was

washed twice with ice-cold acetone. After 20 min centrifugation at 2000 *g*, the pellet was re-dissolved in 0.1 M Tris/HCl (pH 8.0), 1 % SDS and 50 mM dithiothreitol and heated to 100 °C. Samples were analysed by SDS/PAGE (7.5 % polyacrylamide).

Size-exclusion chromatography

Gel-filtration experiments were performed on a Superdex 200 10/300 GL column on a FPLC system (Amersham Biosciences). The column was equilibrated and run in 20 mM Bis-Tris propane buffer (pH 8) at the desired urea concentration at 25 °C. The enzyme solutions (1 μ M) were incubated at the desired urea concentrations for 12 h at 25 °C; 200 μ l of this sample was loaded on to the column and run at 25 °C with a flow rate of 0.2 ml/min. The column was equilibrated with standard markers of known molecular mass.

Refolding experiments

For the kinetic determination of the renaturation rate, MalY was first denatured at the desired urea concentration. The denatured enzyme was diluted in 20 mM Bis-Tris propane (pH 8) without denaturant at 25 °C to attain a final urea concentration of ≤ 0.18 M. At the indicated times, aliquots were removed for enzyme activity assays and for analysis of their molecular dimensions by size-exclusion chromatography studies. To probe the reversibility of the unfolding process, the enzyme (6 μ M) was incubated at 25 °C in 20 mM Bis-Tris propane (pH 8) in the presence of 8 M urea. After 12 h of incubation, the refolding was started by a 45-fold dilution with the same buffer as that used for unfolding, containing decreasing denaturant concentrations. The final protein concentration was 0.13 μ M. The spectra were recorded after equilibrium had been reached (12 h).

Data analysis

The K_d value of the enzyme–coenzyme complex was obtained using a tight-binding hypothesis according to the equation

$$Y = Y_{\max} \frac{[E]_t + [PLP]_t + K_d - \sqrt{([E]_t + [PLP]_t + K_d)^2 - 4[E]_t[PLP]_t}}{2[E]_t} \quad (1)$$

where $[E]_t$ and $[PLP]_t$ represent the total concentration of MalY dimer and PLP respectively, Y refers to the enzymatic activity change at the PLP concentration $[PLP]$ and $Y_{\max}$ refers to the enzymatic activity when all enzyme molecules are complexed with the coenzyme.

The urea-unfolding curves corresponding to a two-state model ($N \leftrightarrow U$, where N and U are native and unfolded states) were analysed using the equation [19–21]:

$$Y = \frac{Y_N + (Y_U + Y_N)e^{-m(C_m - C)/RT}}{1 + e^{-m(C_m - C)/RT}} \quad (2)$$

where Y is the observed variable parameter, Y_N and Y_U are the values characteristic of the native and fully unfolded conformations respectively, C denotes the urea concentration, C_m is the midpoint concentration of urea required for unfolding and m stands for the slope of the unfolding curve at C_m .

When experimental data could not be reconciled with a two-state mechanism, a three-state treatment was therefore employed. A unimolecular, three-state unfolding process of a dimeric protein is described as follows: $N \leftrightarrow I \leftrightarrow U$, where N , I and U are native, intermediate and unfolded states respectively. According to the

method of Ayed and Duckworth [22], the urea-unfolding curves corresponding to a three-state model were fitted to the equation

$$Y = \frac{Y_{N-I}[\text{urea}]^{n1}}{C_{m1}^{n1} + [\text{urea}]^{n1}} + \frac{Y_{I-U}[\text{urea}]^{n2}}{C_{m2}^{n2} + [\text{urea}]^{n2}} \quad (3)$$

where C_{m1} and C_{m2} are the midpoint concentrations of urea for the $N \leftrightarrow I$ and $I \leftrightarrow U$ transitions respectively; Y_{N-I} and Y_{I-U} represent the change in the signal associated with the $N \leftrightarrow I$ and $I \leftrightarrow U$ transitions respectively, and exponents $n1$ and $n2$ reflect the steepness of the transition between states as a function of urea concentration.

Due to the fact that the denaturation of MalY is not fully reversible, it is not possible to calculate significant thermodynamic parameters from the denaturation curves. Therefore the above equations have been used to obtain the best estimate of C_m values.

Data were fitted using MicroCal Origin 7.03 (MicroCal Software, Northampton, MA, U.S.A.).

RESULTS AND DISCUSSION

Spectroscopic and molecular properties of MalY

At pH 8, native purified MalY exhibits absorption maxima at 280 and 420 nm with an A_{280}/A_{420} ratio of 11 (Figure 1A). The PLP content was found to be 2 mol/mol of dimer. A 320 nm absorption peak characterizes the NaBH_4 -reduced holoMalY. The CD spectrum of the holoenzyme in the visible region displays a positive dichroic band at a wavelength corresponding to the absorption band (Figure 1A, inset). The apoenzyme does not exhibit either absorbance or dichroic bands in the visible region. In the near-UV region, holoMalY displays negative dichroic bands at 298 and 270 nm, whereas both apoMalY and NaBH_4 -reduced holoenzyme exhibit, in addition to a small negative dichroic signal at 298 nm, a consistent positive dichroic band at 278 nm (Figure 1A, inset). The lack of an internal aldimine could be responsible for the difference in the dichroic signals of holoenzyme in the near-UV region with respect to the reduced holo and apo forms. This implies that, in comparison with the holoenzyme, either the reduced enzyme or apoMalY displays an altered asymmetry of certain aromatic amino acids, most probably those associated with the active site, with respect to that of the holoenzyme. The emission spectra of holo- and apo-MalY in the native state are shown in Figure 1(B). When excited at 280 nm, holo- and apoenzymatic species show emission maxima at 333 and 334 nm respectively; however, the protein fluorescence of the holoenzyme has a quantum yield 60 % lower than that of the apoenzyme. These data are consistent with a model in which a substantial fraction of the tryptophan emission fluorescence evident in an apoenzyme is quenched in the holoenzyme. On reconstitution of apoMalY with PLP, the absorbance, visible and near-UV dichroic bands and the emission fluorescence features are restored to intensities essentially identical with those of the starting material (results not shown). For NaBH_4 -reduced holoMalY in the native conformation, two emission maxima, one at approx. 333 nm (tryptophan fluorescence) and the other at 360 nm (pyridoxyl emission resulting from fluorescence resonance energy transfer) were observed (Figure 1B). The far-UV CD spectra of holo-unreduced MalY, holo-reduced MalY and apoMalY display minima at 222 and 208 nm, which are characteristic of a protein having a high content of α -helical structure, as revealed by the X-ray structure of MalY [3] (results not shown).

From titration analysis of the apoenzyme with PLP, the data for enzymatic activity versus PLP concentration fitted to eqn (1) yielded $K_d = 1.4 \pm 0.2$ nM for the PLP–MalY complex. This

Figure 1 Spectroscopic and molecular properties of MalY

(A) Absorbance spectra and (A, inset) CD spectra of holoMalY (—), apoMalY (---) and NaBH₄-reduced holoenzyme (.....) at 6 μM concentration. (B) Emission fluorescence (excitation at 280 nm) spectra of holoMalY, apoMalY and NaBH₄-reduced holoenzyme at 1 μM concentration; the symbols are the same as for (A). In all cases, enzymatic solutions were in 20 mM Bis-Tris propane (pH 8). (C) Size-exclusion chromatographic profiles of apoMalY at 0.1, 1 and 10 μM concentrations on a Superdex 200 column in 20 mM Bis-Tris propane (pH 8).

result, indicating a very tight interaction between the coenzyme and the apoprotein (results not shown), is unexpected given the mode of PLP binding. It can be suggested that water molecules involved in PLP phosphate binding contribute remarkably to the anchoring of the coenzyme.

A striking difference between holo and apo forms of MalY concerns their quaternary structure. Both holoMalY and NaBH₄-reduced holoenzyme at concentrations ranging from 0.1 to 10 μM are eluted as a single peak from a pre-equilibrated Superdex 200 column with a retention volume of 12.1 ml, corresponding to an apparent molecular mass of 90 kDa. This value is consistent with the fact that holoMalY, in the unreduced and reduced forms, is a dimer of 2 × 44 kDa [4], at least under the experimental conditions examined. A different behaviour of the apoenzyme could also be observed (Figure 1C). When 0.1 or 1 μM apoMalY was loaded on to the same column and eluted, two peaks with retention volumes of 12.1 and 13.0 ml appear. The latter, which accounts for approx. 40 and 30 % of the total protein at 0.1 and 1 μM apoenzyme respectively, was eluted at a position of approx. 55 kDa, consistent with a monomeric species. At 10 μM, the apoenzyme was eluted as a dimer. This behaviour is evidence for the increased equilibrium dissociation constant in the apoenzyme when compared with the holoenzyme. At all these concentrations, a fully dimeric species can be obtained by reconstitution of the apoenzyme with coenzyme. Taken together, these results demonstrate the importance of the internal aldimine in maintaining not only a correct rigid or asymmetric binding of PLP but also the dimeric integrity of MalY by partially preventing the dissociation of the enzyme into subunits. It has already been reported that, in sheep liver serine

hydroxymethyltransferase, removal of PLP results in changes in its oligomeric structure [23].

Changes in molecular properties of MalY associated with urea-induced unfolding

We have studied the effects of urea-induced changes in the structural and functional properties of MalY using a variety of techniques such as gel filtration, cross-linking, ANS binding and by visible, near- and far-UV CD, fluorescence and NMR spectroscopies. Time-dependent changes in the structural parameters and enzyme activity of MalY at increasing urea concentrations (1, 3 and 5 M) were monitored to standardize the incubation time required for achieving equilibrium under these conditions. Under all the conditions tested, the changes occurred within a maximum of 12 h, with no further alteration up to 30 h (results not shown). These observations demonstrate that an incubation of 12 h is sufficient for achieving equilibrium under any conditions of denaturant studied.

Enzyme activity and dissociation of PLP from the enzyme

Enzyme activity is a sensitive probe of changes in the active-site conformation since it reflects subtle readjustment at the active site, allowing very small conformational variations in an enzyme structure to be detected. As shown in Figure 2, the overall process describing the loss of lyase activity of holoMalY was found to be biphasic. An approx. 40 % loss of enzymatic activity is associated with the first transition ($C_{m1} = 0.65 \pm 0.02$ and 0.71 ± 0.05 M urea

Figure 2 Urea-induced changes in enzyme activity and coenzyme binding of MalY

Changes in enzymatic activity of 0.6 μM (■) and 6 μM (●) holoMalY and 0.6 μM (▼) and 6 μM (▽) apoMalY are shown. Solid lines for apo- and holo-MalY represent the best fit obtained using a two-state (eqn 2) and three-state denaturation model (eqn 3) respectively. Changes in visible CD spectroscopy measured at 420 nm for 0.6 μM (□) and 6 μM (○) holoMalY are also shown. Solid lines represent the best fit obtained using a two-state denaturation model (eqn 2).

at 0.6 and 6 μM enzyme concentration respectively). The remaining enzymatic activity was lost through a steep co-operative transition centred at 1.50 ± 0.05 and 2.10 ± 0.01 M denaturant concentration for 0.6 and 6 μM enzyme concentration respectively. Typical unfolding profiles for apoMalY (0.6 and 6 μM) as monitored by loss of enzymatic activity are also shown in Figure 2. This shows that, for 0.6 and 6 μM apoenzyme, the loss of activity is a two-state process with $C_m = 0.73 \pm 0.05$ and 1.5 ± 0.2 M urea concentration respectively.

The urea-induced changes in the enzyme-bound PLP of MalY were studied by monitoring the changes in the visible CD signal at 420 nm of the enzyme (0.6 and 6 μM) at increasing urea concentrations (Figure 2). Urea denaturation gives rise to an apparent two-state unfolding transition. It is clear that the visible CD-detected C_m exhibits protein concentration dependence ($C_m = 1.50 \pm 0.05$ and 2.27 ± 0.02 M urea for 0.6 and 6 μM enzyme respectively). These transitions are coincident with the second transition observed by enzymatic activity measurements of holoMalY at the corresponding protein concentrations. Taken together, these results suggest that the activity signal could reflect, in addition to loss of PLP from the enzyme, other events such as unfolding and dissociation of the dimeric enzyme.

The C_m values for urea-induced unfolding of holo- and apo-MalY at 0.6 μM concentration monitored by enzymatic activity assays and visible CD measurements are reported in Table 1.

Equilibrium denaturation of MalY involves a populated intermediate

The equilibrium denaturation of MalY with urea is followed by both fluorescence emission and CD spectroscopy, which report on the environment of tryptophans and secondary structure respectively. Because of the large number of tryptophans (26 residues/dimer) in MalY, the overall changes in fluorescence reflect global changes in protein structure, and only the average microenvironments of tryptophans can be assessed. Treatment with urea causes changes both in the emission maximum (Figure 3A) and in the emission intensity (Figure 3A, inset) of tryptophan fluorescence in both holo- and apo-MalY forms. The overall transition of holo- and apo-MalY occurs according to the

Table 1 C_m values for urea-induced unfolding of untreated and NaBH_4 -treated holo- and apo-MalY monitored by various signals, at an enzyme concentration of 1 μM

A single value of C_m resulted from fitting data to a two-state process (eqn 2) and two C_m values resulted from fitting data to a three-state process (eqn 3).

	HoloMalY	ApoMalY	NaBH_4 -reduced holoMalY
Enzyme activity*	0.65 ± 0.02 M 1.50 ± 0.05 M	0.73 ± 0.03 M	—
CD-visible*	1.50 ± 0.05 M	—	—
Tryptophan fluorescence			
λ_{max}	1.6 ± 0.1 M 4.4 ± 0.1 M	0.95 ± 0.04 M 4.2 ± 0.1 M	— —
Relative intensity			~ 3 M
CD-far UV	1.6 ± 0.1 M 6.2 ± 0.4 M	0.95 ± 0.04 M 5.9 ± 0.3 M	3.30 ± 0.04 M 7.1 ± 0.1 M
PLP fluorescence	—	—	
λ_{max}			3.20 ± 0.04 M
Relative intensity			0.80 ± 0.05 M 3.20 ± 0.04 M

* The enzyme concentration was 0.6 μM .

following phases: (i) the emission maximum starts to increase at 0.2 and 0.6 M urea for apo- and holo-MalY respectively. In both cases, the λ_{max} red-shifts in a co-operative manner with a midpoint (C_m) equal to 0.95 ± 0.04 and 1.6 ± 0.1 M urea concentration for apo- and holoMalY respectively. Between 1 and 2 M denaturant concentration, the emission intensity of the holoenzyme increases significantly, while that of the apoenzyme remains almost the same. The significant change observed in the holoenzyme may result from a loss of quenching due to the release of PLP. (ii) An intermediate, characterized by an emission intensity at λ_{max} of approx. 341 nm, is formed at approx. 1 and 2 M urea, for apo- and holo-MalY respectively. (iii) At urea concentrations higher than approx. 2 M, the intensity and maximum emission fluorescence of holo- and apo-enzymes were very similar. The emission maximum displays a sharp increase up to 352 nm ($C_{m2} = 4.2 \pm 0.1$ and 4.4 ± 0.1 M for apo- and holo-MalY respectively) and the emission intensity decreases reaching a minimum value at 6 M urea. These latter changes seem to reflect the complete exposure of the tryptophan residues to the solvent. Furthermore, the position of the first transition observed at low urea concentrations was dependent on protein concentration, whereas no such dependence was observed for the second transition (results not shown). This implies that dimer dissociation into monomers occurs during the first transition.

Although holo- and apo-MalY display similar urea-induced unfolding profiles, the cofactor seems to stabilize the structure of the native dimeric protein. One way of circumventing equilibrium binding conditions between PLP and MalY is to attach covalently the coenzyme to the protein molecule by reducing the internal aldimine between the prosthetic group and the active-site lysine residue (Lys-233) by treatment with NaBH_4 before denaturation of the protein. After this treatment, the enzyme obviously becomes completely inactive. As reported above, excitation at 280 nm of NaBH_4 -reduced holoMalY gives rise to two emission maxima, one around 333 nm (tryptophan fluorescence) and the other at 360 nm (reduced PLP emission resulting from fluorescence resonance energy transfer). Therefore both the environment of aromatic residues associated with the active site and their proximity to the pyridoxyl group can be monitored by observing the protein fluorescence emission at 333 and 360 nm respectively. After incubating the reduced holoMalY at increasing urea concentrations, the emission intensity at both wavelengths

Figure 3 (A) Urea-induced unfolding/refolding equilibrium of MalY detected by means of tryptophan fluorescence and (B) urea-induced unfolding equilibrium detected by far-UV CD at 222 nm

(A) Unfolding profiles of holoMalY (●) and apoMalY (▼) and refolding profile of apoMalY (▽) monitored by maximum emission fluorescence (excitation at 280 nm). Inset: unfolding profiles monitored by emission intensity protein fluorescence. (B) Unfolding profiles monitored by far-UV CD at 222 nm of holoMalY (●), apoMalY (▼) and NaBH₄-reduced enzyme (◆). In the unfolding experiments, protein concentration was 1 μ M, and in the refolding experiment, protein concentration was 0.13 μ M. Solid lines represent the best fit obtained using a three-state denaturation model (eqn 3). A.U., arbitrary units.

does not change significantly up to approx. 2 M urea. Between 2 and 4 M urea, the emission at 333 nm increases and red-shifts concomitantly with the disappearance of the emission at 360 nm (Figure 4A). This transition, which is highly co-operative, indicates the loss of the adequate requirements of distance and orientation between donor and acceptor fluorophores, which gives rise to the energy transfer process in the reduced holoenzyme.

Reduction of the bound PLP results in the formation of a pyridoxyl phosphate amine, a fluorescent probe placed in the critical active-site region of MalY. Therefore the fluorescent properties of this fluorophore as a function of urea concentrations can provide further information on the perturbations introduced in the environment surrounding PLP during unfolding. When excited at 325 nm, NaBH₄-reduced MalY displays an emission spectrum

Figure 4 Fluorescence emission spectra of NaBH₄-reduced holoMalY at various urea concentrations and urea-induced unfolding as detected by means of fluorescence of the reduced coenzyme

(A) Fluorescence emission spectra (excitation at 280 nm) of NaBH₄-reduced holoenzyme unfolded at the indicated urea concentrations. (B) Changes in emission intensity measured at 367 nm (excitation at 325 nm) of NaBH₄-reduced holoenzyme (◆). Inset: effect of urea on the emission maximum of reduced holoenzyme. In all cases, the protein concentration was 1 μ M. Solid line in (B) represents the best fit obtained using a three-state denaturation model (eqn 3) and that in the inset to (B) represents the best fit obtained using a two-state denaturation model (eqn 2). A.U., arbitrary units.

centred at 367 nm. In the presence of increasing concentrations of urea, marked changes occur in the range up to 4 M. The fluorescence intensity increases, reaching a maximum at approx. 2 M urea, followed by a decline, reaching a value coincident with that for the free chromophore (at the same concentration as the reduced coenzyme; Figure 4B). The midpoints of the first and second transitions are at 0.80 ± 0.05 and 3.20 ± 0.04 M urea respectively. Additionally, a steep shift in the emission maximum from 367 to 398 nm could be seen as the urea concentration is raised from 2 to 4 M ($C_m = 3.20 \pm 0.04$), after which no further changes are observed in either the emission maximum or intensity (Figure 4B and inset). The midpoint of the first transition monitored by the emission intensity at 367 nm nearly coincides with the first transition monitored by enzymatic activity measurements of holoMalY. This suggests that both enzymatic activity and the fluorescent PLP cofactor are sensitive probes, revealing that a slight disturbance of the correct spatial geometry of the functional groups in the PLP microenvironment has occurred.

For the apo and holo forms of MalY, a two-step loss of CD signal at 222 nm with increasing concentrations of urea was observed and an intermediate state appeared to be stabilized at

approx. 1 and 2 M urea respectively (Figure 3B). Above 2 M urea concentrations, the denaturation curves for apo- and holo-MalY overlap. The transition midpoints were at 0.95 ± 0.04 and 1.6 ± 0.1 M for the first phase of apo- and holo-MalY respectively and at approx. 6 M for the second phase for both enzymatic species. Approximately 25 % of the CD signal at 222 nm was lost during the first transition and a complete loss of secondary structure occurred with the second transition. The changes in ellipticity of reduced MalY with increasing urea concentrations also highlight two well-separated transitions (Figure 3B). However, the transitions require higher urea concentrations than those for holo- and apo-enzymes. The first transition, which is highly co-operative, has a midpoint at approx. 3.3 M urea and is followed by a plateau between 4 and 6 M urea, whereas the second transition is centred at approx. 7 M urea. The equilibrium intermediate state of reduced MalY has only 30% of the native helical secondary structure. The first transition of the far-UV CD profile of reduced enzyme corresponds to the midpoint of the second transition monitored by the emission intensity and to that monitored by the emission maximum of reduced PLP fluorescence as well as to that observed in protein fluorescence. Thus the transition leading to the formation of the intermediate that accumulates at 4–6 M urea reveals not only gross conformational changes at or near the PLP microenvironment, as detected by the complete exposure to the solvent of the reduced PLP, but also a significant loss of secondary and tertiary structures.

In Table 1, the C_m values for urea-induced unfolding of the holo-enzyme, in the untreated and NaBH₄-treated forms, and of the apoenzyme monitored by fluorescence and far-UV CD spectroscopy are listed.

Evidence for a self-associated intermediate

Both fluorescence and CD spectroscopy identify a stable intermediate for apo- and holo-MalY at approx. 1 and 2 M urea respectively, which retains approx. 75 % of the native CD spectral amplitude, has a maximum fluorescence wavelength of approx. 341 nm, but is enzymatically inactive. Therefore we considered the possibility that the intermediate form produced by urea has some similarity to a classical molten globule, i.e. a partly unfolded form that retained secondary structure but not regular tertiary structure, in which extensive hydrophobic surfaces are exposed to the solvent [24]. ANS binding to the hydrated hydrophobic surfaces [24] of molten globule conformations [25] is a convenient test for these conformations since this binding leads to a large increase in ANS fluorescence and blue shifts its emission maximum from 530 to 470 nm. As a test, we added 10 μ M ANS to holo- and apo-MalY solutions, and measured ANS-associated fluorescence after equilibration with different urea concentrations (Figure 5). At the same protein concentration, the ANS fluorescence intensity observed for holo- and apo-MalY was almost identical, suggesting the binding of ANS molecules to the exposed hydrophobic patches present in the native conformation of both these enzymatic species. However, the ANS fluorescence displays emission maxima at 480 and 490 nm for apo- and holo-MalY respectively, indicating a higher exposure of hydrophobic surfaces in native apoenzyme when compared with native holoenzyme. An increase in ANS fluorescence up to 1 and 2 M urea concentrations occurs for apo- and holo-enzymes respectively, whereas a shift of the emission maximum to 483 nm could be observed in the 0–2 M urea range for both apo- and holo-enzymes. Above 1 and 2 M for apo- and holo-MalY respectively, a decline of ANS fluorescence takes place, reaching the same value at 6 M as if MalY were not present, whereas at concentrations higher than 2 M, a shift in the emission wavelength from 483 to 516 nm could be observed

Figure 5 ANS binding

ANS fluorescence in the presence of holoMalY (●) and apoMalY (▼) as a function of urea concentrations; emission fluorescence intensity is at 483 and 480 nm for holo- and apo-MalY respectively. Inset: changes in the emission maximum. Protein concentration was 1 μ M. A.U., arbitrary units.

for both the enzymatic species (Figure 5 and inset). These ANS binding studies indicate that the unfolding intermediate observed by protein fluorescence and far-UV CD-monitored denaturation profiles at 1 and 2 M urea concentrations for apo- and holo-MalY respectively has exposed hydrophobic patches on structural sites that are accessible to ANS. Such a change in enzyme structure may lead to its aggregation due to hydrophobic interactions. Although the reduced enzyme in the native conformation binds ANS, as revealed by the maximum emission wavelength at 483 nm, only a slight increase in ANS fluorescence could be detected in the first transition zone (results not shown). Thus, unlike the unfolding of holo- and apo-enzymes, unfolding of reduced MalY does not lead to an 'ANS-bound' intermediate.

In order to obtain further insight into possible formation of aggregates during unfolding of MalY, the effect of increasing urea concentrations on the molecular dimensions of MalY was investigated by size-exclusion chromatography studies on a Superdex 200 column in the presence and absence of urea at 25 °C (Figure 6A). As reported above, for native MalY, a single peak with a retention volume of 12.1 ml was observed. When holoMalY treated with 0.5 M urea was loaded on to the same column and eluted, only a single peak with a retention volume identical with that of native enzyme was seen. However, for the 1 and 1.5 M urea-stabilized enzyme, this peak decreases at the expense of a peak with a retention volume of 12.8 ml, corresponding to a monomeric species, as confirmed by the glutaraldehyde cross-linking studies (Figure 6B). At 2 and 2.5 M urea, the dimeric and monomeric species gradually disappear and a broad peak at a significantly lower retention volume, indicating an increase in molecular dimensions (higher aggregation state or increased hydrodynamic radius), appears. When incubated in 3–4 M urea, MalY is eluted with a significantly reduced retention volume of 8.5 ml, which is the void volume of the column. Cross-linking experiments reveal that, from 2 to 4 M urea, aggregates corresponding to tetramers, octamers and decamers accompanied by very large aggregates (> 700 kDa) appear (Figure 6B). Increasing the urea concentration beyond 4 M leads to a single peak whose elution volume gradually decreases, indicating a transition towards a species with increased hydrodynamic radius and corresponding to an unfolded monomer, as revealed by glutaraldehyde-cross-linking

Figure 6 Urea-induced changes in molecular dimension and subunit assembly during holo- and apoMalY unfolding

(A) Chromatographic profiles of native and urea-treated holoMalY on the Superdex 200 column at the indicated urea concentrations. Protein concentration was 1 μ M. (B) SDS/PAGE profiles of glutaraldehyde-cross-linked native and urea-treated holoMalY. Lane 1, standard markers (sizes in kDa); lane 2, native enzyme; lane 3, glutaraldehyde-cross-linked native enzyme; and lanes 4–7, the 1, 3, 4 and 8 M urea-treated cross-linked holoenzymes. (C) Chromatographic profiles of native and urea-treated apoMalY on the Superdex 200 column at the indicated urea concentrations. Protein concentration was 1 μ M.

experiments (Figure 6B). The presence of multiple peaks indicates an equilibrium among protein species under the chromatographic conditions used here. The addition of non-ionic detergents to the chromatographic buffer (20 mM Bis-Tris propane, pH 8, in the presence of various urea concentrations) did not affect the aggregation phenomena.

The effect of increasing urea concentration on molecular dimensions of apoMalY is shown in Figure 6(C). As pointed out above, native apoenzyme at 1 μ M concentration is an equilibrium mixture of dimers and monomers. Over the range 0.5–1 M urea, a progressive decrease in the peaks corresponding to dimers and monomers occurs concomitantly with the appearance of peaks having retention volumes corresponding to aggregates, as detected by glutaraldehyde cross-linking (results not shown). Aggregate species were populated in the 1–4 M urea range. At concentrations $\geq$ 4 M urea, a single peak corresponding to a monomer undergoing unfolding was observed.

Size-exclusion chromatography of reduced holoMalY as a function of urea concentrations shows that, whereas between 0 and 2 M urea the reduced enzyme is eluted as a native dimer, between 2 and 4 M it is eluted with a decreased retention volume (from 12.1 to 11 ml). As revealed by cross-linking studies, this species corresponds to a dimer, which, on the basis of the protein fluorescence and CD denaturation profiles, is a partially unfolded dimer. Unlike the monomeric intermediate formed during the unfolding of holo- and apo-MalY, this dimeric intermediate does not present exposed hydrophobic residues (see above). A completely unfolded monomer is present at urea concentrations $\geq$ 7 M (results not shown). The finding that, unlike the unreduced holoenzyme, the reduced holoenzyme retains its dimeric state up to 4 M is a further indication that dissociation of PLP from the active site results in destabilization of the quaternary structure. It should also be noted that the total peak area of reduced holoMalY in the chromatogram decreased significantly (to $\sim$ 60% of that measured for the untreated enzyme) over the 3–5 M urea range. This result clearly indicates that the solubility of the protein is minimal at the urea concentrations where the partially folded intermediate is present.

NMR spectroscopy reveals a native-like structure below 4 M urea and unfolding transition above 4 M urea

The one-dimensional ^1H NMR spectrum, at 800 MHz, of MalY at 25.0 $^\circ\text{C}$ in $^2\text{H}_2\text{O}$ is shown in Figure 7. Despite resonance broadening due to the slow tumbling of this large dimeric enzyme in solution, the spectrum shows unmistakable signs of folded secondary and tertiary structures. The peaks > 8 p.p.m. are due to the resonance of unexchanged amide protons in MalY. Since exchange with solvent deuterons would occur within 1 s ($k \approx 100 \text{ s}^{-1}$), if these protons were not protected by hydrogen bond formation within the protein [26], the presence of these amide proton signals is a clear evidence for an intact secondary structure in MalY. At the other end of the spectrum, the series of peaks appearing < 0 p.p.m. is characteristic of alkyl groups stacked on aromatic rings in the folded hydrophobic core, indicating the presence of a native tertiary structure in MalY [27]. Similar spectra are observed in the presence of 1.1, 2.5 and 3.0 M urea, and these spectra remain practically unchanged over 14–16 h (results not shown). In contrast, at 4.0 and 4.2 M urea, some changes in the upfield (< 0 p.p.m.) and other alkyl peaks (0.8–2.5) are observed in the first spectrum recorded (dead time = 30 min), indicative of changes in the magnetic environment of these groups (Figure 7). Size measurements performed using diffusion-ordered spectroscopy are consistent with the existence of a dimeric species in 0 M urea and a monomeric species in 4 M urea (results not shown). After overnight incubation in 4.0 M urea, the intensity of the resonances decreased by two-thirds, which is consistent with the formation of large NMR-invisible aggregates. Moreover, the NH peaks (> 8 p.p.m.) exchanged out, the upfield peaks (< 0 p.p.m.) disappeared and other alkyl peaks sharpened, converging towards chemical shift values typical of short unstructured

Figure 7 One-dimensional ^1H NMR spectroscopy of MalY at 800 MHz

Top panel: MalY in $^2\text{H}_2\text{O}$ at pH* 7.3 and 25.0°C. The unexchanged amide groups (NH) and upfield alkyl groups (*) indicative of native secondary and tertiary structures are indicated. Middle panel: initial spectrum recorded of MalY protein in $^2\text{H}_2\text{O}$ and 4.2 M urea. Bottom panel: MalY in $^2\text{H}_2\text{O}$ and 4.2 M urea after incubation overnight.

peptides after overnight incubation in 4 M urea (Figure 7) [27]. All these spectral changes are consistent with the unfolding of the tertiary structure and a destabilization of the secondary structure. Essentially identical spectra indicative of fully denatured protein were observed soon after dissolving MalY in 8 M urea (results not shown).

Secondary-structure content during unfolding

The secondary-structure content of holoMalY in buffer solution detected by CD has been compared with that observed in the X-ray crystal structure (Table 2). The amount of helix found by both methods is very similar. A higher amount of β structure is detected by CD than by X-ray; this is most probably due to the relatively low intensity of the β -sheet CD band, which makes its quantification relative to the coil less precise. On the basis of the CD data, the native apoenzyme exhibits a lower helical content and an increased amount of β -structures with respect to the holoenzyme (Table 2). It should also be noted that one or more tryptophan residues are more solvent-exposed in native apoenzyme (maximum at 333 nm) than in holoMalY (maximum at 334 nm), as shown by intrinsic fluorescence measurements (Figure 1B). It can be suggested that binding of the coenzyme causes conformational changes, as also revealed by the difference in dichroic signals in the near-UV region between apo- and holo-MalY, associated with a reorganization of the secondary structure. Among PLP enzymes, a different composition of secondary-structure elements as well as a different solvent exposure for

Table 2 Secondary-structure content of native holoMalY, apoMalY and refolded holoMalY

Values are percentages.

	α -Helix	3_{10} helix	β -Structure	Random coil
HoloMalY*	40.4	4.7	16.1	38.8
HoloMalY†	43.3	—	27.9	28.8
ApoMalY†	38.8	—	31.5	29.7
Refolded holoMalY†	42.9	—	29.5	27.9

* Values obtained from the crystallographic data in [3].

† Values obtained from the deconvolution of far-UV CD spectra using the CD spectra deconvolution software, version 2.1 [18].

Figure 8 Changes in the secondary-structure content of holoMalY in 4 and 8 M urea with time

(A) HoloMalY (1 μM) was dissolved in 4 M urea, and the content of (■) α -helix, (●) β -structure and (▲) random coil was evaluated at the indicated times. (B) HoloMalY (1 μM) was dissolved in 8 M urea, and the content of (■) α -helix, (●) β -structure and (▲) random coil was evaluated at the indicated times. Solid lines are from a fit of the data to a first-order rate equation.

tryptophan residues for holo- compared with apo-enzyme has been observed only for *O*-acetylserine sulphydrylase [11].

Changes in the secondary-structure content under mild (4 M urea; Figure 8A) and strong (8 M urea; Figure 8B) denaturing conditions were monitored kinetically by recording and analysing a series of CD spectra. The first time point recorded after dissolving holoMalY in 4 M urea shows a secondary-structure content very similar to that present under native conditions. However, with time, the amount of helix shows a slight decrease and the amounts of β -structure and coil show small apparent increases. When fitted to a first-order rate equation (solid lines), the observed changes in helix, β and coil contents all show rates of approx. 1.0 h^{-1} , which suggests that these changes occur simultaneously.

However, it should be noted that part of these changes could be due to release of PLP and the conversion of holo into apo form. This event, measured by the decrease in the 420 nm dichroic band with time, occurs at 4 M urea with a rate constant of 7.2 h^{-1} . Nevertheless, the apparent conversion of α secondary structures into β structures at urea concentrations in which the kinetics of unfolding leads to formation of aggregates indicates that these aggregates are characterized by a higher content of β structures and a lower content of α -helix with respect to the native enzyme.

Unfolding in 8 M urea is both more rapid and complete. Under these conditions, release of PLP occurs in the time required for mixing the enzyme with urea solution. The first time point recorded after dissolving the holoenzyme in 8 M urea reveals a remarkable loss of α -helical secondary structure and an enrichment of β -structure and random coil compared with that of the apoenzyme. Glutaraldehyde-cross-linked experiments together with gel electrophoresis indicate that this species is mainly populated by aggregates. Subsequently, the remainder of the helix continues to unfold rather rapidly at a rate of 7.8 h^{-1} until it is essentially completely denatured. The β -structure unfolds at a lower rate (0.66 h^{-1}). These kinetic results detect intermediates in the unfolding of MalY and are consistent with the equilibrium results, which also detect the presence of partially structured intermediates of MalY. It should be pointed out that the percentage values of β structure and random coil could not be exactly evaluated due to the difficulty in distinguishing these conformations by CD measurements performed at high urea concentrations.

Unfolding pathway of MalY

Several experimental properties that reflect different aspects of the structural integrity of MalY 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 MalY is a complex process involving equilibrium intermediate(s). Based on these studies, the unfolding process itself of holoMalY can be divided into at least three steps. The first step, occurring over the 0–1 M denaturant concentration range, is associated with the conversion of the native folded fully active dimeric form of the enzyme (D) into a partially inactivated state (D'). When the reduced holoenzyme was excited at 325 nm, the fact that the first fluorescent transition is coincident with the first phase of the transition monitored by enzymatic activity measurements strongly suggests that a small conformational change takes place in the dimeric enzyme, which causes inactivation, probably by a distortion of the active site. Nonetheless, D' produces fluorescence, CD and NMR signals that are very similar to those of the native dimer.

The second step of the unfolding process represents the formation of a stable equilibrium intermediate that predominates at moderate concentrations of denaturant (2–4 M urea) and includes a transient monomeric species capable of binding ANS, which shows a slight alteration of the tertiary structure, retains approx. 75 % of the ellipticity of the native enzyme, does not bind PLP, is inactive, but is prone to aggregation. Folding intermediates with these characteristics, often referred to as molten globule-like species, have been reported for many proteins [25]. This transition is characterized by several events such as a nearly complete loss of lyase activity, release of PLP from the holoenzyme (as evidenced by decrease in the 420 nm dichroic signal of holoMalY and by changes in the intrinsic emission fluorescence), dissociation of dimer into monomers and exposure of

new hydrophobic ANS-binding surfaces. In this regard, the crystal structure of MalY reveals that the interaction surface between the two monomers is large and is characterized by a great deal of intimate contacts comprising hydrogen bonds, salt bridges and extensive hydrophobic interactions [3]. The observation that dissociation takes place concomitantly with unfolding is consistent with the observed dependence of this transition on protein concentration, as detected by tryptophan fluorescence and enzymatic activity assays. In addition, size-exclusion chromatography and cross-linking experiments show that the transient inactive monomeric molten globule state gives rise to aggregates consisting of tetramers, octamers, decamers as well as very large soluble aggregates ($> 700 \text{ kDa}$). The idea that these species are on the unfolding pathway of MalY is demonstrated by the following observations: (i) the total peak area in the chromatograms (Figures 6A and 6C) does not decrease from 0 to 8 M urea; (ii) the 3 M urea-stabilized enzyme adjusted to 8 M final urea concentration is completely converted into the unfolded monomer under equilibrium conditions. On the basis of the unfolding kinetics monitored by far-UV CD studies, these aggregates have lost significant amounts of helical structure but are enriched in β -structure when compared with the native enzyme. However, it should be noted that NMR studies do not detect signs of molten globule intermediate. There are at least three possible explanations for this apparent discrepancy: (i) the much higher protein concentration and the presence of $^2\text{H}_2\text{O}$ in the NMR experiment, which could significantly stabilize the native state relative to the molten globule state; (ii) protein motions in the molten globule state might lead to extremely broad and essentially undetectable NMR resonance; and (iii) finally, it is possible that the group responsible for the signal $< 0 \text{ p.p.m.}$ lies in a different part of the protein core, which is more resistant to unfolding. Another possibility to be considered is that the increase in ANS fluorescence at 2 M urea may reflect an exposure of the hydrophobic protein–protein interface upon dimer dissociation, and the decrease in the CD signal at 222 nm centred at 2 M urea could reflect loss of a helix involved in the dimeric interface that unfolds upon dissociation without affecting the global native-like packing of the remainder of the protein. Consistent with this view is the fact that the contacts between the subunits of MalY are chiefly hydrophobic interactions and that helix 3 of the N-terminal segment is pivotal for dimerization [3].

The subsequent event corresponds to the unfolding of the stable intermediate into an almost completely unfolded protein, which, as revealed by size-exclusion chromatography and cross-linking studies, is a monomer.

Thus the global unfolding of MalY involves several steps including perturbation of the active site, release of the coenzyme and dissociation of dimer into monomers, in addition to unfolding of each subunit. The presence of PLP bound to the active site of the enzyme increases its stability, in particular with regard to the step of dimer dissociation. This is clearly demonstrated by the fact that, even in the absence of denaturant, the presence of the PLP is necessary to attain a native dimeric fold. It should be emphasized that the dimeric state is required for the function of MalY either in exhibiting cystathionase activity or in repressing the maltose system. The available stoichiometry data on the union of MalY and MalT point to a complex containing one MalY dimer and two MalT subunits [4]. Therefore PLP has not only a structural importance but also a functional relevance in MalY. Moreover, almost complete dimer dissociation of holoMalY occurs at 2 M urea, whereas, for apoMalY, it is obtained at 1 M urea. Yet, if PLP is not allowed to diffuse from its binding site as when irreversibly attached to the protein through reduction of the internal Schiff-base linkage, the dimer dissociation starts at urea concentrations

≥ 4 M, confirming the significant role of coenzyme attachment to the active site in the stabilization of MalY. The PLP stabilizing effect has already been reported for most of the PLP enzymes so far examined regardless of whether they belong to fold type I (glutamate decarboxylase [28], aspartate aminotransferase [5,6,29–31], sheep liver serine hydroxymethyltransferase [32] and tryptophanase [7,33,34]) or type fold II (tryptophan synthase [10,35–37] and *O*-acetylserine sulphydrylase [11]). However, no stabilizing effect of PLP has been observed for dopa decarboxylase [12] and *E. coli* serine hydroxymethyltransferase [13]. Thus the PLP stabilizing effect does not seem to be associated either with the fold type or with the PLP binding mode of PLP enzymes.

Holo- and apo-MalY lose a large portion of their secondary structure at denaturant concentrations higher than that in which dimer dissociation occurs. A similar equilibrium unfolding mechanism has already been proposed for other PLP enzymes studied so far. These enzymes belong either to fold type I (*E. coli* aspartate aminotransferase [5,38,39], *Bacillus subtilis* [8] and *E. coli* serine hydroxymethyltransferase [13], 5-aminolaevulinate synthase [9] and dopa decarboxylase [12]) or to fold type III (alanine racemase [40]). Nonetheless, monomerization of *O*-acetylserine sulphydrylase (fold type II) has been found to occur once the structure is sufficiently destabilized [11]. The equilibrium unfolding curve for reduced holoMalY, as monitored by the denaturant dependence of the ellipticity at 222 nm, is significantly right-shifted compared with those obtained for holo- and apoMalY. The gel filtration and cross-linking experiments suggest that the reduced holoenzyme undergoes monomerization after disruption of a large portion of its native secondary structure. This implies that, in the presence of irreversibly bound PLP, strong intermonomer interactions require that a high degree of unfolding is to be achieved to allow monomerization. Although PLP is located in a cleft that is assembled by residues from both domains, it mostly makes contacts with the large domain. This suggests that the two structural domains could be differently stabilized by the interaction with the coenzyme whose binding to the enzyme is relevant for dimerization. As shown in Figure 9, the cofactor, accommodated in the large domain, interacts with Tyr-121, Asp-201, Asn-173 and His-204, in addition to the covalent bonding of its 4'-aldehyde group with the ϵ -amino group of Lys-233. All these residues belong to the large domain. Unique to the active site of MalY is the binding mode of the PLP phosphate group, which is bound primarily by five water molecules. The only interaction with a protein residue is with Tyr-61 of the neighbouring subunit (Tyr-61* in Figure 9), which is located on the interdomain hinge. Furthermore, Tyr-61 is a residue of helix 3 that is a structural element playing a key role in dimerization. The unfolding profile of the reduced holoMalY could be interpreted on the basis of these observations concerning the mode of PLP binding in MalY. When PLP is irreversibly fixed to the protein, MalY, although losing approx. 70 % of its native secondary structure, remains in its dimeric state. This could reflect the presence of a part of the protein core more resistant to unfolding, possibly a portion of PLP domain essential for intersubunit interaction. Monomerization of reduced MalY begins at a denaturant concentration in which the reduced coenzyme is completely solvent-exposed, thus suggesting that, under these conditions, the network of interactions between the coenzyme and the protein matrix has been mainly disrupted. This could imply that, in MalY, PLP stabilizes the large domain to a higher extent with respect to the small domain.

An important role for domains in the stabilization of the native dimeric structure has already been suggested for other PLP enzymes. Despite the fact that the active site of *O*-acetylserine sulphydrylase is located between the N- and C-terminal domains,

Figure 9 PLP binding site of MalY

Close-up view of the active-site structure of *E. coli* MalY showing the residues that interact with the PLP. The asterisk denotes the residue coming from the neighbouring subunit. The spheres represent water molecules.

it has been proposed that different interactions of the coenzyme with residues belonging to the two domains could account for a stronger stabilizing effect on the C-terminal domain [41]. Again, N- and C-terminal domain swapping between mesophilic and thermophilic serine hydroxymethyltransferase enzymes and studies of their unfolding profiles clearly demonstrate that the C-terminal domain of the mesophilic enzyme plays a vital role in the stabilization of the oligomeric structure and in modulating its unfolding pathway [42]. Based on this result, it can be argued that, besides the degree of sequence identity as well as sequence and structural homology, other factors such as different degrees of stability of individual domains and/or the stabilizing effects elicited by their interaction with the coenzyme could play a role in determining the folding mechanism of PLP enzymes. This could explain why global structural stabilization varies among the PLP enzymes and does not seem to be linked to the particular fold type.

Reversibility of the unfolding process

We have studied the kinetics of the refolding process to assess if the re-activation pathway can be described as a simple reversal of the denaturation reactions. After complete unfolding of holo-MalY by incubation in 8 M urea, whereas step dialysis has been proved to be unsuccessful in re-activation, a simple dilution allows the enzymatic activity to reappear and increase with refolding time. The maximum recovery of enzymatic activity was complete within 12 h (Figure 10). Identical results were obtained for the refolding of apoMalY. These results suggest that PLP does not seem to facilitate refolding by acting as a nucleation site and support the interpretation that PLP binds only at the end of the folding pathway to the apoenzyme and plays no significant role in the folding mechanism of single subunits. The effect of PLP on folding varies among the different PLP-dependent enzymes analysed to date. The isolated β_2 subunit of tryptophan synthase [43] and the cytosolic aspartate aminotransferase [44] require a coenzyme for re-activation *in vitro*, whereas refolding of *E. coli*

Figure 10 Kinetics of re-activation of holoMalY after its inactivation by incubation in 8 M urea and regain of activity after renaturation of native enzyme exposed to 3 M urea for various time periods

(■) HoloMalY (6 μ M) was denatured for 12 h in 8 M urea. Refolding was performed by 45-fold dilution in 20 mM Bis-Tris propane (pH 8) containing 100 μ M PLP, and the enzyme activity was measured at the indicated times. (□) HoloMalY (6 μ M) was denatured in 3 M urea for different time periods indicated on the x-axis, and enzyme activity was measured after dilution and 12 h refolding time.

Figure 11 Refolding yield of holo-MalY

HoloMalY (6 μ M) was denatured for 12 h in various concentrations of urea as indicated on the x-axis. Refolding was induced by 45-fold dilution. Enzyme activity measurements (●) were performed after 12 h of refolding. The activity data are expressed relative to the final activity of samples of holoenzyme incubated in the absence of denaturant under the same conditions.

serine hydroxymethyltransferase [45] and mitochondrial aspartate aminotransferase [46] can proceed in the absence of coenzyme. The dependence of the refolding yield on MalY concentration was also examined. At a high MalY concentration (60 μ M), only 30 % of the active holoenzyme was recovered, thus suggesting that aggregation is probably the cause of the incomplete recovery of the active enzyme upon refolding.

To investigate the oligomeric state of the refolded holo- and apo-MalY denatured in 8 M urea, the refolded species were passed through a Superdex 200 column equilibrated with 20 mM Bis-Tris propane (pH 8). The holoenzyme is essentially dimeric, whereas the apoenzyme eluted as a mixture of dimers and monomers in a ratio similar to that observed for the native apoenzyme at the same concentration. However, in addition to these species, 10–15 % of the total protein is eluted as aggregates for both holo- and apo-refolded species. These results are consistent with the incomplete recovery of activity of the refolded enzymes. The dimer species collected after gel filtration and concentrated up to 0.4 μ M has been subjected to far-UV CD analysis. The secondary-structure content of the refolded holoMalY is almost identical with that of the native holoenzyme (Table 2). The observation that the process was not completely reversible was also indicated by the refolding curve of apoenzyme as monitored by the intrinsic protein fluorescence (Figure 3B). Unfortunately, under the experimental conditions of unfolding/refolding giving the highest yield recovery of active enzyme, the refolding process cannot be monitored by far-UV CD and NMR spectroscopies, because of the low concentration of the refolded species (0.13 μ M).

The existence of an apparent intermediate of unfolding with a high tendency to aggregate, which predominates at urea concentrations of 1–4 and 2–4 M range for apo- and holo-MalY respectively prompted a more detailed analysis of this denaturation step. To understand better the events leading to aggregation, changes in molecular mass of holoMalY on exposure to 3 M urea were followed as a function of time. A progressive conversion of the dimeric species into monomer and aggregates could be observed during the unfolding time and, after 12 h, oligomers and larger aggregates were the only species present. During 12 h of unfolding of holoMalY denatured in 3 M urea, aliquots were withdrawn, diluted into refolding buffer (20 mM Bis-Tris propane, pH 8), and the enzyme activity recovery was measured after further

Scheme 1 Summary of the folding and misfolding pathways of holoMalY

D and D' indicate folded dimeric forms of the enzyme which are fully active and partially reversible inactivated species respectively; I represents the monomeric intermediate prone to aggregate; A shows the irreversible aggregates and U is the unfolded monomer.

incubation of the enzyme under these conditions at 25 °C for 12 h. As shown in Figure 10, regain of activity decreases as a function of the time that the enzyme is exposed to 3 M urea. Concomitant with the decrease in recovery of activity, an increase in the molecular mass of holoMalY was observed. The species refolded after 12 h of unfolding in 3 M urea regains approx. 40 % of the activity. On a Sephadex-200 column, it is eluted in the form of a native dimer and of aggregates in relative amounts of 40 and 60 % of the total protein respectively. When native holoMalY was equilibrated with different concentrations of urea and then diluted 45-fold, the yield of re-activation was strongly influenced by the urea concentration used (Figure 11). At concentrations below 2 M urea in the denaturation step, the reversibility of the unfolding of holoMalY was nearly 100 %. Between 2.5 and 4 M urea, the recovery of catalytic activity was low, and finally, partial reversibility was observed in samples incubated at higher concentrations of urea (5–8 M). Thus the partially unfolded molecule can be returned to its fully functional state only before the formation of aggregates occurs. London et al. [47] reported similar results for urea-denatured *E. coli* tryptophanase. Taken together, these results indicate that the partial reversibility observed when unfolding was carried out at urea concentrations higher than 2 M is due to the fact that part of the protein becomes trapped in a 'wrong' structure, probably represented by large misfolded soluble aggregates that cannot be refolded.

Scheme 1 summarizes the folding and misfolding pathways of holoMalY. This model is the simplest plausible mechanism that would account for most of the data obtained up to now. However, more detailed kinetic studies are needed to draw a parallel between the unfolding and refolding pathways of MalY.

Conclusions

The overall urea-induced unfolding process of *E. coli* MalY is a multistate process including active-site perturbation, release of coenzyme and dissociation of dimer into monomers, followed by the complete unfolding of monomers. Evidence is provided for the formation of a self-associated intermediate that, on the basis of kinetic unfolding CD data, is characterized by a lower content of helix and a higher content of β structure with respect to the corresponding native enzyme. It will be interesting to investigate if these structural changes represent the basis for the development of amyloid structures. Characterization of the interplay of MalY folding and PLP binding allowed us to establish that coenzyme strongly increases the protein stability. In particular, PLP is essential for the step involving dissociation of dimer into monomers and is not required for refolding of single monomers, but it is necessary to attain the native dimeric structure critical for the biological activities of MalY.

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