

# The folding of dimeric cytoplasmic malate dehydrogenase

## Equilibrium and kinetic studies

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Porcine heart cytoplasmic malate dehydrogenase (s-MDH) is a dimeric protein (2 × 35 kDa). We have studied equilibrium unfolding and refolding of s-MDH using activity assay, fluorescence, far-UV and near-UV circular dichroism (CD) spectroscopy, hydrophobic probe-1-anilino-8-naphthalene sulfonic acid binding, dynamic light scattering, and chromatographic (HPLC) techniques. The unfolding and refolding transitions are reversible and show the presence of two equilibrium intermediate states. The first one is a compact monomer (M<sub>C</sub>) formed immediately after subunit dissociation and the second one is an expanded monomer (M<sub>E</sub>), which is little less compact than the native monomer and has most of the characteristic features of a 'molten globule' state. The equilibrium transition is fitted in the model: 2U ↔ 2M<sub>E</sub> ↔ 2M<sub>C</sub> ↔ D.

The time course of kinetics of self-refolding of s-MDH revealed two parallel folding pathways [Rudolph, R.,

Fuchs, I. & Jaenicke, R. (1986) *Biochemistry* **25**, 1662–1669]. The major pathway (70%) is 2U → 2M\* → 2M → D, the rate limiting step being the isomerization of the monomers ( $K_1 = 1.7 \times 10^{-3} \text{ s}^{-1}$ ). The minor pathway (30%) involves an association step leading to the incorrectly folding dimers, prior to the very slow D\* → D folding step.

In this study, we have characterized the folding-assembly pathway of dimeric s-MDH. Our kinetic and equilibrium experiments indicate that the folding of s-MDH involves the formation of two folding intermediates. However, whether the equilibrium intermediates are equivalent to the kinetic ones is beyond the scope of this study.

**Keywords:** equilibrium denaturation; folding, unfolding; molten globule; malate dehydrogenase.

To answer the protein folding problem, a general assumption was made ≈ 28 years ago that a protein folds through several intermediates, and that each intermediate has an increasing number of native-like structural features [1]. Later on, evidence from several *in vitro* studies established the above hypothesis [2–6]. These intermediates usually occur in the kinetic pathway of protein folding; however, they are often formed so fast that it is difficult to characterize them by standard biophysical methods. Therefore efforts have been made to obtain these intermediate states under equilibrium conditions in the hope that they will mimic the states present under the kinetic conditions at least to some extent [7–10].

The first direct experimental evidence in support of the above prediction came in 1981 [11], which revealed the equilibrium intermediate state as the molten globule state [2,3,6,12]. This state was found to be similar to an intermediate state observed in experiments of folding kinetics [13–15]; a lot of attention has since focused on its study. The original formulation of this molten globule state suggested that a globular protein can exist not only in the compact native and the unfolded random coiled state, but also in a rather compact state with significant secondary structure but highly disrupted tertiary structure. It has been observed that low urea, guanidine hydrochloride (GdnHCl) treatment, slightly elevated temperature, moderately acidic or alkaline pH induces molten-globule-like intermediate in many proteins [2,16–18]. There is also evidence for the existence of more than one equilibrium folding state, which depicts the folding or unfolding pathway of a protein in finer detail [10].

In the case of the oligomeric proteins, the folding problem is even more complex because subunit association plays a vital role here in addition to folding, and the sequences of these two actions are not similar in different systems. Yet there is good evidence for the presence of the intermediates, especially molten globule intermediates, whose characterization can help in understanding the rules that govern their folding [9].

Porcine heart cytoplasmic or supernatant malate dehydrogenase (s-MDH) is a homodimeric protein (molecular mass 2 × 35 kDa), each subunit containing 333 amino acids and an equivalent cofactor (NAD<sup>+</sup>/NADH) binding site.

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**Abbreviations:** ANS, 1-anilino-8-naphthalene sulfonic acid; D\*, inactive dimer; DLS, dynamic light scattering; GdnHCl, guanidine hydrochloride; M\*, partially folded monomer; M, folded monomer; M<sub>C</sub>, compact monomeric intermediate; M<sub>E</sub>, expanded monomeric intermediate; N or D, native dimer; s-MDH, porcine heart supernatant or cytoplasmic malate dehydrogenase; U, unfolded state.

**Enzyme:** porcine heart cytoplasmic malate dehydrogenase (EC 1.1.1.37).

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The subunits are associated in the dimer by noncovalent bonds and dissociation of the subunits results in the loss of its activity [19]. This enzyme is different from its mitochondrial isozyme with respect to the amino acid composition [20] and follows a totally different kinetic pathway during self-folding [21,22] though they show essentially identical biochemical activity.

In this article we report the detailed study of GdnHCl induced equilibrium denaturation and reversible renaturation of s-MDH using different biochemical and biophysical techniques. The data fits best to the model  $2U \leftrightarrow 2M_E \leftrightarrow 2M_C \leftrightarrow D$  where  $M_C$  and  $M_E$  are two equilibrium intermediates between the native and the unfolded states. The first intermediate in the unfolding transition is a 'compact monomer ( $M_C$ )' resulted by subunit dissociation of the native dimer. This intermediate further unfolds to form the 'expanded monomer ( $M_E$ )' state, which shows most of the properties of a 'molten globule state'. This intermediate retains secondary structure similar to the compact monomer but has lost most of the native tertiary structure. It is the most potent binder of the hydrophobic probe 1-anilino-8-naphthalene sulfonic acid (ANS) and is little less compact than the native monomeric subunits as detected by size exclusion chromatography and dynamic light scattering. While studying equilibrium renaturation of s-MDH no aggregation was detected.

The self-folding pathway of s-MDH was reported in 1986 by Rudolph *et al.* [22]. Our reactivation and chemical cross-linking experiments reconfirm their results. The unassisted folding of s-MDH revealed two parallel kinetic pathways. The major pathway (70–75%) is  $2U \rightarrow 2M^* \rightarrow 2M \rightarrow D$  and the rate limiting step is  $M^* \rightarrow M$ , with a first order rate constant of the order of  $10^{-3} \text{ s}^{-1}$ . The minor pathway ( $2U \rightarrow D^* \rightarrow D$ ) involves the association of the incompletely folded monomers to produce an inactive dimers ( $D^*$ ), which that folds to form the active dimers ( $D$ ) in a very slow folding kinetics ( $K_2 =$  in the order of  $1.2 \times 10^{-5} \text{ s}^{-1}$ ). In this article we report the detailed study of GdnHCl induced equilibrium denaturation and reversible renaturation of s-MDH using different biochemical and biophysical techniques. The data fits best to the model  $2U \leftrightarrow 2M_E \leftrightarrow 2M_C \leftrightarrow D$  where  $M_C$  and  $M_E$  are two equilibrium intermediates between the native and the unfolded states. The first intermediate in the unfolding transition ( $M_C$ ) is a 'compact monomer' resulted by subunit dissociation of the native dimer that unfolds further to the 'expanded monomer ( $M_E$ )' state, which shows most of the properties of a 'molten globule state'. This intermediate retains secondary structure similar to the compact monomer but has lost most of the native tertiary structure. It is the most potent binder of hydrophobic probe and is little less compact than the native monomeric subunits. The relative stabilities of different conformational states were derived from the thermodynamic analysis of the equilibrium renaturation profiles. With respect to the unfolded state the relative stabilities of the 'N', ' $M_C$ ', ' $M_E$ ' state are 24, 21.8 and  $11.5 \text{ kJ} \cdot \text{mol}^{-1}$ , respectively.

Our equilibrium and the kinetic studies indicate that folding of this dimeric protein goes through a four-state folding pathway, which involves two intermediate states. The equilibrium intermediates are thoroughly characterized in this study. One of these intermediates ( $M_E$ ) has molten globule features. However, very short lifetime of the kinetic

intermediates make them unavailable for this study. Further experimental data on the kinetic intermediate states are needed to draw parallel between the equilibrium and the kinetic intermediates.

## MATERIALS AND METHODS

**Enzyme.** Porcine heart supernatant or cytoplasmic malate dehydrogenase (s-MDH) (EC 1.1.1.37), bought from Sigma (St Louis, MO, USA), was obtained as a precipitate in 3.2 M  $(\text{NH}_4)_2\text{SO}_4$ , added as a stabilizing salt during its storage. To remove this high salt the enzyme solution was dialysed against 100 mM potassium buffer phosphate buffer (pH 7.6) containing 5 mM 2-mercaptoethanol. After dialysis, s-MDH had a specific activity of  $350 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , as determined at 25 °C, pH 7.6, in the presence of 0.5 mM oxaloacetate and 0.2 mM NADH. Enzyme concentrations were determined spectrophotometrically at 280 nm by using an extinction coefficient of  $\epsilon^{0.1\%} = 1.08$  [23]. Molar concentrations refer to a subunit molecular mass of 35 000.

### Reagents and buffers

All experiments were generally performed in 100 mM sodium phosphate buffer pH 7.6 containing 1–5 mM 2-mercaptoethanol. Monobasic and dibasic sodium phosphate salts, 2-mercaptoethanol, ultrapure GdnHCl, oxaloacetate and NADH were purchased from Sigma and ANS was from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were of analytical grade.

### Equilibrium denaturation of s-MDH

Denaturation of s-MDH was generally performed by 18-h incubation at 20 °C in 100 mM sodium phosphate buffer (pH 7.6), containing various concentrations of denaturant GdnHCl (pH adjusted to 7.6) so that equilibrium was achieved.

### Equilibrium renaturation of s-MDH

s-MDH was first denatured to equilibrium in 6 M GdnHCl at 20 °C and subsequently diluted (60 fold) in 100 mM sodium phosphate buffer (pH 7.6) containing 1–5 mM 2-mercaptoethanol and GdnHCl in the desired concentration. All samples were incubated at 20 °C for 24 h for equilibrium refolding.

### Biochemical activity assay

The enzymatic activity of each equilibrium denatured/renatured sample (concentration range  $20\text{--}200 \mu\text{g} \cdot \text{mL}^{-1}$ ) was measured following the standard procedure of s-MDH assay, monitoring the rate of the fall of absorbance of 0.2 mM NADH at 340 nm at 25 °C in 150 mM sodium phosphate buffer (pH 7.6) containing 0.5 mM oxaloacetate and 2 mM 2-mercaptoethanol in the presence of respective amount of GdnHCl as was in the unfolding/refolding mixture. In the control set native s-MDH samples were assayed in the same way in the presence of GdnHCl (0–1 M). All assays were done for a brief period of 15 s only, within which even the strongest denaturant used (1 M)

had no detectable effect on the activity of the native enzyme.

### Fluorescence spectroscopy

Fluorescence measurements were carried out on a Hitachi F-3010 spectrofluorometer at 20 °C with a protein concentration 20–400  $\mu\text{g}\cdot\text{mL}^{-1}$ . The samples were excited at 285 nm and the fluorescence emission at 340 nm and the emission  $\lambda_{\text{max}}$  were monitored. All fluorescence values were corrected by subtraction of the apparent fluorescence of the respective concentrations of GdnHCl in the same buffer.

### Circular dichroism spectroscopy

CD spectral measurements were done on a Jasco J-600 spectropolarimeter at 20 °C using a 0.1-cm pathlength cuvette for far-UV and 1.0 cm pathlength cuvette for near-UV region. Protein concentration was typically 100  $\mu\text{g}\cdot\text{mL}^{-1}$  for far-UV and 200  $\mu\text{g}\cdot\text{mL}^{-1}$  for near-UV CD measurements. In all the sets CD spectra were corrected for background absorbance.

### Binding of hydrophobic probe

All equilibrium denatured and renatured samples were incubated with a potent hydrophobic probe ANS (30  $\mu\text{M}$ ) for 5 min at 20 °C and the binding was measured by monitoring ANS fluorescence at 482 nm. To avoid the inner filter effect excitation was done at 420 nm. The emission  $\lambda_{\text{max}}$  was also noted for each set.

### Size exclusion chromatography

To measure the compactness of the different folding states high-pressure liquid chromatography (HPLC) was used. The equilibrium denatured/renatured samples (200  $\mu\text{g}\cdot\text{mL}^{-1}$ ) were run in a Protein pack I<sup>25</sup> gel filtration column pre-equilibrated with the respective amount of GdnHCl (as in the sample), in 100 mM Na-phosphate buffer (pH 7.6) and 1 mM 2-mercaptoethanol, at a flow rate of 1  $\text{mL}\cdot\text{min}^{-1}$  at 4 °C, and the elution profiles were obtained. The apparent molecular masses and Stoke's radii of the peaks were determined from the calibration curves made with the proteins of known molecular mass and Stoke's radius (BSA, 66.3 kDa; 33.9 Å; ovalbumin, 43.5 kDa; 31.2 Å; myoglobin, 16.9 kDa; 20.2 Å and cytochrome *c*, 11.7 kDa; 17.0 Å) [24].

### Dynamic light scattering (DLS)

In addition to the HPLC experiments, DLS was used to measure the hydrodynamic volumes of different folding states during equilibrium unfolding and refolding. This was carried out to check if any aggregation occurred during refolding. The equilibrium unfolding experiments were designed at a protein concentration of 1  $\text{mg}\cdot\text{mL}^{-1}$  and incubated in different GdnHCl concentrations for 24 h. To study equilibrium refolding, 10  $\text{mg}\cdot\text{mL}^{-1}$  s-MDH was denatured with 6 M GdnHCl, at 20 °C for 2 h. Refolding was initiated by 10-fold dilution of the unfolding mixture in the refolding buffer. The carry-over GdnHCl concentration during refolding was 600 mM. Additional GdnHCl was added in the other refolding sets to achieve the required

denaturant concentrations. Equilibrium refolding was achieved by incubating these samples for 24 h at 20 °C.

A 100- $\mu\text{L}$  sample from each reaction was centrifuged at 16 000 *g* for 30 min and then filtered through a 0.1  $\mu\text{m}$  Anatox filter. The protein concentrations of the samples, before and after these treatments, were measured using a 1  $\mu\text{L}$  sample with the Biorad Protein Estimation Kit. No significant loss of sample was observed. The samples are then injected into the Dynapro DLS instrument and 20–30 readings were taken for each sample at 20 °C, with an acquisition time 5 s. The data was analyzed using the 'regularization histogram' and 'cumulant' methods.

### Kinetic study of s-MDH renaturation

Biological activity of any protein depends strictly on its properly folded three-dimensional conformation. Therefore reactivation experiments were used as the most sensitive tool to study refolding. However, these experiments do not provide direct evidence for subunit reassociation, which is essential for the renaturation of this dimeric protein. Therefore, in order to elucidate the assembly mechanism, the functional analysis (reactivation) was supplemented by a direct kinetic analysis of the reassociation process using a chemical cross-linking technique.

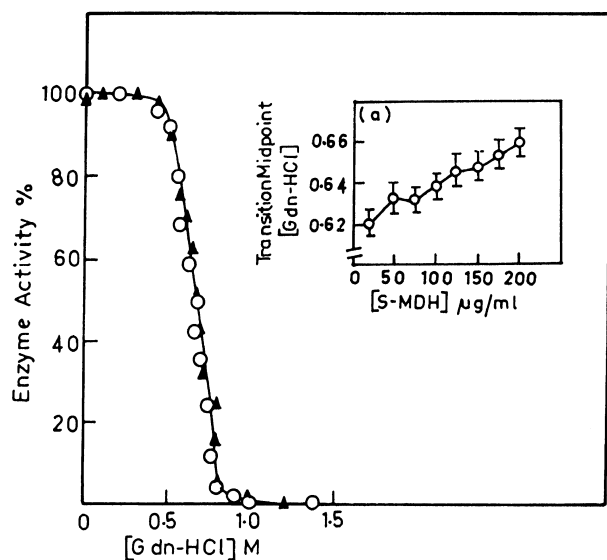
**Reactivation.** The reactivation of s-MDH was initiated using an 80-fold dilution of the 6 M GdnHCl equilibrium denatured samples in 100 mM sodium phosphate pH 7.6, containing 5 mM 2-mercaptoethanol at 20 °C. The recovery of activity was studied by sampling aliquots of refolding mixture (enzyme concentration 0.5–5  $\mu\text{g}\cdot\text{mL}^{-1}$ ) at different time points and measuring the biochemical activity following the standard procedure of the s-MDH assay as described above.

**Chemical cross-linking with glutaraldehyde.** For cross-linking experiments, denaturation of native s-MDH was performed at a concentration of 2  $\text{mg}\cdot\text{mL}^{-1}$  in 6 M GdnHCl at 20 °C for 18 h. No 2-mercaptoethanol or EDTA were present in the buffer. Reconstitution was initiated by 200-fold dilution of the denaturation mixture in 100 mM sodium phosphate pH 7.6 at 20 °C, so that the residual denaturant concentration was 30 mM (above which no successful cross-linking could occur). Chemical cross-linking with glutaraldehyde was carried out using a method modified from Zettlmissl *et al.* [28]. The cross-linking products were run in SDS/PAGE for separation. Then individual lanes were scanned with Biorad gel-documentation system and the profiles were plotted to obtain the relative proportions of different species formed at different times of folding.

## RESULTS

### Enzyme activity

The inactivation profile of s-MDH showed a single transition in the GdnHCl concentration range 0.5 M to 0.8 M above which no enzyme activity was observed (Fig. 1). Upon varying the enzyme concentration (20–200  $\mu\text{g}\cdot\text{mL}^{-1}$ ), the transition midpoints showed a shift towards the right (inset, Fig. 1). This result indicates that the loss of activity could be due to subunit dissociation along with unfolding



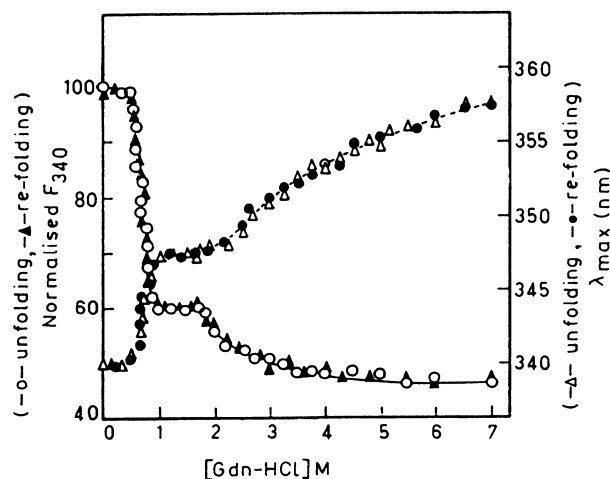
**Fig. 1.** Relative changes of the enzymatic activity of s-MDH as a function of GdnHCl concentration. The enzyme ( $20 \mu\text{g mL}^{-1}$ ) was incubated for more than 18-h at  $20^\circ\text{C}$  in the presence of GdnHCl at different concentrations and the equilibrium denatured samples were assayed in the presence of same concentrations of denaturant in the assay mixture (○). While studying reactivation, 6 M GdnHCl denatured protein was diluted 60-fold (final concentration  $20 \mu\text{g mL}^{-1}$ ) in the presence of different concentrations of GdnHCl and assayed in the same way (▲). The solid line is a nonlinear least-square fit to the data. The inset (a) shows the protein concentration dependence of the inactivation transition midpoint.

because the enzyme monomers are not biochemically active. The reversibility of this inactivation transition was studied by assaying equilibrium refolded samples in the similar way. The maximum recovery was about 60% of the native enzyme activity. Assuming this maximum recovery to be 100%, the data were normalized; the resulting curve overlapped the inactivation profile (Fig. 1).

### Intrinsic fluorescence properties

Fluorescence emission spectra of tryptophan residues are conventionally used as very sensitive probe to the tertiary structure of the proteins. The s-MDH has 10 tryptophan residues, five in each subunit. When excited at 285 nm, it exhibited an emission maximum at 339.6 nm. The fluorescence spectra showed progressive red shift along with a decrease in fluorescence intensity upon exposure to gradually increasing concentration of the denaturant.

Figure 2 shows the change in fluorescence intensity at 340 nm and the emission  $\lambda_{\text{max}}$  shift at different GdnHCl concentrations both during equilibrium unfolding and refolding of s-MDH. The equilibrium refolding transition curve closely matches the unfolding transition showing the process to be perfectly reversible. From these plots it can be seen that the overall unfolding process involves two transitions separated by a plateau region. The first transition occurs between 0.5 and 1 M GdnHCl, which involves a significant drop of  $F_{340}$  (about 80% of total intensity fall) and a red shift of  $\lambda_{\text{max}}$  from 339.6 nm (native  $\lambda_{\text{max}}$ ) to 347 nm. Following this transition, a plateau region is



**Fig. 2.** GdnHCl-dependent unfolding and refolding of s-MDH ( $20 \mu\text{g mL}^{-1}$ ) measured by fluorescence emission. The excitation wavelength was 285 nm. The change in fluorescence intensity at 340 nm ( $F_{340}$ ) during unfolding (○) and refolding (▲) and shift of emission maxima during unfolding (△) and refolding (●) as a function of GdnHCl concentration is shown.

observed extending from 1 M to  $\approx 1.5$  M GdnHCl, within which almost no change in any of the fluorescence parameters takes place. This region between the two transitional zones is a clear indication of the presence of an intermediate state. The second transition of  $F_{340}$  is complete at about 3 M GdnHCl. This transition is small and not as sharp as the first one. However, the second transition involves a red shift in emission maxima from 347 nm to about 356 nm in the GdnHCl concentration range 1.3–5 M.

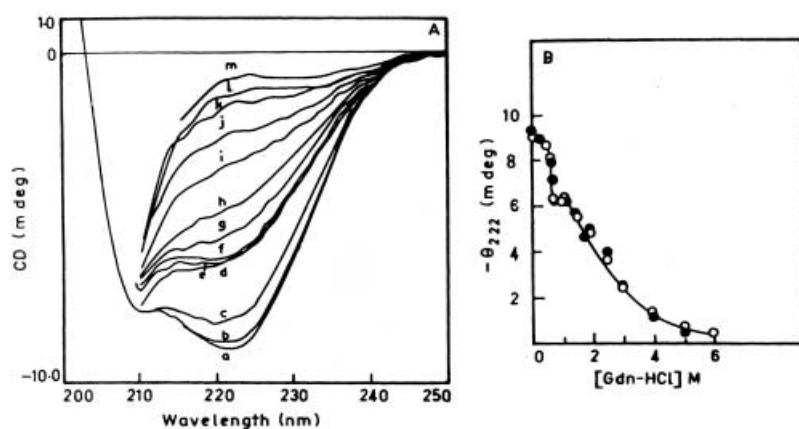
The first transition shows a protein concentration dependence. In the concentration range  $20\text{--}400 \mu\text{g mL}^{-1}$ , the first transition midpoint gradually shifts to the right indicating that this transition may involve subunit dissociation along with unfolding. On the other hand, no change is observed in the second transition zone in the concentration range tested (Table 1).

### CD spectra analysis

The helical content in any protein molecule can be estimated from its far-UV CD spectrum. The far-UV CD spectra of s-MDH in the presence of various GdnHCl concentrations are shown in Fig. 3A. The profile displays minima at 208 nm and 222 nm, which is characteristic of a protein with a high content of  $\alpha$  helical structure. From the value of  $\theta_{222}$  the  $\alpha$  helical content of the native protein is estimated to

**Table 1.** Effect of the variation of the protein concentration in GdnHCl induced equilibrium unfolding transition of s-MDH (detected by fluorescence emission  $\lambda_{\text{max}}$ ).

Protein concentration ( $\mu\text{g mL}^{-1}$ )	Transition mid-points in terms of [GdnHCl] (M)	
	I	II
20	0.67	3.35
400	0.75	3.36



**Fig. 3.** Relative changes of far-UV CD ellipticity of s-MDH due to GdnHCl induced equilibrium denaturation and renaturation.

(A) The far-UV CD spectra of  $100 \mu\text{g}\cdot\text{mL}^{-1}$  s-MDH in the presence of (a) 0 M (b) 0.5 M (c) 0.6 M (d) 0.75 M (e) 1.0 M (f) 1.25 M (g) 1.5 M (h) 2.0 M (I) 2.5 M (j) 3.0 M (k) 4.0 M (l) 5.0 M (m) 6.0 M GdnHCl after correction for background absorbance (average of 10 readings). (B) Change in relative ellipticity or  $\theta_{222}$  (mdeg) as a function of GdnHCl concentration during unfolding (○) and refolding (●).

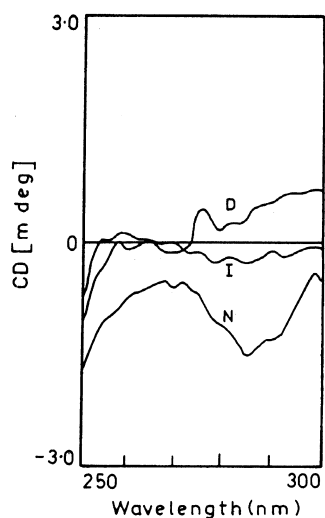
be around 39%, which is in good agreement with the previous reports [29]. When incubated with increasing concentrations of GdnHCl there is a decline in the far-UV CD signals reflecting the gradual loss of the secondary structure of the protein. Figure 3B shows the change in the mean residue ellipticity ' $\theta_{222}$ ', with increasing GdnHCl concentrations during unfolding as well as during refolding. The overall transition process appears to be biphasic. The first phase is brief and ranges from 0.5 to 0.75 M GdnHCl, which involves only 25% of total  $\theta_{222}$  drop. The second phase ranges from 1.25 to 6 M GdnHCl that involves major secondary structure change. At 6 M or higher denaturant concentrations the equilibrium denatured s-MDH samples are practically devoid of any secondary structures. Between these two transitions (to 0.75–1.25 M GdnHCl concentrations) the CD value remains same indicating the presence of an intermediate.

The near-UV CD spectrum is considered to be a sensitive tool to probe the tertiary structure though the information is mostly qualitative. We have studied the near-UV CD spectrum of the native s-MDH and equilibrium denatured s-MDH in the presence of 1.1 M and 6 M GdnHCl where the intermediate and fully unfolded states are expected to occur, respectively, as suggested by intrinsic fluorescence

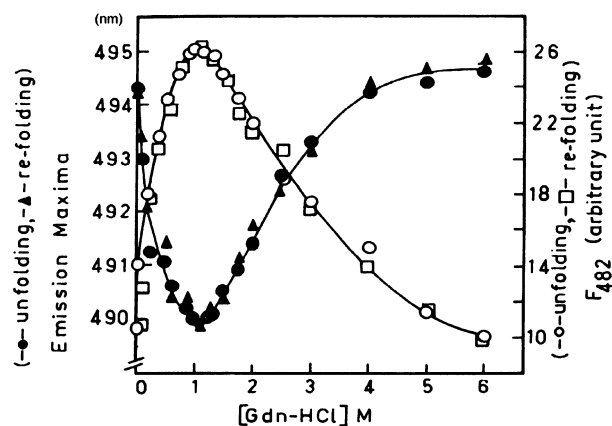
and far-UV CD experiments. The native state has a negative near-UV CD signal where as the fully denatured state shows a positive signal. Figure 4 shows that the near-UV CD spectrum of the 1.1 M GdnHCl equilibrium denatured sample lies in between the native and the denatured spectra depicting its intermediate feature.

#### Binding of hydrophobic probe

The large loss of fluorescence intensity and little change in the far-UV CD signal are often seen in the transitions of native structure to molten globule state [3,6,9,14,15,30]. Similar is our observation in the case of the equilibrium denaturation/renaturation of s-MDH, which indicated the molten globule nature of the intermediate. One of the characteristic features of the molten globule state is the increased access to the interior hydrophobic patches by hydrophobic probes such as ANS and Bis-ANS. Figure 5 shows the binding of  $30 \mu\text{M}$  ANS to equilibrium denatured s-MDH as a function of GdnHCl concentration. As free ANS does not contribute significantly to the total fluorescence, the fluorescence intensity is a reflection of bound ANS. From Fig. 5 it can be seen that the fluorescence intensity at 480 nm gradually increases till 0.9 M GdnHCl



**Fig. 4.** Near-UV CD spectra of s-MDH ( $200 \mu\text{g}\cdot\text{mL}^{-1}$ ) in the presence of (N) 0 M (I) 1.15 M and (D) 6 M GdnHCl (average of 10 readings).



**Fig. 5.** Effect of GdnHCl on ANS binding of s-MDH detected by fluorescence. The excitation wavelength is 420 nm. The ANS fluorescence at 482 nm ( $F_{482}$ ) [unfolding (○) and refolding (□)] and the emission maxima [unfolding (●) and refolding (▲)] are indicated as a function of GdnHCl concentration.

and then remains more or less the same in the GdnHCl concentration range 0.9–1.25 M and then declines at higher denaturant concentrations. The emission  $\lambda_{\text{max}}$  also undergoes a blue shift from 492 nm at 0 M GdnHCl to 490.2 nm at 0.9 M GdnHCl. Beyond 1.25 M GdnHCl, it shows a red shift up to 494 nm at 5 M GdnHCl. Because one intermediate state has been already identified by other spectroscopic methods in the GdnHCl range 0.9–1.25 M where maximum ANS fluorescence was obtained, the conclusion is that this intermediate is the most potent binder of ANS with exposed hydrophobic patches. This result suggests this intermediate state is has a molten globule nature. The intermediate state detected during equilibrium refolding also showed similar ANS binding behavior.

However, the lack of a clear plateau region in the ANS binding experiments compared to the fluorescence and CD experiments suggested additional intermediate species could be present between the native and the molten globule intermediate state (at GdnHCl 0.7–0.9 M), which also has quite high ANS binding capacity. This was investigated by HPLC and DLS studies.

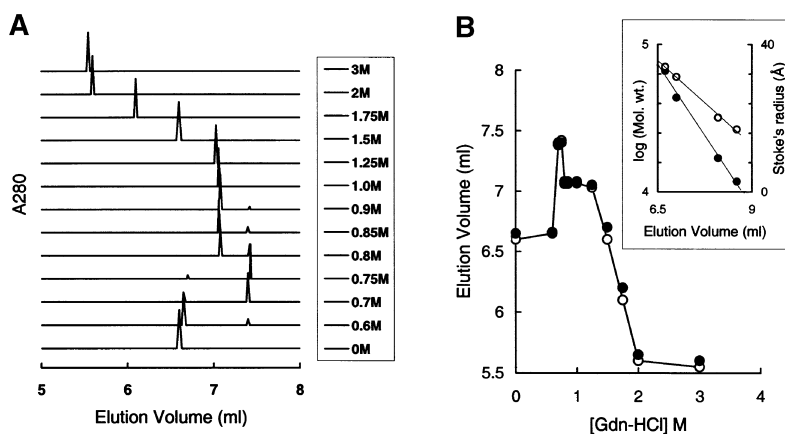
### HPLC measurements

The hydrodynamic properties of the intermediate state are of great importance for its characterization [31,32]. Changes in the hydrodynamic volume of s-MDH during unfolding and refolding process were investigated using size-exclusion HPLC. Figure 6A shows the elution profile of different equilibrium denatured samples. Figure 6B is the plot of the elution volume (major peak) against GdnHCl concentration. Calibration curves of log molecular mass and log Stoke's radius were drawn using BSA, ovalbumin, myoglobin and cytochrome *c* (Fig. 6B, inset) [24]. The native s-MDH has a retention volume of 6.6 mL, consistent with an apparent molecular mass of 68 kDa, which is very close to that expected for homodimeric enzyme. As the GdnHCl concentration is increased (from 0.5 to 0.75 M GdnHCl) another peak appears at 7.4 mL, which sharply increases and the previous peak for the native dimer decreases. This peak corresponds to an apparent molecular

mass of 34.43 kDa, which is in good agreement with the true subunit molecular mass of 35 kDa. Therefore, this sharp transition is due to subunit dissociation of dimeric s-MDH into monomers. We identify this state as the 'compact monomer' ( $M_C$ ) state. With a further increase in the denaturant concentration beyond 0.75 M, the protein peak at 7.4 mL decreases sharply and another peak appears at 7.08 mL that remains unchanged up to 1.25 M GdnHCl. This is the second intermediate state. The lower retention volume of this state compared to that of the  $M_C$  state corresponds to an apparently larger Stoke's radius. Hence this intermediate state (0.85–1.25 M GdnHCl) is called an 'expanded monomer' ( $M_E$ ). Increasing the denaturant concentration beyond 1.25 M led to a further decrease of elution volume indicating complete unfolding of the  $M_E$  state. During refolding the unfolding profile was retraced.

### DLS measurements

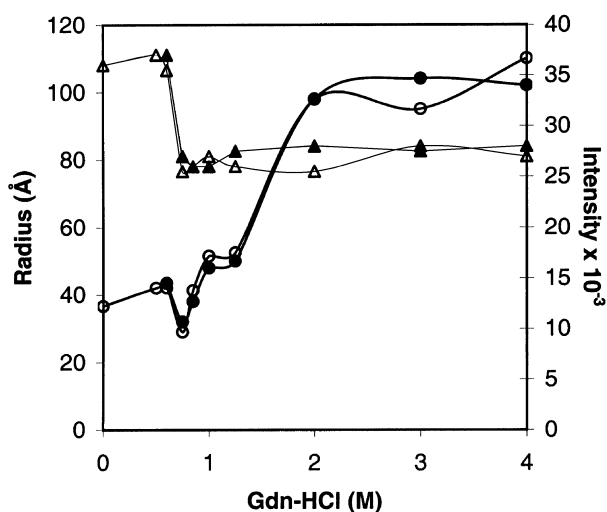
Analysis of the autocorrelation function by cumulants led to the result shown in Table 2. The data points with polydispersity < 25% were monomodal. The other data points, having polydispersity greater than 30%, were analyzed using a bimodal distribution model and the relative fractions of the two populations were determined using an apparent fraction calculator. The apparent radii and the intensities as a function of GdnHCl concentration are plotted in Fig. 7. The radius of the native s-MDH was estimated to be 36.6 Å, which is in good agreement with the result from HPLC measurements. As shown in the figure, the particle size increases slightly between 0 and 0.6 M GdnHCl; a decrease in particle size is seen at 0.75 M. The intensity also dropped at this point and didn't increase further. Because the product of the molecular mass ( $m$ ) and concentration is constant, the change in intensity ( $I \propto C \cdot m$ ) suggests that the decrease in size between 0.6 to 0.75 M GdnHCl is due to particle dissociation, rather than a shift in structural conformation. Therefore, this must be reflecting the compact monomer. Increasing GdnHCl concentration beyond 0.75 M, the particle size expands and remains more



**Fig. 6.** Equilibrium 'dissociation and unfolding', and 'association and refolding' of s-MDH, as measured by size-exclusion HPLC. Experimental conditions were as described in Materials and methods. (A) Elution profiles of s-MDH at the indicated concentrations of GdnHCl during unfolding. (B) Changes in the elution volume (major peak) as a function of GdnHCl concentration [unfolding (○) and refolding (●)] are shown. The inset shows the calibration curves using standard proteins BSA (66.3 kDa, 33.9 Å), ovalbumin (43.5 kDa, 31.2 Å), myoglobin (16.9 kDa, 20.2 Å) and cytochrome *c* (11.7 kDa, 17.0 Å) [24]. The log molecular wt (●) Stoke's radii ( $R_s$ ) in Å (○) are plotted against elution volume.

**Table 2.** Summary of DLS results by method of cumulants. SOS, sum of the square fitting; Polyd, polydispersion.

	[GdnHCl] (M)	Counts per s	Baseline	SOS error	%Polyd	Radius (Å)
Native s-MDH	0	36128	0.999	5.07	15.1	36.62
Equilibrium denaturation	0.5	37005	1.040	4.98	17.0	42.16
	0.75	25526	1.018	4.90	17.6	29.24
	0.85	26719	1.099	5.51	32.5	41.32 (90%) 30.31 (10%)
	1.0	27398	1.002	5.12	18.2	51.50
	4.0	27431	1.014	4.78	21.0	110.2
Equilibrium renaturation	0.6	37046	1.003	5.3	19.9	43.52
	0.75	27139	1.001	5.1	20.2	32.02
	1.0	26298	1.002	4.97	18.7	48.25
	4.0	28375	1.017	5.02	24.6	102.4

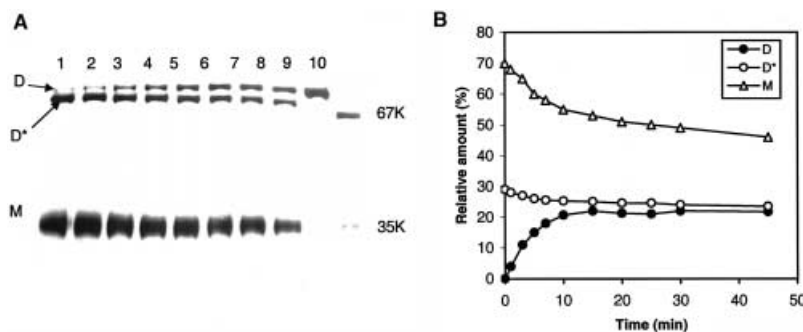
**Fig. 7.** Apparent radius [equilibrium unfolding (○) and refolding (●)] and total intensity [equilibrium unfolding (△) and refolding (▲)] data from DLS measurement as a function of denaturant concentration.

or less constant up to 1.25 M, which is the 'expanded monomer' state identified above. With a further increase in denaturant concentration, the molecule fully unfolds and polydispersity increases to some extent (within the limit of the monomodal distribution). While studying renaturation, the denaturation transition is retraced. No aggregation was seen during renaturation.

#### Kinetics of reassociation

Chemical cross-linking with glutaraldehyde and subsequent analysis of the cross-linked material by SDS/PAGE allows identification and relative quantitation of the different intermediate species reflecting the actual particle distribution at different time points.

Figure 8A shows the band pattern of different molecular species with increasing time. Figure 8B reflects the kinetics of reassociation of s-MDH as estimated from the relative peak areas of the scanned profile of the individual time points. It shows two parallel pathways. Most ( $70 \pm 5\%$ ) of the monomers ( $M^*$ ) folded slowly to form folded monomers ( $M$ ) with a rate constant of  $K_1 = 1.7 \times 10^{-3} \text{ s}^{-1}$ , which then

**Fig. 8.** The kinetics of reassociation and folding of s-MDH at 25 °C as determined from the chemical-cross linking reactions with the nonspecific cross-linking reagent glutaraldehyde. The enzyme concentration used was  $1 \mu\text{g mL}^{-1}$ . (A) The photograph of the SDS/PAGE (10%) showing the different folding populations during the time course of refolding of s-MDH. M is the band of the monomers (35K), D\* represents the slightly faster migrating inactive dimer species and D is the active native dimer. The time points at which the cross-linking was done are as follows: Lane 1, 1 min; lane 2, 3 min; lane 3, 5 min; lane 4, 7 min; lane 5, 10 min; lane 6–15 min; lane 7–25 min; lane 8, 45 min; lane 9, native dimeric s-MDH (cross-linked); lane 10, molecular mass markers. (B) Individual time points were scanned using gel-documentation system and the kinetics of reassociation and folding of s-MDH was determined from the peak-areas. The data were fitted in two parallel first order reactions with rate constants  $K_1 = 1.74 \times 10^{-3} \text{ s}^{-1}$  (relative amplitude  $75 \pm 5\%$ ) and  $K_2 = 1.2 \times 10^{-5} \text{ s}^{-1}$  (relative amplitude  $25 \pm 5\%$ ).

associated quickly to form the active dimers. The rest of the monomers ( $25 \pm 5\%$ ) rapidly associated to form a presumably dimeric intermediate ( $D^*$ ), with a slightly higher electrophoretic mobility in comparison to the folded dimers ( $D$ ). These dimers fold to form the active dimers by a very slow first order reaction with a rate constant of  $K_2 = 1.2 \times 10^{-5} \text{ s}^{-1}$ . So the former pathway is obviously the 'major' folding pathway. This result agrees well with the previous report [22].

### Reactivation time course analysis

The kinetics of reactivation of s-MDH is resolved by the same parallel folding reactions as seen for the chemical cross-linking study (Fig. 9). The reactivation starts from zero activity at the first time point immediately after dilution of the denaturant. The rate and the yield of reactivation do not depend on enzyme concentration in the range  $0.5\text{--}5 \mu\text{g}\cdot\text{mL}^{-1}$ .

### DISCUSSION

Previously it was thought that folding of a protein involved two states: native ( $N$ ) and unfolded ( $U$ ), the transition being  $N \rightarrow U$ . It is now well established that several intermediates accumulate in the folding pathway and again there can be multiple pathways of folding. Therefore, to explore the folding mechanism two approaches are most commonly used: (a) characterization of the intermediates to understand the structural changes involved in each transition, and (b) analysis of the kinetic mechanism that enables determination of the rate constants of individual reactions occurring in the pathway. The intermediate states need to be sufficiently populated to be detectable for their characterization. But the kinetic intermediates are so transient in nature that it is very difficult to trap them

under kinetic conditions. So, the only possible alternative is to create similar situations under equilibrium conditions so that the kinetic intermediates can be trapped and characterized.

Among these equilibrium intermediates the molten globule state is perhaps the most characterized. Fink, Goto, Ptitsyn and others have shown that a number of proteins can be transformed into the molten globule state either at low pH [17,18,33] or at low concentrations of GdnHCl [34] or other denaturants. There are also reports of kinetic intermediate states of folding virtually identical with the equilibrium molten globule state [13–15]. This frequent occurrence of the equilibrium molten globule state together with the observation that it serves as a universal kinetic intermediate in protein folding and is involved in a number of physiological processes emphasizes its important role in the folding pathway [8]. There is now evidence for multiple equilibrium intermediate states, which are thought to be involved in the kinetic process [10]. These intermediates show different degrees of structural parameters and stabilities.

Many dimeric proteins like aspartate aminotransferase [35], platelet factor 4 [36], brain derived neurotropic factor [37,38], 3,4-dihydroxyphenyl alanine decarboxylase [9], tubulin [39] are reported to have molten globule intermediates that are partially melted inactive monomers. In the present study we have taken several experimental approaches to probe into the structural integrity of another dimeric protein, porcine heart cytoplasmic malate dehydrogenase through the course of its GdnHCl induced equilibrium denaturation and renaturation. The time course of reactivation and reassociation of s-MDH are also studied by activity assay and chemical cross-linking with glutaraldehyde to get an insight to the kinetics of its folding.

Table 3 shows the summary of the results obtained from equilibrium denaturation and renaturation studies of s-MDH by various methods. Like many other dimeric proteins [40], it also undergoes subunit dissociation first. This is evident from the first transition of HPLC and DLS studies (GdnHCl concentration range  $0.5\text{--}0.75 \text{ M}$ ). Because this enzyme is only active in dimeric form, subunit dissociation leads to its total inactivation [19]. That is why the inactivation transition overlaps the dissociation transition observed by HPLC and DLS. The first transition of far-UV-CD, indicating melting of the secondary structure also overlaps this transition (20–25% of total drop of  $\theta_{222}$ ). This change in secondary structure in this transition can be due to local unzipping on the surface of the s-MDH molecule or due to partial relaxation of the building blocks because of subunit dissociation. This transition results in the formation of a 'compact monomeric intermediate ( $M_C$ )' with an apparent Stoke's radius of  $2728 \pm 1.12 \text{ \AA}$ , that show distinctly higher ANS binding capacity compared to the native state.

The fate of this intermediate is determined in the next transition (traced by HPLC and DLS) at the GdnHCl concentration range  $0.75\text{--}0.9 \text{ M}$ , where its tertiary structure gets mostly dissolved (as indicated by tryptophan fluorescence), it becomes less compact (Stoke's radius  $30.86 \text{ \AA}$ , from HPLC) and the hydrophobic core becomes more solvated and hence the accessibility to the hydrophobic probe ANS increases. In this transition, the compact

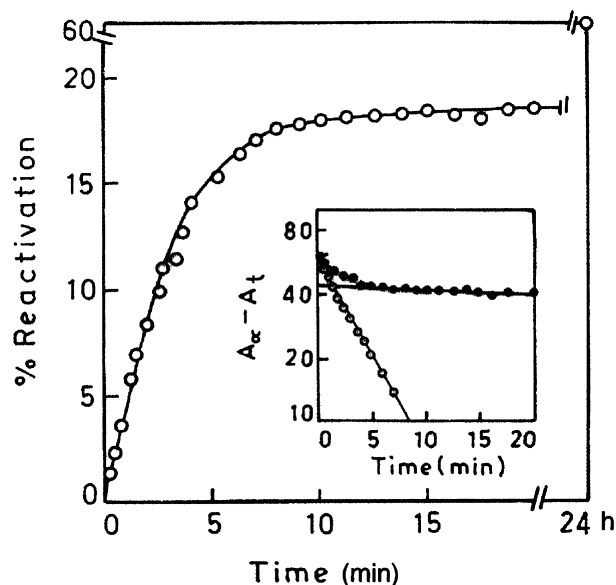


Fig. 9. Time course and kinetics of self-folding of s-MDH after 40 min denaturation by  $6 \text{ M GdnHCl}$  at  $20 \text{ }^\circ\text{C}$ . The enzyme concentration used  $0.5$  was  $0.5\text{--}5 \mu\text{g}\cdot\text{mL}^{-1}$ . The inset shows the parallel kinetics.



**Table 3. Summary of the equilibrium denaturation/renaturation transitions of s-MDH.**

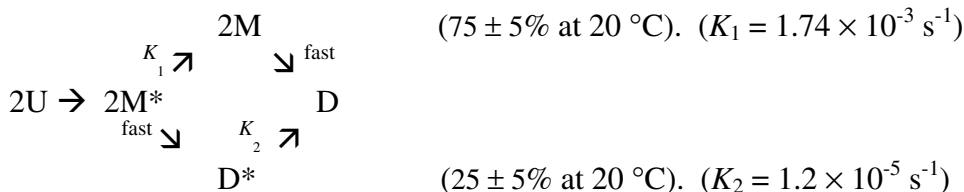
Parameters	[GdnHCl] (M)	Observed changes/special features
Activity	0.5–0.8	Total inactivation
$F_{340}$ and emission $\lambda_{\max}$	0.5–1.0	80% of total decrease of $F_{340}$ . $\lambda_{\max}$ shifts from 339.6 to 347 nm
	1.0–1.5	No change of $F_{340}$ and emission $\lambda_{\max}$
	> 1.5	$F_{340}$ decrease complete, peak shifts from 347 to 356 nm
	0.5–0.75	Minor change, 25% of total drop of $\theta_{222}$
$\theta_{222}$	0.75–1.25	No change of $\theta_{222}$
	1.25–6.0	Major change, 75% of total drop of $\theta_{222}$
ANS binding	0.5–0.9	Increases, indication of intermediate species around 0.75 M
	0.9–1.25	Most potent binding of ANS
	1.25–6	Decreases
Hydrodynamic volume (HPLC and DLS)	0.5–0.75	Subunit dissociation leading to formation of $M_C$
	0.75–0.85	Partial melting of $M_C$ , leading to formation of $M_E$
	0.85–1.25	$M_E$ intermediate state retained
	> 1.25	$M_E$ further unfolds to U state

monomer transforms to an expanded monomer ( $M_E$ ) state. This state occurs up to 1.25 M GdnHCl concentration during which no change in any of the biophysical parameters was observed. So this is the most prominent equilibrium intermediate state which retains most of the native secondary structure but has an almost completely disrupted tertiary structure, it is a little less compact than the native or compact monomeric species ( $M_C$ ) and is the most potent binder of hydrophobic probe ANS. This observation is in good agreement with the original formulation of the molten globule state [2,3,6,11,12,30,41]. With a further increase in denaturant concentration all of its residual structures get dissolved. Hence the equilibrium denaturation and/renaturation pathway of s-MDH can be summarized as  $D \leftrightarrow 2M_C \leftrightarrow 2M_E \leftrightarrow 2U$ .

The refolding of s-MDH was extensively studied by Rudolph *et al.* [22]. They showed that the refolding of s-MDH followed two parallel pathways. The rate limiting steps in both the pathways were first order isomerization reactions ( $M^* \rightarrow M$  with rate constant  $K_1 = 1.3 \times 10^{-3} \text{ s}^{-1}$  and relative amplitude 70%, hence called 'major pathway' and  $D^* \rightarrow D$  with rate constant  $K_2 = 7 \times 10^{-5} \text{ s}^{-1}$  and relative amplitude 30%, hence called 'minor pathway'). Our results of reactivation and chemical cross-linking studies agree well with the reported results (major pathway ' $M^* \rightarrow M$ ' with rate constant  $K_1 = 1.74 \times 10^{-3} \text{ s}^{-1}$  and relative amplitude  $75 \pm 5\%$  and minor pathway, ' $D^* \rightarrow D$ ' with rate constant  $K_2 = 1.2 \times 10^{-5} \text{ s}^{-1}$  and relative amplitude  $25 \pm 5\%$ ). The association reactions in both the pathways are so fast that rate constants cannot be determined by these techniques. Schematically the pathway can be represented as:

where U = unfolded,  $M^*$  = partially folded monomer, M = folded monomer,  $D^*$  = incompletely folded dimer and D = native dimer.

In summary, our results indicate that the folding of s-MDH goes through a four-state pathway. The equilibrium unfolding transitions are fully reversible except for the reactivation transition, which recovers 60% of native state activity. However, this is not an unusual case and has been reported previously in other oligomeric proteins [21,22,42,43]. This is probably because reactivation needs finer tuning of the folding in the active site of the protein than the other transitions. From the kinetic studies we can see that the unfolded molecules taking the minor pathway undergo fast association leading to incorrectly folding dimers. These misfolded dimers fold so slowly compared to the active ones that they effectively do not contribute to reactivation. Nevertheless it should be mentioned that they don't aggregate and are indistinguishable from the active dimers in terms of most of their structural parameters (fluorescence, CD, hydrodynamic radius measurement). As we have shown that  $\approx 30\%$  of the unfolded molecules take this 'unproductive' folding pathway, this can't account fully for the 40% inactive population. We assume that the rest of the inactive population is the contribution of the other incorrectly folded dimers, which originate as a by-product of the major folding pathway, when monomers associate prematurely, before they reach the correct state of folding needed for the active dimer formation. The equilibrium and the major kinetic folding pathway of s-MDH is apparently similar. However, experimental data on the kinetic intermediates are needed to draw parallel between them.



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## REFERENCES

- Ptitsyn, O.B. (1973) Stages in the mechanism of self organisation of protein molecules. *Dokl. Akad. Nauk SSSR* **210**, 1213–1215.
- Ptitsyn, O.B. (1987) Protein folding: hypothesis and experiments. *J. Protein Chem.* **6**, 273–293.
- Ptitsyn, O.B. (1992) The molten globule state. In *Protein Folding* (Creighton, T.E., ed.), pp. 243–300. W. H. Freeman and Company, New York.
- Kim, P.S. & Baldwin, R.L. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Annu. Rev. Biochem.* **51**, 459–489.
- Kim, P.S. & Baldwin, R.L. (1990) Intermediates in the folding reaction of small proteins. *Annu. Rev. Biochem.* **59**, 631–660.
- Kuwajima, K. (1989) The molten globule as a clue for understanding the folding and cooperativity of globular protein structure. *Proteins: Struct. Funct. Genet.* **6**, 87–103.
- Ptitsyn, O.B. (1994) Kinetic and equilibrium intermediates in protein folding. *Protein Eng.* **7**, 593–596.
- Ptitsyn, O.B. (1995) Structures of folding intermediates. *Curr. Opin. Struct. Biol.* **5**, 74–78.
- Dominici, P., Moore, P.S. & Borri Voltattorni, C. (1993) Dissociation, unfolding and refolding trials of pig kidney 3,4 dihydroxy phenyl alanine decarboxylase. *Biochem. J.* **295**, 493–500.
- Uversky, V.N. & Ptitsyn, O.B. (1994) "Partly folded state", a new equilibrium state of protein molecules: four state guanidinium chloride induced unfolding of  $\beta$ -lactamase at low temperature. *Biochemistry* **33**, 2782–2791.
- Dolgikh, D.A., Gilmanshin, R.I., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminov, S.Y. & Ptitsyn, O.B. (1981) Alpha lactalbumin: compact state with fluctuating tertiary structure. *FEBS Lett.* **136**, 311–315.
- Christensen, H. & Pain, S.R. (1991) Molten globule intermediates and protein folding. *Eur. Biophys. J.* **19**, 221–229.
- Dolgikh, D.A. (1983) *PhD Thesis*, Institute of Protein Research, Academy of Sciences of the USSR, Pushchino.
- Dolgikh, D.A., Kolomiets, A.P., Bolotina, I.A. & Ptitsyn, O.B. (1984) Molten globule' state accumulates in carbonic anhydrase folding. *FEBS Lett.* **165**, 88–92.
- Dolgikh, D.A., Abaturon, L.V., Bolotina, I.A., Brazhnikov, E.V., Bychkova, V.E., Bushuev, V.N., Gilmanshin, R.I., Lebedev, Yu. O., Semisotnov, G.V., Tiktupulo, E.I. & Ptitsyn, O.B. (1985) Compact state of a protein molecule with pronounced small scale mobility: Bovine (alpha) lactalbumin. *Eur. Biophys. J.* **13**, 109–121.
- Damaschun, G., Gernat, C., Damaschun, H., Bychkova, V.E. & Ptitsyn, O.B. (1986) Solvent dependence of dimensions of unfolded protein chains. *Int. J. Biol. Macromol.* **8**, 226–230.
- Goto, Y., Calciano, L.J. & Fink, A.L. (1990a) Acid-induced folding of proteins. *Proc. Natl Acad. Sci. USA* **87**, 573–577.
- Goto, Y., Takahashi, N. & Fink, A.L. (1990b) Mechanism of acid-induced folding of proteins. *Biochemistry* **29**, 3480–3488.
- Birktoft, J.J., Rhodes, G. & Banaszak, L.J. (1989) Refined crystal structure of cytoplasmic malate dehydrogenase at 2.5-Å resolution. *Biochemistry* **28**, 6065–6081.
- Banaszak, L.J. & Bradshaw, R.A. (1975) Malate dehydrogenase. In *The Enzymes*, 3rd edn. XI. (Boyer, P.D., ed.), pp. 369–396, Academic Press, New York.
- Jaenicke, R., Rudolph, R. & Heider, I. (1979) Quaternary structure, subunit assembly and *in vitro* association of mitochondrial malic dehydrogenase. *Biochemistry* **18**, 1217–1223.
- Rudolph, R., Fuchs, I. & Jaenicke, R. (1986) Reassociation of dimeric cytoplasmic malate dehydrogenase is determined by slow and very slow folding reactions. *Biochemistry* **25**, 1662–1669.
- Frieden, C., Honegger, J. & Gilbert, H.R. (1978) Malate dehydrogenases. The lack of evidence for dissociation of the dimeric enzyme in kinetic analyses. *J. Biol. Chem.* **253**, 816–820.
- Corbett, R.J.J. & Roche, R.S. (1984) Use of high speed size exclusion chromatography for the study of protein folding and stability. *Biochemistry* **23**, 1888–1894.
- Dautrevaux, M., Boulanger, Y., Han, K. & Biserte, G. (1969) Structure covalente de la myoglobine de cheval. *Eur. J. Biochem.* **11**, 267–277.
- Finn, E.B., Chem, X., Jennings, P.A., Saalau-Bethell, S.M. & Mathews, C.R. (1992) In *Protein Engineering. A Practical Approach*, pp. 167–189. IRL Press, Oxford.
- Press, W.H., Flannery, B.P., Teulolsky, S.A. & Vetterline, W.T. (1989) In *Numerical Recipes: the Art of Scientific Computing*. Cambridge University Press, Cambridge.
- Zettlmeissel, G., Rudolph, R. & Jaenicke, R. (1982) Rate-determining folding and association reactions on the reconstitution pathway of porcine skeletal muscle lactic dehydrogenase after denaturation by guanidine hydrochloride. *Biochemistry* **21**, 3946–3950.
- Siegel, J.B., Steinmetz, W.E. & Long, G.L. (1980) A computer assisted model for estimating protein secondary structure from circular dichroic spectra: comparison of animal Lactate dehydrogenases. *Anal. Biochem.* **104**, 160–167.
- Dolgikh, D.A., Abaturon, L.V., Brazhnikov, E.V., Lebedev, Yu.O., Chirgadz, Yu.N. & Ptitsyn, O.B. (1983) Acid form of carbonic anhydrase: 'Molten globule' with a secondary structure. *Dokl. Akad. Nauk. SSSR* **272**, 1481–1484.
- Uversky, V.N., Semisotnov, G.V., Pain, R.H. & Ptitsyn, O.B. (1992) All-or-none mechanism of the molten globule unfolding. *FEBS Lett.* **314**, 89–92.
- Uversky, V.N. (1993) Use of fast protein size exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule. *Biochemistry* **32**, 13288–13298.
- Goto, Y. & Fink, A.L. (1989) Conformational states of  $\beta$ -lactamase: mother globule states at acidic and alkaline pH with high salt. *Biochemistry* **28**, 945–952.
- Hagihara, Y., Aimoto, S., Fink, A.L. & Goto, Y. (1993) Guanidine hydrochloride-induced folding of proteins. *J. Mol. Biol.* **231**, 180–184.
- Herold, M. & Kirschner, K. (1990) Reversible dissociation and unfolding of aspartate amino transferase from *Escherichia coli*: characterization of a monomeric intermediate. *Biochemistry* **29**, 1907–1913.
- Mayo, K.H., Barker, S., Kuranda, M.J., Hunt, A., Myers, J.A. & Maione, T.E. (1992) Molten globule monomer to condensed dimer: role of disulphide bonds in platelet factor-4 folding and subunit association. *Biochemistry* **31**, 2255–12265.
- Philo, J.S., Rosenfeld, R., Arakawa, T., Wen, J. & Narhi, L.O. (1993) Refolding of brain derived neurotrophic factor from guanidine hydrochloride: kinetic trapping in a collapsed form which is incompetent for dimerization. *Biochemistry* **32**, 10812–10818.

38. Narhi, L.O., Rosenfeld, R., Wen, J., Arakawa, T., Prestrelski, S.J. & Philo, J.S. (1993) Acid-induced unfolding of brain derived neurotrophic factor results in the formation of a monomeric 'a state'. *Biochemistry* **32**, 10819–10825.
39. Guha, S. & Bhattacharyya, B. (1995) A partially folded intermediate during tubulin unfolding: Its detection and spectroscopic characterization. *Biochemistry* **34**, 6935–6931.
40. Kelly, S.M. & Price, N.C. (1991) The unfolding and refolding of pig heart fumerase. *Biochem. J.* **275**, 745–749.
41. Kuwajima, K. (1992) Protein folding *in vitro*. *Curr. Opin. Biotechnol.* **3**, 462–467.
42. Jaenicke, R. & Rudolph, R. (1986) Refolding and association of oligomeric proteins. *Methods Enzymol.* **131**, 218–250.
43. Zettlmeissl, G., Rudolph, R. & Jaenicke, R. (1982) Rate-determining folding and association reactions on the reconstitution pathway of porcine skeletal muscle lactic dehydrogenase after denaturation by guanidine hydrochloride. *Biochemistry* **21**, 3946–3950.