Remarkably slow folding of a small protein

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Abstract Equilibrium denaturation of the 72 amino acid α/β-protein MerP, by acid, guanidine hydrochloride, or temperature, is fully reversible and follows a two-state model in which only the native and unfolded states are populated. A *cis-trans* equilibrium around a proline peptide bond causes a heterogeneity of the unfolded state and gives rise to a slow- and a fast folding population. With a rate constant of 1.2 s^{-1} for the major fast folding population, which has none of the common intrinsically slow steps, MerP is the slowest folding protein of this small size yet reported.

Key words: MerP; Protein folding; Tyrosine fluorescence; Proline isomerization

1. Introduction

To thoroughly investigate the protein folding process and to find clues that might help in solving the protein folding problem, we need to expand the database of studied proteins further. Small proteins are particularly interesting in this regard, since they are likely to be less complex than large ones and may provide the simplest starting point for answering these questions. MerP is a water soluble protein of 72 amino acids and one of the proteins involved in the detoxification of mercuric ions. It is located in the periplasm of Gram-negative bacteria that are resistant to mercuric ions, and binds the ions when they diffuse across the outer membrane [1–3]. The reduced form of MerP, without a disulfide bond between Cys^{14} and Cys^{17}, is probably the biologically active form, and the two cysteins are thought to be involved in the binding of mercuric ions [4,5]. The sequential assignment of the ^1H NMR spectrum has led to an estimate of the secondary structure and global fold of oxidized MerP, and the determination of a high-resolution structure is under way [6]. The secondary structure comprises four β-strands and two α-helices in the order $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$, and the protein folds into an antiparallel β-sheet, $\beta_2\beta_3\beta_1\beta_4$, with the two α-helices on one side of the sheet (Fig. 1).

We think that MerP is an interesting addition to the current repertoire of very small, one-domain proteins investigated with respect to folding and stability [7–17]. In this article we characterize spectroscopic properties of oxidized MerP, and investigate the stability and reversibility of the folding/unfolding reaction to denaturation with guanidine hydrochloride (GuHCl), pH and temperature. The folding rate of oxidized and reduced MerP is measured with intrinsic fluorescence.

2. Materials and methods

Guanidine hydrochloride (GuHCl, sequanal grade) was purchased from Pierce. All other chemicals were of reagent grade. Oxidized MerP was expressed and purified as previously described [4].

The stability of MerP was determined at a concentration of 20 μM (0.15 mg/mL). GuHCl-stability was determined by incubating MerP for 24 h at 25°C in various concentrations of GuHCl. This was done at three different pH values: 0.1 M Na-acetate (pH 4.9), 0.1 M Tris-SO_4 (pH 7.5), and 0.1 M Tris-SO_4 (pH 9.0). Thermal stability was determined in 0.1 M MOPS (pH 7.5) between 25°C and 90°C. pH stability was monitored at 20 different pH values: 0.1 M glycine-Cl in the pH range 1.0–3.0, 0.1 M Na-acetate in the pH range 3.5–6.0, 0.1 M Tris-Cl in the pH range 7.0–9.0, and 0.1 M Na-glycine in the pH range 9.5–12.0. The stability of the samples was monitored by intrinsic fluorescence (Shimadzu RF-5000 Spectrofluorophotometer) and circular dichroism (CD) (Jasco J720 Spectropolarimeter and Jobin-Yvon CD6 spectrodichrograph). The fluorescence of the single tyrosine was measured between 290 and 450 nm, using an excitation wavelength of 276 nm (the entrance bandpass was 10 nm and the exit bandpass was 3 nm). CD spectra were the average of three scans obtained by collecting data at 0.5 nm intervals with an integration time of 2 s. Far-UV CD spectra were recorded using a 1 mm quartz cell and near-UV CD spectra with a 4 mm quartz cell.

Thermal stability was measured by following the change in ellipticity at 222 nm. At each new temperature the signal was stable after roughly 2 min, but a time scan was carried out for a total of 10 min and the value used was the average of the last 5 min. The pH-stability profile was obtained by measuring the far-UV CD spectrum of each sample, in the same way as for the GuHCl-containing samples.

The reversibility of the folding/unfolding reaction was investigated by incubating different concentrations of MerP in 6 M GuHCl, 0.1 M Tris-SO_4 (pH 7.5) for 24 h at 25°C. The samples were thereafter diluted with 0.1 M Tris-SO_4 (pH 7.5) to several different GuHCl concentrations and 20 μM MerP after which they were incubated at 25°C for another 24 h. The intrinsic fluorescence and far-UV CD were measured and plotted in the same way as for the stability samples.

After measuring thermal stability as described above, the temperature was lowered stepwise, and the values used for each temperature were the average of the last 5 min of a 15-min period.

All kinetic experiments were performed at 25°C and 0.1 M Na-acetate (pH 4.9) was present at all times. To study the folding kinetics, 220 μM MerP was first denatured by incubation in 3 M GuHCl for 24 h. Renaturation was achieved by an 11-fold dilution, giving a protein concentration of 20 μM and GuHCl concentrations of 0.27 M. To measure the folding kinetics of reduced MerP, 10 mM dithiothreitol was also present at denaturing conditions and in the diluting buffers. The reaction was monitored by the change in intrinsic fluorescence-emission intensity at 304. Fluorescence data were collected with an Applied Photophysics SX-17MV stopped flow instrument in the time range 2 ms–20 s (excitation wavelength 276 nm with bandpass 10 nm and emission wavelength 304 nm with bandpass 30 nm), and with a Shimadzu RF-5000 Spectrofluorophotometer between 20 and 1000 s (excitation wavelength 276 nm with bandpass 3 nm and emission wavelength 304 nm with bandpass 30 nm). With the latter instrument the samples were excited for only 2 s per min to minimize photo bleaching. The unfolding reaction was monitored in exactly the same way as the folding reaction. 220 μM native MerP was diluted 11-fold with a buffer containing 3.3 M GuHCl.

Double-jump experiments, where the protein was allowed to unfold (or refold) for shorter periods of time before the onset of folding (or unfolding), were also performed. Double-jump folding was achieved by first mixing 440 μM MerP with 6 M GuHCl in a 1:1 ratio and allowing the unfolding reaction to proceed for 2 s in the stopped-flow

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apparatus, before folding was initiated by a 11-fold dilution to 0.27 M GuHCl. Double-jump unfolding, where the protein was allowed to refold for shorter periods of time before the onset of unfolding, was executed by first mixing 440 μM MerP in 6 M GuHCl with buffer in a 1:1 ratio. The folding reaction proceeded for 0.02, 0.1, 0.5, 1, 2, 4, 10 or 16 s, whereafter a 11-fold dilution with 3.3 M GuHCl started the unfolding reaction. These samples were monitored with intrinsic tyrosine fluorescence as described above.

Data were fitted with non-linear least-square analysis, using the computer program GraFit 3.0 (Erithacus Software Ltd., Staines, UK). The stability data obtained in GuHCl indicated a two-state process, and were fitted to the equation:

$$y = \frac{\alpha_N + \beta_N[\mathrm{GuHCl}] + a\alpha_U + a\beta_U[\mathrm{GuHCl}]}{1 + a} \quad (1)$$

where [18]

$$a = \exp\left\{\frac{m_{eq}[\mathrm{GuHCl}] - \Delta G_N^{H_2O}}{RT}\right\}$$

in which y is the measured parameter's value at a certain GuHCl concentration, α_N and α_U are this parameter's values at 0 M GuHCl, whereas β_N and β_U are its linear dependencies on GuHCl for the native and unfolded forms, respectively; $\Delta G_N^{H_2O}$ is the free energy of unfolding in water, m_{eq} is a constant that is proportional to the increase in the degree of exposure of the protein on denaturation, and R is the gas constant. To determine $[\mathrm{GuHCl}]_{1/2}$ with good accuracy, the concentration of GuHCl at which half of the protein is denatured, the data were also fitted to Eq. 1, but in this case:

$$a = \exp\left\{\frac{m([\mathrm{GuHCl}] - [\mathrm{GuHCl}]_{1/2})}{RT}\right\}$$

Thermal stability data were fitted to the equation:

$$y = \frac{\alpha_N + \beta_N T + b\alpha_U + b\beta_U T}{1 + b} \quad (2)$$

where [19]

$$b = \left(\frac{T}{T_m}\right)^{\Delta C_p/R} \exp\left\{\frac{1}{R}\left(\frac{T_m^* \Delta C_p^* \Delta H_m}{T} + \frac{\Delta H_m}{T} - \Delta C_p\right)\right\}$$

in which ΔH_m is the enthalpy change for unfolding measured at the melting temperature, T_m, and ΔC_p is the difference in heat capacity between the native and unfolded states.

3. Results and discussion

3.1. Stability and reversibility of MerP

The intrinsic fluorescence of MerP increases markedly upon denaturation in GuHCl (Fig. 2A), probably reflecting the movement of the single tyrosine from the proximity of a specific quencher in the native state to the relatively less quenching environment of the surrounding water. The near-UV CD spectrum (Fig. 2B), whose fine structure is a reflection of the asymmetry in the environment of the aromatic side chains, indicates that MerP is a protein with a well ordered tertiary structure. The near-UV CD spectrum in 3 M GuHCl, on the other hand, lacks this fine structure which indicates that the aromatic side chains are in a symmetric environment in the unfolded state. Since MerP only has one tyrosine and no tryptophan, the intensity of the near-UV CD spectrum is very low and, unfortunately, it is therefore not a practical method for monitoring the folding/unfolding reaction; it would require large amounts of protein. Far-UV CD, reporting mainly on secondary structure content, is better suited for this purpose. The CD spectrum between 178 and 260 nm (Fig. 2C) was used to predict the secondary structure content of

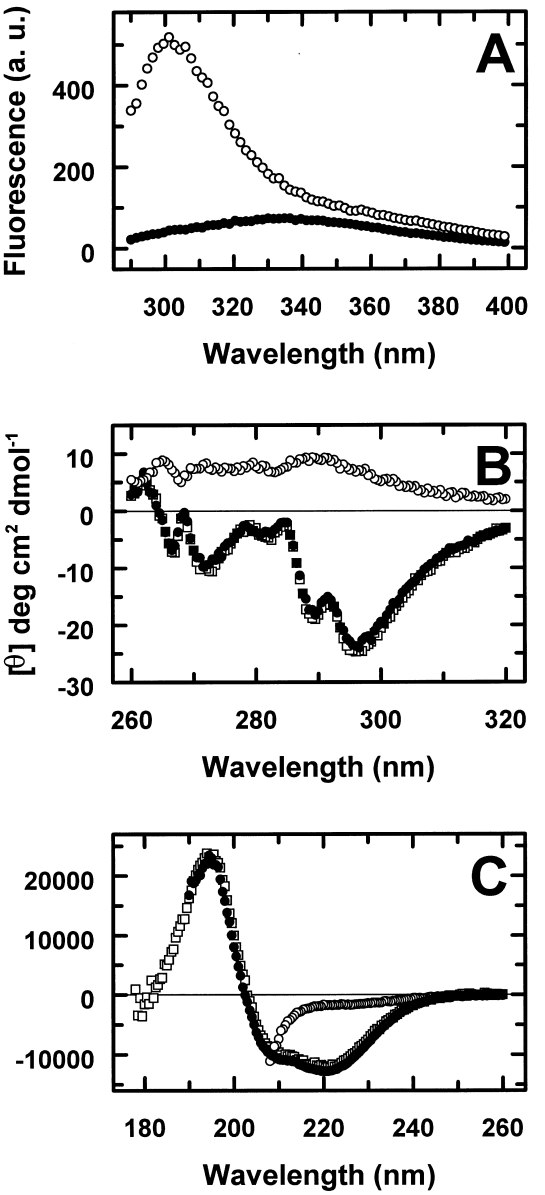

Fig. 2. Intrinsic fluorescence (A), near-UV CD (B), and far-UV CD spectra (C) of native (filled circles) and denatured (open circles) MerP. For intrinsic fluorescence and far-UV CD the protein concentration was 20 μM, and for near-UV CD 500 μM, in 0.1 M Tris-H_2SO_4 (pH 7.5). Denaturation was achieved with 3 M GuHCl. CD spectra at pH 4.9 (open squares) overlaps with those recorded at pH 7.5 (filled circles).

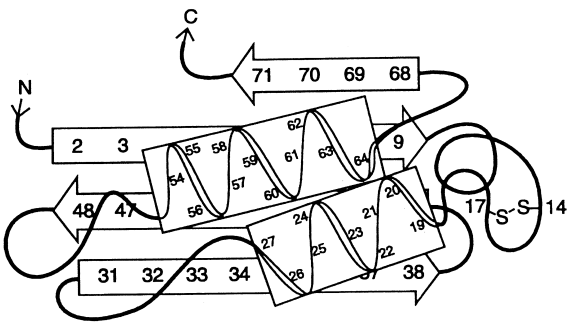

Fig. 1. The global fold of MerP (from [6]). The protein folds into an antiparallel β-sheet, $\beta_2\beta_3\beta_1\beta_4$, with two α-helices on one side of the sheet.

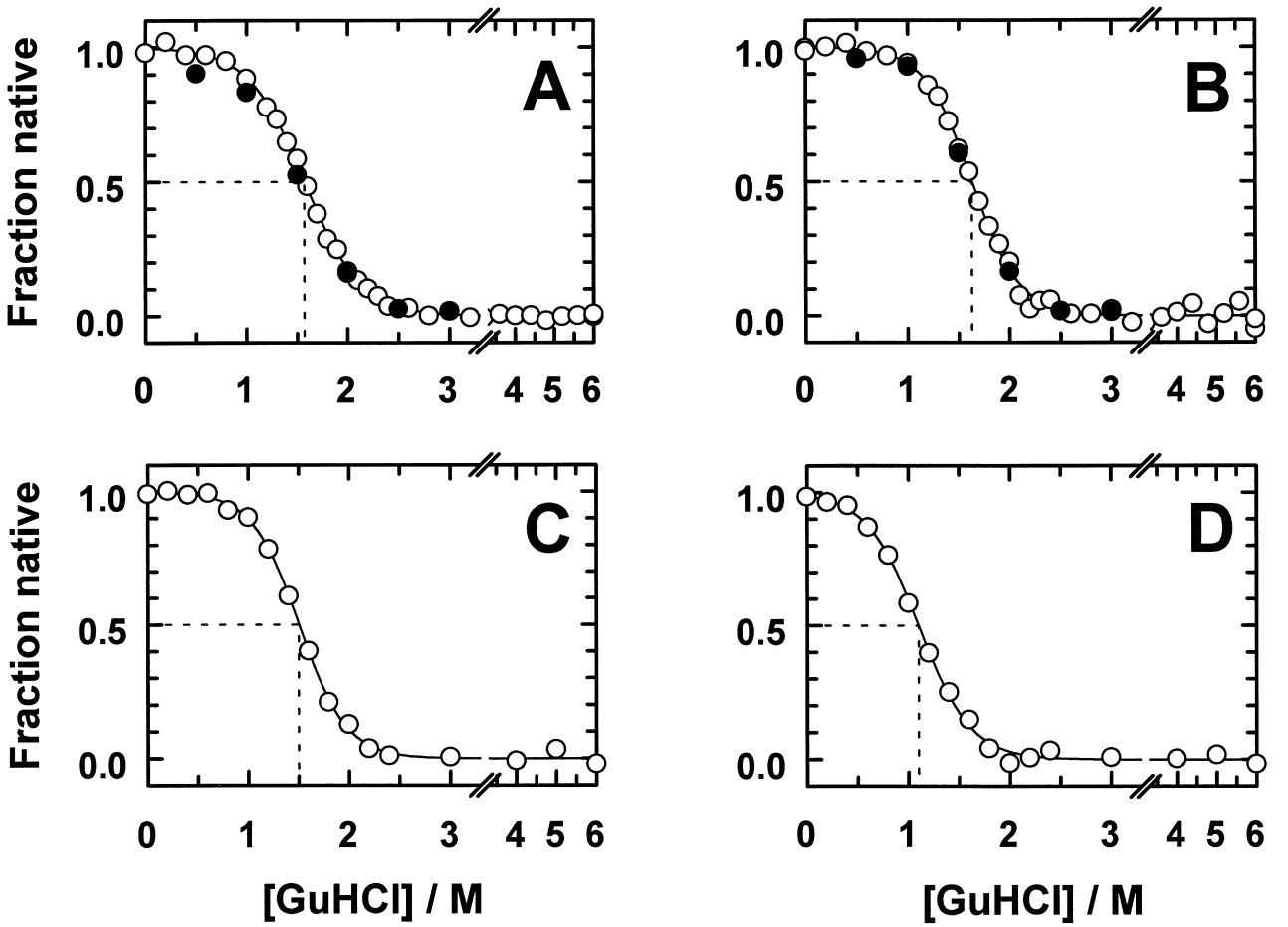

Fig. 3. GuHCl stability and reversibility of MerP at different pH values. The stability of MerP at pH 7.5, measured with the change in ellipticity at 222 nm (A) and the intrinsic-fluorescence emission of the tyrosine at 304 nm (B), give rise to very similar GuHCl-denaturation curves (open circles). The reaction is reversible, and samples exposed to 6 M GuHCl regain their native properties when diluted to lower concentrations of GuHCl (filled circles). The stability at pH 4.9 (C) is unaltered as compared to pH 7.5, but at pH 9.0 (D) it is slightly lower. The lines are fits of the data to Eq. 1.

MerP by using a variable selection method [20,21]. The α-helical content was estimated to 37%, while the errors were very high in the predictions of the other secondary structure elements. The α-helical content as judged from the preliminary global fold [6] is approximately 30%, and the amount of β-sheet is around 40%. The native far-UV CD spectrum has vanished in 3 M GuHCl (Fig. 2C), and the signal that is left is what would be expected for a random coil, showing the disappearance of the secondary structure upon denaturation.

The concentration of GuHCl at which half of the protein is denatured, $[GuHCl]_{1/2}$, is identical when measured either with the change in ellipticity at 222 nm (Fig. 3A) or with the change in fluorescence at 304 nm (Fig. 3B, Table 1). Since these two independent probes give the same result, it shows that the transition is due to a global denaturation of the protein. The denaturation curves perfectly obey a two-state model, according to Eq. 1, in which only the native state and the unfolded state are populated at equilibrium. The denaturation process is fully reversible and the protein regains its native fluorescence- and ellipticity properties when the concentration of denaturant is lowered (Fig. 3A and B). At pH 4.9 the stability towards GuHCl is the same as for pH 7.5 (Fig. 3C), and at pH 9.0 the stability is somewhat lower (Fig. 3D, Table 1). The *m*-values obtained from equilibrium unfolding of MerP, 8.9–10.7 kJ mol^{-1} M^{-1} (Table 1), is what is expected for a protein of this size [22]. The *m*-value is a measure of the difference in surface accessible area between two states [23], in this case the native state and the unfolded state

Table 1
Equilibrium unfolding parameters of MerP

	ΔG^{H_2O} (kJ mol^{-1})	m_{eq} (kJ mol^{-1} M^{-1})	$[GuHCl]_{1/2}$ (M)
Circular dichroism pH 7.5	14.1 ± 0.7	8.9 ± 0.4	1.57 ± 0.03
Fluorescence pH 7.5	17.4 ± 1.6	10.7 ± 0.9	1.63 ± 0.03
Fluorescence pH 4.9	15.9 ± 1.5	10.6 ± 0.9	1.50 ± 0.03
Fluorescence pH 9.0	10.6 ± 1.8	9.6 ± 1.2	1.10 ± 0.07

Unfolding was acquired with GuHCl at 25°C, and the process was monitored with circular dichroism and fluorescence.

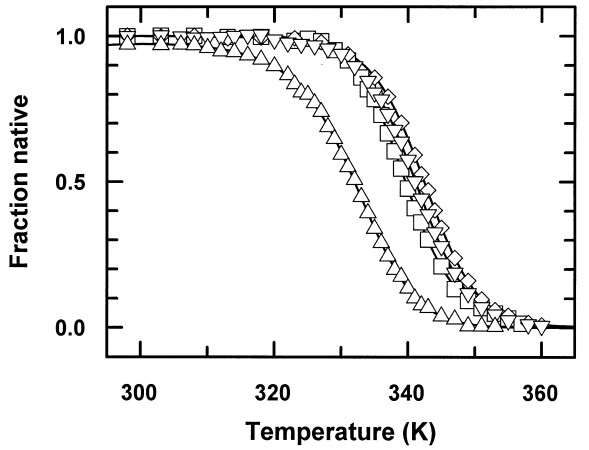

Fig. 4. Temperature denaturation of MerP, measured by the ellipticity at 222 nm, was measured at four different pH values: pH 4.0 (open triangles), pH 4.9 (open inverted triangles), pH 6.5 (open diamonds), and pH 7.5 (open squares). The lines are fits to Eq. 2.

of MerP. Thus, small proteins should have smaller *m*-values than larger proteins on account of their smaller volume-to-surface ratio.

The stability towards temperature is highest around pH 6.5 where MerP melts with a T_m of 342.5 ± 0.5 K (Fig. 4A). At all pH values, the data agree with the two-state model of Eq. 2. Temperature denaturation is fully reversible if MerP is exposed to temperatures above T_m for only 10 min (data not shown). After incubation for 3 h above T_m, however, 80–90% returns to the native state, and one possibility is the cleavage of peptide bonds which often occurs during prolonged incubation at high temperatures [24]. Circular dichroism measurements show that within the pH range 4–10 MerP retains the native conformation and acid denaturation at pH 1.0 is fully reversible (data not shown).

3.2. Folding of MerP

When refolded from 3 M into 0.27 M GuHCl, MerP regains its native intrinsic fluorescence properties as the sum of two exponential phases (Fig. 5). The first exponential phase has an apparent rate constant of 1.2 s^{-1}, and this phase accounts for 81% of the total signal. The rate of the second folding phase is 0.053 s^{-1}, with an amplitude of 17%. In Fig. 5 the signals are compared to native and denatured references that were recorded independently from the kinetic experiments, and this is why the amplitudes do not add up to exactly 100%. It is important to note, that, within the small experimental error, the two fluorescence folding phases account for the entire change in signal between the native and unfolded states. Thus, there are no burst or hidden phases before the experimental dead time of 2 ms.

The folding experiment in Fig. 5 was performed on long term denatured (24 h) MerP. In another experiment, where the protein was allowed to unfold for only 2 s before folding was initiated, there was only one exponential change in fluorescence (data not shown); the slow phase observed earlier was completely abolished, and only one phase with a rate of 1.3 s^{-1} remained. Two seconds is enough time for the polypeptide chain to become unfolded (see below), but not enough time for any significant *cis-trans* isomerization around peptide bonds [25,26]. Thus, when jumping back to native conditions all peptide bonds will have their native conformation, and *cis-trans* isomerizations will not be rate limiting steps. Since the slow folding phase is abolished in the double-jump experiment, it is most certainly due to *cis-trans* isomerization around a proline peptide bond. The *cis-trans* equilibrium of X-Pro peptide bonds are usually around 10–30% in favor of the *cis* conformation in a free peptide [27], and so the small amplitude of the slow folding phase, 17% of the total signal, indicates a *cis* to *trans* conversion of a single proline peptide bond and that isomerization at the other two proline peptide bonds in MerP not are limiting the rate of folding. This is consistent with the fact that two prolines (number 10 and 18) are located in a loop with highly dynamic structure, and that the third proline (number 67) is part of a rigid tight turn (Ann-Christin Brorsson, unpublished results). Consequently, in 17% of the molecules a *cis* conformation around one pro-

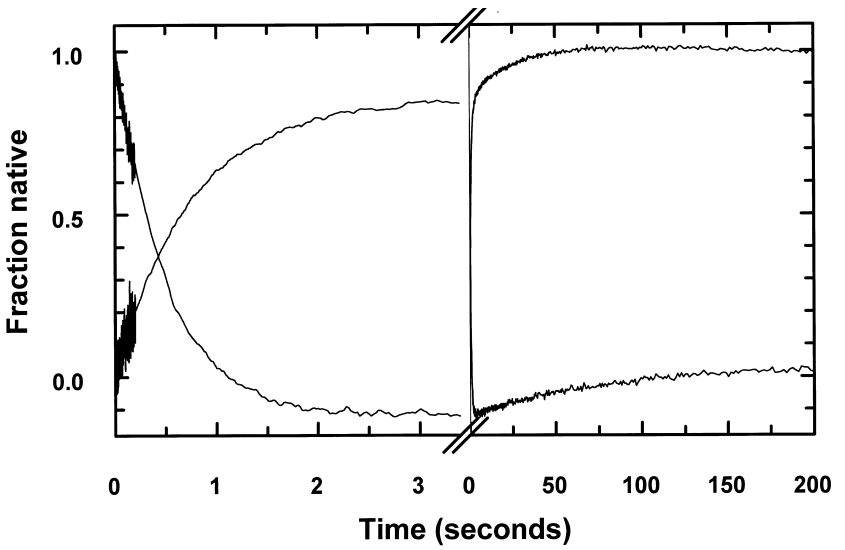

Fig. 5. Kinetics of folding and unfolding monitored with intrinsic fluorescence at 304 nm. Folding was initiated by diluting 220 μM MerP in 3 M GuHCl tenfold to 20 μM MerP and 0.27 M GuHCl. Unfolding was initiated by adding 10 volumes of 3.3 M GuHCl to one volume of 220 μM MerP, giving a final GuHCl concentration of 3 M and 20 μM MerP.

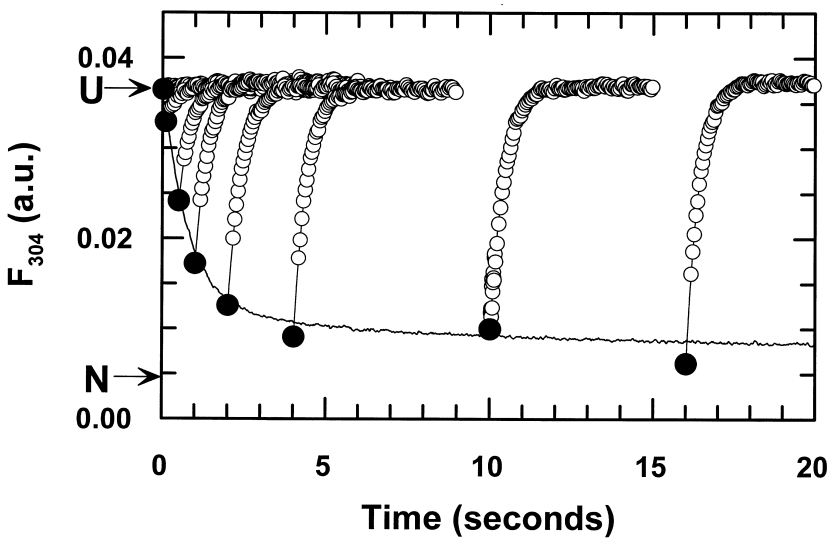

Fig. 6. Double-jump experiments in which unfolded MerP was allowed to refold for only a short period of time before the onset of unfolding (open circles). The fluorescence signals of the native (N) and unfolded (U) references are indicated by arrows. The delay times are 0.02, 0.01, 0.5, 1, 2, 4, 10 and 16 s, and the extrapolated values at zero time (filled circles) overlaps well with a regular refolding trace (line, as in Fig. 5) that is included for comparison. All unfolding rates are 2.6 ± 0.2 s^{-1}, except for the 0.02 s delay time for which the amplitude is too small to determine any unfolding rate.

line peptide bond locks this fraction in the unfolded state, and the only way for these molecules to reach the native state is to first convert the peptide bond in question into the *trans* conformation, which occurs with a rate of 0.053 s^{-1}. In the remaining 81% of the population the crucial proline peptide bond is in the native configuration from the start, and the folding from this species to the native state is controlled by other rate limiting steps.

3.3. Unfolding of MerP

When unfolding of MerP into 3 M GuHCl is monitored with intrinsic fluorescence there is both a fast and a slow phase as well (Fig. 5). First there is a rapid change in fluorescence with a rate of 2.7 s^{-1}. This phase over shoots with an amplitude of −109%, and the signal is for a while outside the end points, but the fluorescence returns to the level of the unfolded reference in a slower reaction with a rate of 0.012 s^{-1} and an amplitude of 11%. In a double-jump experiments, where fully unfolded MerP was refolded for shorter periods of the time, in the range 0.1–16 s, before the onset of unfolding, there was only one exponential change in fluorescence with a rate of 2.6 s^{-1} and no over shoot was observed (Fig. 6). These results show that the fast unfolding phase reflects that the entire population of native molecules unfolds, but retains the native conformations around the proline peptide bonds, and that the slower reaction reflects the subsequent divergence of this population into 81% *trans* and 17% *cis* conformation at the crucial proline peptide bond, that is the previously discussed fast- and slow folding populations, respectively. Tyr^{66}, the only fluorophore in MerP, is joined to Pro^{67} via a peptide bond that is in the *trans* conformation in the native state. When the protein becomes unfolded this bond is no longer restricted to the *trans* conformation, and so the slow unfolding phase most likely is due to changes in local environment of the Tyr^{66} side chain upon *trans-cis* isomerization. This is similar to Ribonuclease A [28], and in both cases the *trans* conformation around the Tyr-Pro peptide bond gives the higher fluorescence quantum yield.

3.4. An unfolding assay

An assay of the biological function would perhaps be the best way to test whether a species with native fluorescence properties on the folding pathway really has the native structure, or reflects an intermediate state which is converted to the native state in a slower undetected reaction. However, the assay for biological activity of MerP it too slow in comparison with the folding rate to be practical in this regard. Another commonly used test for this purpose is an unfolding assay [15,29–31] which is based on the idea that a native protein unfolds much more slowly than any partially folded intermediate species. Consequently, if a mixture of native molecules and folding intermediates is exposed to strongly unfolding conditions, only the unfolding kinetics of the native molecules can be detected since the unfolding of the intermediate states occur too fast. We used this unfolding assay with intrinsic fluorescence as the probe to monitor the native state of MerP. As indicated by the fluorescence spectra (Fig. 2) Tyr^{66} is affected by one or more quenchers in the native state. The structure around Tyr^{66} would have to be very native like indeed to give rise to a native fluorescence, since fluorophores are very sensitive to its proximity to quenchers [32]. The unfolding rate of MerP, after various folding times within the range 100 ms–16 s, is identical with a single exponential phase (2.6 ± 0.2 s^{-1}) and in all cases do the amplitude span the entire gap between the reference signals (Fig. 6). This shows that the fluorescence of Tyr^{66} monitors the native state, or more specifically, that the fluorescence not is a reporter for an intermediate state that more slowly is converted to the native state in an undetected reaction.

3.5. Concluding remarks

Small proteins that perfectly obey a two-state mechanism

are not uncommon [7,11–15,17], but there are also some very small proteins that fold in a complex manner via intermediate states [8–10,16]. Although the stability measurements of MerP at equilibrium perfectly comply with a two-state model, it does not at all exclude the possibility of intermediate states on the folding pathway of MerP. Intermediate states are usually metastable and therefore only transiently detectable in kinetic experiments, and more rigorous tests will have to be carried out to validate the apparent two-state behavior of MerP. However, we have shown that the intrinsic fluorescence monitors the major folding event of MerP. Moreover, the intrinsic fluorescence is totally unperturbed before this major folding phase, relative to the unfolded reference, and any preceding folding step would therefore only involve local rearrangements that occur far from Tyr^{66}. Thus, although structural rearrangements may occur before 2 ms of folding, those should only involve local parts of the molecule and do not constitute major folding events. This is not to say that early local rearrangements are unimportant. On the contrary, such early events would yield important information and should be searched for.

Premature formation of disulfide bonds can retard folding and make it substantially slower. Two examples are the investigations of Weissman and Kim [33] and Mücke and Schmid [34], and in these cases the disulfide bonded cysteins are far away in the primary structure of the polypeptide chain. Oxidized MerP contains one disulfide bond between Cys^{14} and Cys^{17}, situated in the loop between β_1 and α_1 (Fig. 1). Separated by two amino acids this extra covalent bond only locally creates an eye which should have little influence on the overall structure. Reduced MerP has been proposed to be the biologically active form, and the two cysteins are thought to be involved in the binding of mercury ions, rather than having a structural role [4,5]. Whit this in mind, it is not surprising that the folding rate of reduced MerP is 1.0 s^{-1} (data not shown), as compared to 1.2 s^{-1} for oxidized MerP in 0.27 M GuHCl. This small difference is within experimental error, and, thus, the disulfide bond in oxidized MerP does not have any measurable effect on the folding rate.

The major folding event of MerP (i.e. the faster of the two phases) is remarkably slow, considering the fact that it lacks intrinsically slow folding steps, such as *cis-trans* isomerization of proline peptide bonds, formation of disulfide bonds, isomerization of disulfide bonds, or the ligation of prosthetic groups and metal ions. With a rate of 1.2 s^{-1} in 0.27 M GuHCl for the major folding event, it is, to our knowledge, the slowest of all small proteins with 100 amino acids or less yet examined. A protein with the same topology, U1A, folds 200-fold faster [17], which seems to exclude the topology itself as the reason for the slow folding of MerP. It will be very interesting to further characterize the folding pathway of MerP, and to uncover the nature of the rate limiting folding steps of this small, yet remarkably slow folding protein.

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