Refolding and unfolding kinetics of the equilibrium folding intermediate of apomyoglobin

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Little is known about the kinetic process in which stable intermediates in protein folding are formed: whether their folding is highly cooperative (two-state) or weakly cooperative is controversial. We report here that the folding and unfolding kinetics of the pH 4-stable intermediate (I_1) of apomyoglobin are measurable, in the millisecond time range, when monitored by stopped-flow measurements of tryptophan fluorescence. The kinetics confirm that folding of I_1 is strongly cooperative, but there is a burst phase (missing amplitude) in unfolding. If the faster steps in unfolding of I_1 can be measured directly by suitable fast-reaction methods, they will give information about the nature of the folding transition.

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Folding to the native conformation is a highly cooperative process for small, single-domain proteins[1,2]. Transient intermediates in the folding process are often observed[3–5], but it is not yet known at what stages of folding the cooperativity arises. Theoretical simulations of the folding process suggest the existence of different classes of folding intermediates at different stages in folding[6]: loosely folded 'molten globule' intermediates at early stages of folding and discrete intermediates, with properties beginning to resemble those of native proteins, at late stages of folding. In a few cases[7,8], including the apomyoglobin folding intermediate[9,10] studied here, a prominent intermediate in the kinetics of folding can be studied at equilibrium as a stable species at low pH. Stable folding intermediates are of particular interest for understanding the mechanism of folding because detailed measurements can be made on them, while the results provide information about the nature of the kinetic folding process.

Our laboratory reported recently[11] that the pH 4 intermediate of apoMb is stabilized by packing interactions involving specific residues and that this intermediate is formed cooperatively, as judged by the coincidence of the unfolding transition curves monitored by probes of secondary structure (far-UV CD (circular dichroism)) and tertiary structure (Trp fluorescence). The I_1 intermediate contains the A, G and H helices of myoglobin but not the B, C and E helices[10]; the A, G and H helices form a compact subdomain of myoglobin. A slightly different form of the intermediate, containing stable A, B, G and H helices is present when 20 mM trichloroacetate is added[12].

A particularly sensitive test for folding intermediates is provided by the kinetics of folding and unfolding[13–15]. If the kinetic folding process is a two-state reaction without observable intermediates, folding will follow a single exponential time course. A populated intermediate can be detected by the presence of an additional kinetic phase. Comparison of the folding kinetics monitored by different probes, especially probes of secondary *versus* tertiary structure, provides a different and sensitive test for folding intermediates[14]. Until now, the unfolding and refolding kinetics have not yet been reported for a stable folding intermediate; instead, formation of these intermediates has been found to be too fast to measure by stopped-flow methods[7,8].

The folding and unfolding kinetics of the I_1 intermediate are measured here within the urea-induced unfolding transition at pH 4.2 (Fig. 1); Trp fluorescence is used to monitor the unfolding and refolding kinetics. It was reported earlier[8] that I_1 is formed within the dead time of the stopped-flow CD apparatus used to follow the refolding kinetics of the U→N reaction at pH 6. The U→I_1 reaction is faster at pH 6 than at pH 4.2 (our unpublished results) and the folding reaction slows down inside the urea-induced unfolding transition (Figs 2, 3); also, the stopped-flow dead time is shorter when Trp fluorescence is monitored instead of CD. Equilibrium unfolding experiments with mutants[11] show that Trp fluorescence monitors unfolding of the AGH subdomain and appears to be a probe of tertiary structure. Very fast unfolding steps in the unfolding process of the I_1 intermediate have been detected by using a probe of secondary structure (infrared absorbance in the amide I region) to monitor unfolding following a laser induced temperature jump[16]. Thus, some unfolding events occur in very fast time ranges but the time range in which the tertiary structure forms and unfolds is unknown.

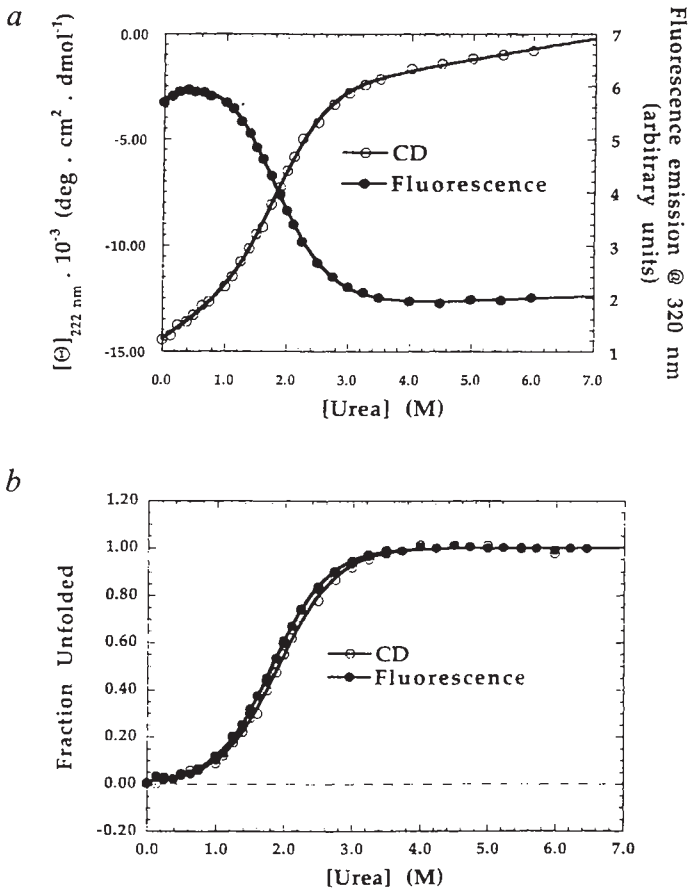


Fig. 1 Equilibrium transition curves for urea-induced unfolding of the apoMb intermediate monitored by probes of secondary structure (far-UV CD) and of tertiary structure (tryptophan fluorescence). Conditions: pH 4.2, 2 mM sodium citrate, 30 mM NaCl, 13 μM protein, 4.5 °C. The transition curves are reversible and independent of protein concentration in this range. *a*, Raw data; *b*, normalized transition curves obtained by fitting the raw data to the two-state model by a procedure which assumes linear baselines and fixes the baselines by using data inside as well as outside the transition zone.

Folding and unfolding kinetics

Fig. 1*a* shows the equilibrium transition curve for urea-induced unfolding of I_1 in low salt conditions: 2 mM sodium citrate, 30 mM NaCl, pH 4.2, 4.5 °C. Unfolding is monitored both by far-UV CD and by Trp fluorescence. The two Trp residues of myoglobin are present in the A helix, which is part of the structure of I_1, and results with Trp→Phe single mutants show that both Trp residues help to stabilize I_1 (ref. 11). Thus, the increase in Trp fluorescence and the blue shift of the maximum in the emission spectrum upon the formation of I_1 probably reflect partial burial of the Trp residues away from solvent. Fig. 1*b* compares the normalized transition curves monitored by these two probes. Both sets of data are fitted to a two-state reaction ($U \rightleftharpoons I_1$) by a procedure[17] that uses data inside as well as outside the transition zone to fix the baselines, which are assumed to be linear. The two transition curves are nearly superimposable (see also ref. 11) and the small differences between the two curves may be caused by error in determining the baselines.

The kinetics of unfolding and refolding were monitored by Trp fluorescence after stopped-flow mixing at various urea concentrations (same conditions as Fig. 1). The starting material for unfolding experiments is I_1 in 0.7 M urea and for refolding it is U in 4.0 M urea. Fig. 2*a* shows the refolding kinetics at 1.1 M and 2.0 M urea. About half the reaction occurs within the stopped-flow mixing time (2 ms); the measured part of the reaction follows a single exponential time course. Refolding becomes faster with decreasing urea molarity and at 0.7 M the refolding reaction is too fast to measure. The unfolding kinetics (Fig. 2*b*) are similar, except that unfolding becomes faster with increasing urea molarity and there is a minor additional reaction in the seconds time range (the minor slow reaction in the unfolding kinetics is discussed below).

Fit to the two-state model

The rate constants for overall folding and unfolding, k_f and k_u, are determined by combined measurements of K (the equilibrium constant for folding) and τ (the relaxation time of the folding kinetics) and by assuming a two-state reaction

$$U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} I_1 \qquad (1)$$

$$(1/\tau) = k_f + k_u \qquad (1a)$$

$$K = k_f/k_u \qquad (1b)$$

First, k_f, k_u and K are expressed as functions of urea molarity (C) by standard relations[18–20].

$$\ln k = \ln k(H_2O) + (m^{\ddagger}/RT)C \qquad (2a)$$

$$\ln K = \ln K(H_2O) + (m/RT)C \qquad (2b)$$

The coefficients $m^{\ddagger}_f$, $m^{\ddagger}_u$ and m describe the dependences of the rate constants and equilibrium constant on urea molarity; the equilibrium m-value is related to the kinetic m-values by

$$m = m^{\ddagger}_f - m^{\ddagger}_u \qquad (3)$$

The values of k_f and k_u *versus* C were determined by a combined least squares fit of ln$K$ and ln(1/τ) *versus* C (Fig. 3). The values found for C=0 are k_f =3900±450 s^{-1}, k_u=48±5 s^{-1}, with $m^{\ddagger}_f$ = –0.90±0.04 kcal mol^{-1} M^{-1} and $m^{\ddagger}_u$=0.40±0.03 kcal mol^{-1} M^{-1}. Because these values fit both the kinetic and equilibrium data (Fig. 3), the results agree with a two-state reaction. If the rate constants k_f and k_u were measurable over a wider range of urea molarity, they could be determined directly from kinetic data alone.

The next test of the two-state model is to find out if the observed kinetic amplitudes account for the entire changes expected from the equilibrium transition curve. The results are shown in Fig. 4. The final kinetic values of the fluorescence fit the equilibrium transition curve both in refolding and unfolding: this agreement indicates that there are no slower steps than the ones studied. The initial fluorescence values

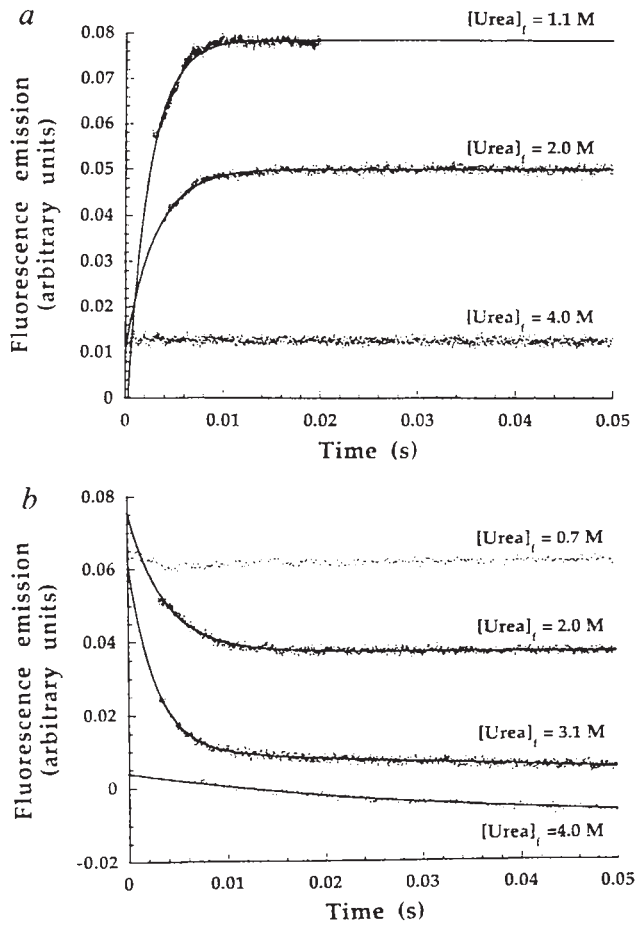


Fig. 2 *a*, Refolding kinetics and *b*, unfolding kinetics measured at various urea concentrations inside the transition zone (see Fig. 1) and monitored by Trp fluorescence. The refolding kinetics are fitted to a single exponential; the unfolding kinetics are fitted to a single exponential plus a slower linear phase. The slow reaction represents dissociation of dimers or oligomers: see text. Conditions: as above, except protein 3.5 μM; initial protein 20 μM, in 4.0 M urea for refolding experiments, in 0.7 M urea for unfolding experiments.

are known less accurately because they are obtained by extrapolation (Fig. 2) and they are affected by any variation in the stopped-flow mixing time. In refolding experiments the initial fluorescence values agree satisfactorily with the equilibrium baseline, but in unfolding experiments there appears to be a burst phase (missing amplitude). This finding suggests that at least one faster reaction in unfolding occurs within the stopped-flow mixing time.

The burst phase in unfolding

A more clearcut demonstration of a burst phase in unfolding is provided by the behaviour of a less stable mutant, M131A. The equilibrium unfolding properties of this mutant were reported recently[11]. Fig. 5 shows the unfolding and refolding kinetics. In Fig. 5*a* the apparent rate constant for folding or unfolding is shown at different positions in the transition zone and the results are compared to those for wild type. The kinetic *m*-values (eqn. 2) used to fit the mutant data are the same as those of wild type and the curve is shifted to lower urea concentrations, as expected from the reduced stability of the mutant.

Fig. 5*b* shows the kinetic amplitudes and also the equilibrium transition curve for the mutant protein. The equilibrium baselines and also the equilibrium *m*-value are the same as those used to fit the data for wild type. The final kinetic values of the fluorescence fit the equilibrium transition curve, both in unfolding and refolding. The initial kinetic values scatter around the equilibrium baseline in the refolding experiments while the burst phase in unfolding is unmistakable, especially at higher urea concentrations. The results indicate that the burst phase in unfolding becomes more pronounced as the stability of the folding intermediate decreases, either by mutation or at higher denaturant concentrations.

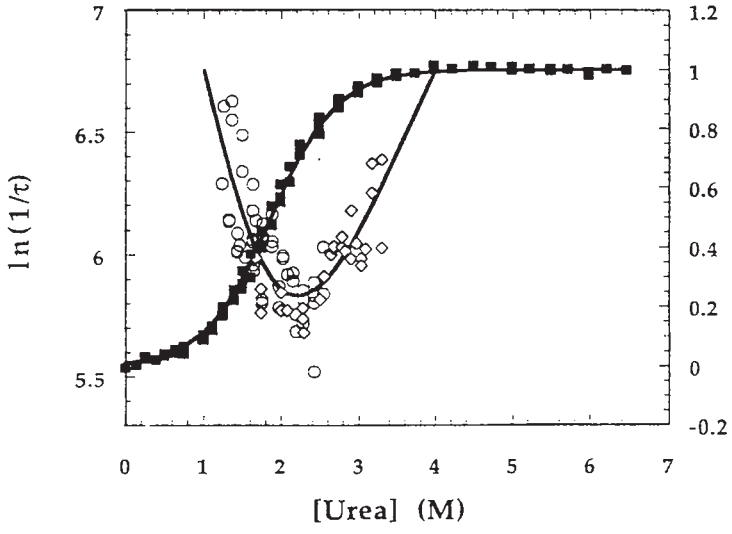


Fig. 3 Unfolding and refolding rates versus position in the equilibrium transition curve. The reciprocal of the relaxation time τ (in seconds) is shown on the left ordinate (logarithmic scale). The open circles (○) are for the refolding kinetics and open diamonds (◇) are for the unfolding kinetics. The urea-induced unfolding transition curve is measured by Trp fluorescence in an independent experiment (see Fig. 1). The optical settings, such as bandpass, of the equilibrium experiments are made to be the same as in the stopped-flow experiments. The fraction unfolded at equilibrium is shown on the right ordinate (■). Both quantities depend on the microscopic rate constants for unfolding and refolding (see eqns 1a, 1b) and a combined fit gives these rate constants as a function of urea molarity (see text). The solid curves represent the fits with $k_f(H_2O)$=3900 s^{-1}, $k_u(H_2O)$=48 s^{-1}, $m^{\ddagger}f$= −0.90 kcal mol^{-1} M^{-1} and $m^{\ddagger}u$=0.40 kcal mol^{-1} M^{-1}.

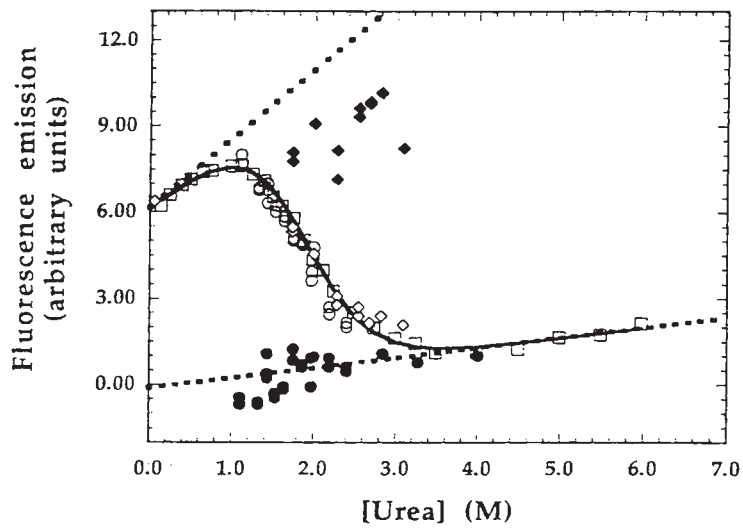
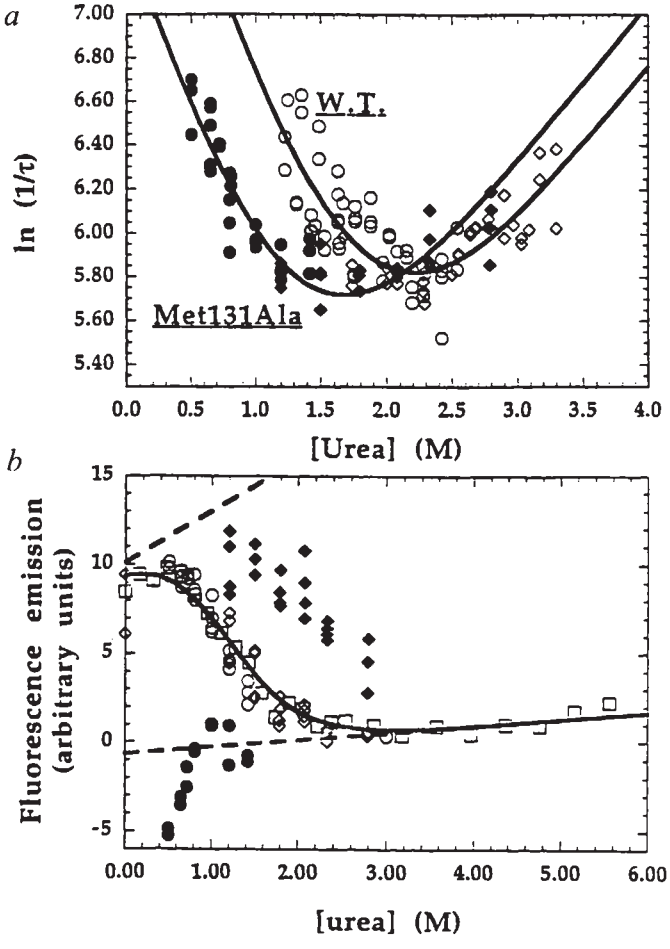


Fig. 4 Comparison between the equilibrium transition curve and the initial and final values of fluorescence emission in the kinetic experiments. The solid line and (□) show the equilibrium transition curve, as measured by the integral of the emission between 320 and 380 nm; the dotted lines are the equilibrium baselines determined by fitting the data to the two-state model (see text). The (○) and (◇) show the final kinetic values of fluorescence emission in refolding and unfolding respectively. These values are normalized for comparison with the equilibrium transition curve by superimposing the final kinetic value from refolding at 1 M urea with the equilibrium value. The (●) and (◆) show the initial flourescence values in the refolding and unfolding kinetics, obtained by fitting the kinetic trace to a single exponential.

Anion effects and association

Two general concerns in the study of folding intermediates are (i) the formation of dimers or higher oligomers, and (ii) specific anion effects. Some dimers or oligomers may be present in these conditions, but their presence does not affect the protein stability at the protein concentrations used here (3.5 μM for Figs 2–4). The equilibrium transition curve monitored by far-UV CD is independent of apoMb concentration from 13 μM (shown in Fig. 1) up to 54 μM (data not shown), indicating that dimers or oligomers have little effect on the measured equilibrium transition curve. In Fig. 2*b*, the minor slow reaction in unfolding appears to result from the dissociation of I_1 dimers or oligomers because it is also seen in the absence of the unfolding process when the protein solution at pH 4.2 is diluted at constant urea molarity, and because the relative amplitude of the reaction increases with the initial protein concentration in experiments in which the protein is diluted at constant urea concentration (data not shown). Equilibrium centrifugation experiments (Dr. James C. Lee, personal communication) on I_1 at 6 μM, pH 4.2, 2 mM sodium acetate, 20 °C, indicate that it is chiefly monomeric. With regard to specific anion effects, titration calorimetry experiments (M. Antalik and D.W. Bolen, personal communication) show that I_1 does interact with citrate. Some of the experiments shown here have been repeated with the more weakly interacting anion acetate (5 mM) in place of citrate and again approximate two-state behaviour is observed (data not shown). The kinetics of formation and unfolding of I_1 are more complex in pH-jump experiments of unfolding and refolding than in the urea-jump experiments shown here (data not shown).



Cooperativity of folding

The presence of a burst phase in unfolding implies that I_1 consists of an ensemble of structurally related forms that are rapidly interconvertible when a shift is made

Fig. 5 Unfolding and refolding kinetics of the mutant M 131A. *a*, Comparison of the apparent rate constant (1/τ) for unfolding or refolding between the M 131A mutant (filled symbols) and wild type (open symbols); (○,●), refolding, (◇,◆) unfolding. *b*, Comparison between the equilibrium transition curve (solid line, □) and the final kinetic values of fluorescence emission (refolding, ○; unfolding,◇). The initial kinetic values of fluorescence emission (●, refolding; ◆, unfolding) can be compared to the equilibrium baselines (dashed lines). Conditions are the same as in Fig. 2.

to unfolding conditions. The existence of such an ensemble was suggested earlier[21] on the basis of the thermal unfolding transitions monitored by far-UV CD. The absence of a significant burst phase in refolding implies that there is a slow initial step, such as a nucleation reaction, in forming the tertiary structure. The term nucleation is used here in the classical sense: once a nucleus has been provided, folding proceeds extremely rapidly. The observation of a large *m*-value for the refolding rate constant (70% of the total) implies that substantial structure is present in the transition state for refolding.

The kinetics of unfolding and refolding oligonucleotide $rA_n \cdot rU_n$ double helices display a burst phase in unfolding but not in refolding[22]. The absence of a burst phase in refolding is explained because nucleation of the double helix is the first step in refolding: this is a pre-equilibrium step with a highly unfavourable equilibrium constant[22]. The burst phase in unfolding is caused by rapid initial unzippering of base pairs at either end of the double helix; this fast step has been measured directly with a special temperature-jump apparatus[23]. Adaptation of the nucleation-growth model to the folding and unfolding kinetics of native proteins was suggested in 1972[24,25]) but fell into disuse for lack of supporting evidence. A different kind of nucleation model was proposed recently[26] for the small protein chymotrypsin inhibitor 2. No populated kinetic intermediates have been observed either in unfolding or refolding of this protein. The concept of a folding nucleus is based in this case on evidence that a few residues strongly affect the folding rate, according to mutational evidence[26,27].

Other approaches have been proposed for determining whether or not stable folding intermediates are formed cooperatively. Measurement of an appropriate order parameter as a function of temperature can in principle distinguish a first-order from a second-order phase transition[28]. Proline residues have been substituted into individual α-helices of α-lactalbumin (B.A. Schulman and P.S. Kim, personal communication) to find out whether preventing the formation of an isolated helix is sufficient to destabilize the entire folding intermediate. This intermediate appears to be formed noncooperatively.

A classic test for whether or not small proteins obey the two-state model of folding is to compare the values of the van't Hoff and calorimetrically determined enthalpies of unfolding[2]. There are problems, however, in applying this test to stable folding intermediates. Their thermal transitions are quite broad, which makes it difficult to measure the enthalpy of unfolding. They sometimes show cold denaturation[21] in addition to thermal unfolding, in overlapping temperature ranges (see also ref. 29), which adds to the difficulty of measuring the enthalpy change. No definite peak of unfolding enthalpy was found in a differential scanning calorimetry study of the pH 4 apoMb intermediate[30], but a broad peak was found for thermal unfolding of the anion-stabilized form of the horse apoMb intermediate at pH 2 (ref. 21).

Another approach to the problem is based on testing the hypothesis that all stable folding intermediates are formed cooperatively and thus they provide a homologous series[31]. Data from the denaturant-induced unfolding transitions are plotted against molecular weight: these results should fall on a single line if the hypothesis is correct. Scatter in the data, taken from the literature, makes it difficult to decide if the hypothesis is correct[31]. Although folding of the apoMb pH 4 intermediate fits the two-state model by the test of finding coincident transition curves for unfolding monitored by different probes (see ref. 11 and Fig. 1 above), the unfolding curve deviates from two-state behavior when the B helix is recruited into the intermediate by making two Gly→Ala mutations[32].

Methods

Protein expression and purification. A synthetic gene for sperm whale apomyoglobin was expressed in *Escherichia coli* and the protein was purified as described[12]. The concentration of apoMb was determined by absorbance in 6.0 M GdmCl (20 mM sodium phosphate, pH 6.5) as described[33] using ε (280 nm)=15,200 M^{-1} cm^{-1} and ε (288 nm)=10,800 M^{-1} cm^{-1}.

Fluorescence and CD measurements. Fluorescence data were collected on a SLM AB2 spectrofluorimeter using 1 cm cuvettes. The excitation wavelength was 288 nm (bandpass 1 nm) and the emission wavelength was 320 nm (bandpass 8 nm). CD data were collected at 222 nm on an AVIV 60 DS spectropolarimeter using 1 mm cuvettes. The protein concentration was 13 μM.

The urea-induced unfolding transition was analyzed by assuming a two-state model and a linear dependence of the Gibbs energy of unfolding on the urea concentration[20]. The data were fitted by a procedure which assumes linear baselines and fixes the baselines by using data inside as well as outside the transition zone[17].

Fluorescence stopped-flow experiments. Data were collected on a sequential mixing stopped-flow instrument (model DX.17 MV) from Applied Photophysics (Leatherhead, UK). Excitation was made at 288 nm (slit width 1 mm) and an optical cut-off filter (50% transmittance at 320 nm) was used to detect the emission. The stopped flow mixing time was determined by measuring a standard second-order reaction[34] at varying initial concentrations of the reactants.

Data processing. The kinetic traces obtained in the stopped-flow experiments were fitted to a single exponential or to a single exponential plus baseline with the software provided by Applied Photophysics. The curve of relaxation time versus urea molarity and the equilibrium transition curve of fluorescence intensity versus urea molarity were fitted jointly using equations (1–3) with SigmaPlot (Jandel Scientific, San Rafael, CA, USA).

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