A Kinetic Method To Evaluate the Two-State Character of Solvent-Induced Protein Denaturation†

Matthias Mücke and Franz X. Schmid*

Laboratorium für Biochemie, Universität Bayreuth, D-95440 Bayreuth, Germany

Received June 21, 1994; Revised Manuscript Received August 16, 1994®

ABSTRACT: We present a kinetic method to determine the concentration of native molecules in protein folding transitions. It is based on the observation that frequently native protein molecules unfold slowly when transferred to unfolding conditions, whereas folding intermediates unfold rapidly. The fraction of native molecules in a folding transition can thus be determined by kinetic unfolding assays in a two-step procedure. Aliquots of the protein are first equilibrated at different concentrations of denaturant and then transferred to constant unfolding conditions to determine the amplitude of unfolding. This amplitude is a direct measure for the concentration of native molecules in the sample. The two-state character of a solvent-induced unfolding transition can thus be examined. When the fractional change of a spectral property in a transition follows the decrease in the concentration of the native molecules, as measured by the unfolding assays, then the presence of intermediates that differ from the unfolded protein in this property can be definitely excluded. This test complements the calorimetric test for intermediates in thermal unfolding transitions. By using this method, we show that the NaCl-induced folding transition of the reduced and carboxymethylated form of a variant of ribonuclease T1 is well described by the two-state approximation. In the unfolding of apo-α-lactalbumin, the measured profile for the native protein coincides with the fluorescence-detected transition, but not with the transition that is monitored by amide circular dichroism. This confirms that a partially folded intermediate is present in the folding transition of apo-α-lactalbumin.

Many proteins undergo reversible transitions between a highly ordered native state and a disordered unfolded state. In the simplest case, such a transition could be a two-state reaction as in eq 1, which involves only native (N)[1] and unfolded (U) molecules.

$$N \rightleftharpoons U \tag{1}$$

Alternatively, partially folded states (I_i) could be populated on the pathway as in eq 2, or unfolding could be a gradual melting process in every molecule, thus resembling a second-

$$N \rightleftharpoons I_i \rightleftharpoons U \tag{2}$$

order rather than a first-order phase transition. The elucidation of the nature of these transitions is an obligatory prerequisite for kinetic and thermodynamic analyses of protein folding and is of central importance for our understanding of the energetic principles of protein folding.

The Gibbs free energy of denaturation (ΔG_D) is generally small, and most proteins can be unfolded by changing the temperature or the composition of the solvent (Tanford, 1968; Pace, 1975; Privalov, 1979, 1992). The thermodynamic properties of heat-induced unfolding are well understood. The reaction is driven by the changes in enthalpy (ΔH) and in heat capacity (ΔC_p), and powerful criteria are available to examine whether a protein unfolding transition is adequately described by a two-state mechanism as in eq 1. ΔH and ΔC_p can be measured directly in a calorimeter, as well as by using the van't Hoff relation. The coincidence of the calorimetric enthalpy (ΔH_{cal}) and the van't Hoff enthalpy (ΔH_{vH}) proves that intermediates which differ from the unfolded protein in enthalpy are not populated at equilibrium (Privalov, 1979, 1992; Privalov & Potekhin, 1986).

For the analysis of solvent-induced unfolding transitions, similar stringent criteria do not yet exist. Often, the coincidence of transitions as obtained by using different probes to monitor unfolding is taken as good evidence for a two-state process. This is, however, not a sufficient criterion, because intermediates that show a similar degree of native properties by all probes would not be detected. On the other hand, noncoincident transitions are definite evidence for intermediates. For several proteins, the changes in amide circular dichroism occur at a higher concentration of denaturant than the changes in spectral properties of the aromatic residues, such as fluorescence, absorbance, and CD, and it was suggested that folding intermediates of the "molten globule" type with ordered secondary structure but little, if any, specific tertiary interactions are present in the unfolding transitions of these proteins (Kuwajima, 1989; Ptitsyn, 1992).

To determine the nature of a solvent-induced unfolding transition, it is not sufficient to follow solely changes in physical properties, because usually they comprise contributions from all species, N, I, and U, which are not easily separated. Instead, it would be highly desirable to measure directly and independently the concentrations of N, U, and I molecules as a function of the denaturant concentration.

We introduce here a kinetic method to determine the concentration of N molecules in the course of an unfolding transition. It is based on the observation that the unfolding of a protein is usually a monophasic reaction that accounts for the entire difference in signal between the native and the

† This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

* To whom correspondence should be addressed. Telephone: ++49 921 553660. FAX: ++49 921 553661.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

[1] Abbreviations: RNase T1, ribonuclease T1; RCM(-Pro55)-T1, S54G/P55N variant of ribonuclease T1 with the substitutions Ser54→Gly and Pro55→Asn, in which both disulfide bonds are reduced and the cysteine residues carboxymethylated; U, unfolded form of a protein; N, native protein; I, folding intermediate; GdmCl, guanidinium chloride; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid; DTE, dithioerythritol.

0006-2960/94/0433-12930$04.50/0

unfolded states. Only the native molecules unfold slowly, whereas partially folded molecules, populated either at equilibrium or transiently during refolding, unfold rapidly when exposed to unfolding conditions (Schmid, 1992).

Experimentally, the concentration profile for the N molecules in a transition is determined by a series of two-step experiments. In the first step, samples of the protein are incubated at various concentrations of denaturant until the equilibrium between folded and unfolded molecules is established. In the second step, every sample is transferred to identical unfolding conditions, and the rates and the amplitudes of the resulting unfolding reactions are determined. Comparison with the kinetics of the respective unfolding of the native protein gives a 2-fold information. (1) A conserved rate of unfolding indicates that the N molecules which are populated in the transition region are indistinguishable from the N molecules as they exist under native conditions. (2) The relative amplitude of unfolding provides a direct measure for the fraction of the protein molecules that were in the N state at a particular concentration of denaturant. A concentration profile for the native protein can thus be measured and compared with the fractional change in a spectroscopic property in the same transition. When the two curves coincide, then the occurrence of folding intermediates which differ from U in this property can be excluded. Alternatively, when the concentration of native molecules decreases more rapidly than the spectroscopic property, then an intermediate is present which is distinct from U in this property and which unfolds rapidly in the unfolding assay.

We apply this method to the unfolding of two proteins: the reduced and carboxymethylated form of S54G/P55N-RNase T1 [RCM(-Pro55)-T1] and apo-α-lactalbumin. Both proteins are only marginally stable, because they lack tertiary interactions [two disulfide bonds in RCM(-Pro55)-T1 and a Ca^{2+} ion in apo-α-lactalbumin] that are present in the respective unmodified proteins. The NaCl-induced refolding transition of RCM(-Pro55)-T1 (Pace et al., 1988; Mücke & Schmid, 1992, 1994) is indeed a two-state reaction, and for apo-α-lactalbumin (Kuwajima, 1977, 1989; Ikeguchi et al., 1986a,b), the presence of an intermediate in the GdmCl-induced unfolding transition could be confirmed.

MATERIALS AND METHODS

Materials. The S54G/P55N variant of RNase T1was purified as described (Mayr and Schmid, 1993a) from *Escherichia coli* cells transformed with a plasmid carrying a chemically synthesized gene which was cloned and expressed in *E. coli* as described (Quaas et al., 1988). GdmCl (ultrapure) and urea (ultrapure) were from Schwarz/Mann (Orangeburg, NY); dithioerythritol (DTE), iodoacetate, α-lactalbumin, and Hepes (Na^+ salt) were from Sigma (St. Louis, MO). Ethylene glycol bis(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA) was from Fluka (Buchs, Switzerland). All other chemicals were from Merck (Darmstadt, Germany). RCM-(-Pro55)-T1 was produced as described by Mücke and Schmid (1994). The concentrations of GdmCl, urea, and NaCl were determined by the refraction of the solutions. The equations correlating the refractive index with the GdmCl and urea concentrations are given by Pace et al. (1989); the respective equation for the NaCl concentration was determined experimentally and is given in eq 3.

$$[\mathrm{NaCl}] = 105.74\Delta n_D - 89.37\Delta n_D^2 + 7259\Delta n_D^3 \quad (3)$$

Production of Apo-α-lactalbumin. To produce the apo form of α-lactalbumin, the protein was depleted of Ca^{2+} by dissolving the lyophilized protein in 100 mM Hepes-Na^+ (the Na^+ salt of Hepes acid)/1 mM EGTA, pH 7.0. EGTA chelates Ca^{2+} with very high efficiency (Schmid & Reilley, 1957) and does not interfere with fluorescence and CD measurements.

Spectroscopic Methods. The concentrations of RCM (-Pro55)-T1 and apo-α-lactalbumin were determined spectrophotometrically by using the absorption coefficient $A_{278nm}^{0.1\%} = 1.9$ of the native wild-type RNase T1 [Takahashi et al., 1970; see also Yu et al. (1994)] for RCM(-Pro55)-T1 and $A_{278nm}^{0.1\%} = 2.0$ for apo-α-lactalbumin (Kronman & Andreotti, 1964). For optical measurements, a JASCO J-600A spectropolarimeter, a Hitachi F4010 fluorescence spectrometer, and a Kontron Uvikon 860 spectrophotometer were used.

NaCl/Urea-Induced Unfolding Transitions of RCM(-Pro55)-T1. The protein was incubated at 10 °C in the presence of 0.1 M Tris/HCl, pH 8.0, and varying concentrations of NaCl and urea for at least 4 h. For fluorescence measurements the protein concentration was 0.7 μM, and the emission of the samples was measured at 320 nm (10 nm bandwidth) after excitation at 268 nm (1.5 nm bandwidth). For measurements of the CD in the amide region, the protein concentration was 8 μM. The CD of the samples was measured in a 0.1 cm thermostated cuvette at 215 nm and at 250 nm with a time constant of 0.5 s. The resolution was 0.2 s; the bandwidth was 1 nm. The fluorescence at 320 nm and the difference in the CD signal between 215 and 250 nm were then plotted as a function of NaCl or urea concentration. The folding transitions were analyzed by assuming a two-state transition between the folded (N) and the unfolded (U) conformations (Santoro & Bolen, 1988).

GdmCl-Induced Unfolding Transitions of Apo-α-lactalbumin. α-Lactalbumin was incubated at 5 °C in the presence of 0.1 M Hepes-Na^+/1 mM EGTA, pH 7.0, and varying concentrations of GdmCl for 4 h. To measure the fluorescence-detected transition, the protein concentration was 1 μM, and the emission of the samples was measured at 350 nm (10 nm bandwidth) after excitation at 278 nm (1.5 nm μM bandwidth). The data were analyzed as described above. For the CD measurements, the protein concentration was 10 μM. The CD signal of the samples was measured at 222 and at 250 nm; the other parameters were as described for RCM(-Pro55)-T1. The data were modeled by using a spline function.

Kinetic Unfolding Assays. The unfolding assays for RCM-(-Pro55)-T1 were initiated by a 20-fold dilution of samples of the protein (equilibrated at a concentration of 14 μM at various concentrations of NaCl within the equilibrium transition) to constant assay conditions of 0.1 M Tris/HCl, pH 8.0, 10 °C, 0.6 M urea, and 0.1 M NaCl. The dead time of mixing was about 3 s. The kinetics of unfolding were followed by the decrease in fluorescence at 320 nm (10 nm bandwidth) after excitation at 268 nm (1.5 nm bandwidth). The final protein concentration was 0.7 μM. The observed kinetic curves were analyzed as the sum of two exponential functions by using the program GraFit 3.0 (Erithacus Software, Staines, U.K.).

The unfolding assays for apo-α-lactalbumin measured by fluorescence were carried out by a 40-fold dilution of the protein (equilibrated at a concentration of 40 μM under the conditions of the respective equilibrium transition) to final conditions of 0.1 M Hepes-Na^+, 1 mM EGTA, pH 7.0, and 3.0 M GdmCl. The unfolding kinetics were followed by the change in emission at 350 nm (10 nm bandwidth) after excitation at 278 nm (1.5 nm bandwidth). The final protein concentration was 1 μM. The unfolding assays of apo-α-lactalbumin followed by far-UV CD were initiated by a 20-

fold dilution of the protein (equilibrated at a concentration of 100 μM under the conditions of the respective CD transition) to the same solvent conditions as in the respective fluorescence measurements. The unfolding kinetics were followed by the change of the CD signal at 222 nm in a thermostated 0.5 cm cell at a bandwidth of 1 nm. The dead time of manual mixing was about 13 s. In all cases, the observed kinetics were monophasic and the amplitudes and time constants of unfolding were derived from the experimental data by using the program GraFit 3.0 (Erithacus Software, Staines, U.K.).

RESULTS

Folding Transition of RCM(-Pro55)-T1. RNase T1 is a small protein of 104 residues. The wild-type protein contains two disulfide bonds and two cis prolines (Pro39 and Pro55) (Pace et al., 1991; Martinez-Oyanedel et al., 1991), the trans → cis isomerizations of which are slow, rate-determining steps of folding (Kiefhaber et al., 1990a; Mayr and Schmid, 1993b). Here we use the variant S54G/P55N, with only a single cis proline (Pro39), because its folding kinetics are simplified (Kiefhaber et al., 1990b). The reduction of the disulfide bonds and subsequent carboxymethylation remove two strong tertiary interactions, and the modified protein RCM(-Pro55)-T1 is unfolded at pH 8.0, 10 °C. It can, however, fold reversibly to a nativelike conformation when NaCl is added at increasing concentrations (Oobatake et al., 1979; Pace et al., 1988; Mücke and Schmid, 1992).

The disruption of stabilizing tertiary interactions often decreases the cooperativity of folding, and intermediates become populated in the transition region at equilibrium. Notable examples are provided by α-lactalbumin, in the presence and in the absence of the Ca^{2+} ion (Ikeguchi et al., 1986a,b), and by myoglobin in the presence and in the absence of the heme (Privalov et al., 1986; Griko et al., 1988).

To investigate whether the NaCl-induced folding transition of RCM(-Pro55)-T1 is in fact a two-state reaction, we compared the fractional decreases in tryptophan fluorescence and in amide CD in this transition with the decrease in the concentration of native molecules, as measured by the kinetic unfolding assays. If in the unfolding transitions the observed decreases in fluorescence and CD originate only from the decrease in the fraction of N molecules, then these curves should coincide. If, however, partially folded intermediates accumulate in the transition and contribute to the measured fluorescence and CD, then the fractional changes in fluorescence and CD should differ from the profile for the N molecules in the unfolding transition.

The unfolding transition of RCM(-Pro55)-T1 as a function of decreasing NaCl concentration followed by fluorescence is shown in Figure 1A. To confirm that unfolding is virtually complete at 0 M NaCl and to determine the fluorescence of the unfolded protein additional measurements were made in the presence of 0–0.7 M urea. They are included in Figure 1A. The unfolding assays to measure the concentration of the N molecules are also outlined in Figure 1. Samples of RCM(-Pro55)-T1 were first incubated at various NaCl concentrations under the conditions of Figure 1A for 4 h to establish the equilibrium between the folded and the unfolded forms of the protein. Then these samples were rapidly diluted in the fluorometer cell to give constant final conditions of 0.6 M urea/0.1 M NaCl, 10 °C, for the unfolding assays. Representative unfolding kinetics are shown in Figure 1B. RCM(-Pro55)-T1 is in the folded state at 2.1 M NaCl (Figure 1A), and the unfolding reaction starting from this condition (the uppermost curve in Figure 1B) serves as a reference. Its

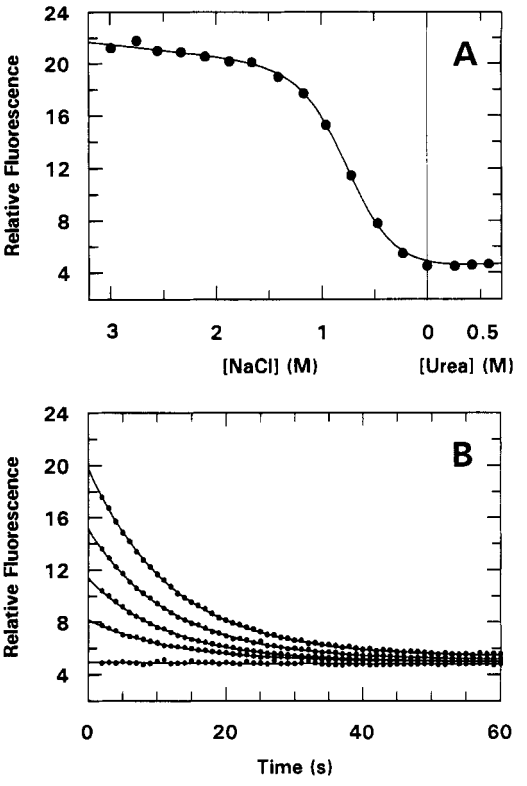

FIGURE 1: (A) Folding transition of RCM(-Pro55)-T1 in 0.1 M Tris/HCl, pH 8.0, 10 °C. The decrease in fluorescence at 320 nm (after excitation at 268 nm) is shown as a function of decreasing NaCl concentration. To obtain the base line for the unfolded protein, the emission of samples in the presence of 0–0.7 M urea was measured in addition. They are included on the right side of panel A. The protein was incubated for at least 4 h in 0.1 M Tris/HCl, pH 8.0, 10 °C, and the indicated concentration of NaCl or urea. (B) Representative unfolding assays of RCM(-Pro55)-T1. Samples of the protein (14 μM) were incubated at 2.1, 1.0, 0.7, 0.5, and 0 M NaCl (from top to bottom) as in panel A, and then unfolding was initiated by a 20-fold dilution to final conditions of 0.1 M Tris/HCl, pH 8.0, 10 °C, 0.6 M urea, and 0.1 M NaCl. The dead time of mixing was about 3 s. The unfolding kinetics in the assays were followed by the decrease in fluorescence at 320 nm. The final protein concentration was 0.7 μM. The observed kinetics were analyzed as described under Materials and Methods. The resulting time constants and amplitudes are found in Table 1.

kinetic data are shown in the first row of Table 1. The fluorescence emission decreases by 14.95 units, and this value is equivalent to the unfolding of 100% N molecules. With decreasing concentration of NaCl, the amplitude of unfolding decreases, and unfolding kinetics were no longer observed when the protein was preincubated at 0 M NaCl.

Under the assay conditions, unfolding is not strictly monophasic. About 95% of the decrease in fluorescence occurs in the major unfolding reaction, which shows a time constant (τ_2) of 12 s. In addition, there is a minor slow reaction (τ_1 = 220 s) with an amplitude of 5%. It originates from the cis ⇌ trans isomerization of Pro39 in the unfolded protein and is a consequence of the kinetic coupling between unfolding and prolyl isomerization near the end of the folding transition (Kiefhaber et al., 1992; Kiefhaber & Schmid, 1992). The kinetic analysis of the folding of RCM(-Pro55)-T1 shows that the amplitude of this minor reaction decreases to zero at higher denaturant concentration (Mücke & Schmid, 1994). Under these conditions, the time constant of the unfolding reaction (τ_2) is, however, further decreased and can no longer be followed after manual mixing as required in the present experiments. Therefore, we used the conditions of Figure 1B for the unfolding assays, where the amplitude of the minor

Table 1: Unfolding Assays To Determine the Fraction of Native Protein in the NaCl-Induced Folding Transition of RCM(-Pro55)-T1: Analyses of Unfolding Kinetics in the Assays[a]

[NaCl][b] (M)	A_2[c]	α_2[d] (%)	τ_2[e] (s)	A_1[c]	τ_1[e] (s)	A_∞[f]	A_{rel}[g]
2.10	14.2	95	12	0.74	220	4.84	1.00
1.87	14.4	95	12	0.72	220	4.88	1.01
1.67	13.5	95	12	0.70	210	4.86	0.95
1.43	13.1	95	12	0.71	170	4.89	0.92
1.19	12.6	95	12	0.64	200	4.90	0.88
0.97	9.8	94	12	0.46	200	4.79	0.69
0.72	6.3	94	11	0.37	230	4.68	0.45
0.50	3.2	93	12	0.25	650	4.76	0.23
0.25	0.9	87	12	0.14	90	4.74	0.07
0	0.2	89	21	0.01	240	4.79	0.01

[a] Samples of RCM(-Pro55)-T1 were incubated in 0.1 M Tris/HCl, pH 8.0, 10 °C, and the indicated concentrations of NaCl for 4 h, and then unfolding was initiated by a 20-fold dilution to final conditions of 0.1 M Tris/HCl, pH 8.0, 10 °C, 0.6 M urea, and 0.1 M NaCl. The unfolding kinetics in the assays were followed by the decrease in fluorescence at 320 nm. The final protein concentration was 0.7 μM. The observed kinetics were analyzed as the sum of two exponential functions. [b] Concentration of NaCl in the incubation step. [c] A_2, A_1, amplitudes of the fast and the slow unfolding reactions, respectively. [d] Relative amplitude of the fast unfolding reaction: $\alpha_2 = 100A_2/(A_1 + A_2)$. [e] τ_2, τ_1, time constants of the fast and the slow unfolding reactions, respectively. [f] Final fluorescence, observed at the end of the unfolding assay. [g] Normalized change in fluorescence in the unfolding assay relative to the value observed at 2.1 M NaCl: $(A_1 + A_2)/(A_1 + A_2)_{2.1M}$.

phase (A_1) is very small already and where the major unfolding reaction can be measured with good accuracy after manual mixing.

The kinetic data for all unfolding assays are shown in Table 1. The amplitudes of unfolding and thus the concentration of N molecules decrease with decreasing NaCl concentration in the incubation step, but the time constants of unfolding are independent of the initial conditions. Deviations occur only below 0.4 M NaCl, conditions under which the protein is more than 90% unfolded already, and the amplitudes of further unfolding in the assay are very small. These unchanged rate constants of unfolding strongly suggest that the N molecules which coexist with other partially or fully unfolded molecules in the transition region do not differ from the N molecules as present in the region of the native base line.

The relative amplitudes of unfolding in the last column of Table 1 provide a direct measure for the fraction of N molecules present in the various samples. Its decrease in the NaCl-induced transition is shown in Figure 2. It coincides with the fractional changes in tryptophan fluorescence and in amide CD. This coincidence demonstrates that the observed changes in fluorescence and in circular dichroism are entirely accounted for by the decrease in the concentration of native molecules. As a consequence, folding intermediates with partially native fluorescence or amide CD cannot be populated in this transition. This result strongly suggests that the NaCl-induced folding transition of RCM(-Pro55)-T1 is a two-state reaction, even though two strongly stabilizing interactions (the disulfide bonds) are absent in this modified form of RNase T1.

Folding Transition of Apo-α-lactalbumin. Apo-α-lactalbumin unfolds in two successive steps and follows a three-state mechanism as in eq 2. The work by Kuwajima and others [reviewed by Kuwajima (1989)] showed that a "molten globule" intermediate I accumulates at intermediate concentrations of GdmCl and noncoincident transitions are observed when unfolding is measured by different probes (Ikeguchi et al., 1986a,b). The intermediate I is thought to have lost the specific tertiary structure of the native protein, but most of the secondary structure is still retained. Spectroscopic probes that are sensitive to changes in tertiary structure, such as absorbance, fluorescence, and near-UV CD, follow the N ⇌ I transition; probes that are sensitive to changes in secondary structure, such as far-UV CD, follow mainly the I ⇌ U transition (see eq 2), which occurs at a slightly higher concentration of GdmCl (Permyakov et al., 1985; Ikeguchi et al., 1986a,b). Because of its well-studied three-state

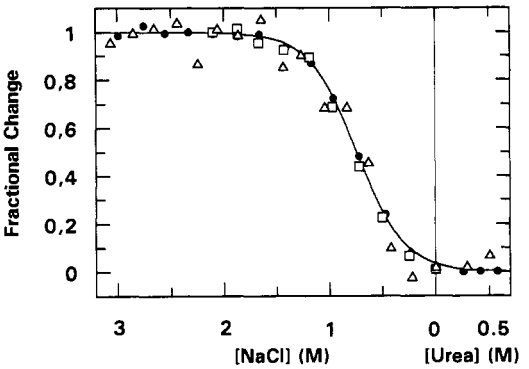

FIGURE 2: Comparison of the normalized unfolding transitions of RCM(-Pro55)-T1 measured by fluorescence (●), by amide CD (▲), and by the kinetic unfolding assays for N molecules (□) in 0.1 M Tris/HCl, pH 8.0, 10 °C. Unfolding was induced by decreasing the NaCl concentration. To obtain the base line for the unfolded protein, additional samples were measured that contained 0–0.7 M urea. Fluorescence was measured at 320 nm (0.7 μM protein) and amide CD at 215 nm (8 μM protein). The unfolding assays were carried out as described in the legend to Figure 1. The fluorescence and the CD transitions were analyzed on the basis of a two-state model, and a linear dependence of the base lines in the pre- and posttransitional regions was assumed to determine the fractional change in signal. To normalize the kinetic amplitudes from the unfolding assays, the value observed in the unfolding reaction of the folded protein (preincubated at 2.1 M NaCl) was taken as 1.0. The line represents the best fit for the fluorescence-detected transition (cf. Figure 1A), which is virtually identical to the fit of the CD transition (not shown).

unfolding mechanism, apo-α-lactalbumin provides an excellent system to test our kinetic method of determining the concentration of N molecules in a complex unfolding transition.

The GdmCl-induced unfolding transitions of apo-α-lactalbumin were measured at pH 7.0, 5 °C, and are shown in Figure 3. As observed previously (Ikeguchi et al., 1986a,b), the loss of tertiary structure, measured here by the increase in tryptophan fluorescence, precedes the loss of secondary structure, measured by the change in CD at 222 nm. To determine the decrease in the concentration of N molecules in this transition we incubated samples of the protein for 4 h in the presence of 0–2.4 M GdmCl and then measured the unfolding kinetics under constant final conditions of 3.0 M GdmCl, pH 7.0, and 5 °C, by both tryptophan fluorescence and amide CD. The results are summarized in Table 2. Unfolding of the native protein after a jump from 0 to 3.0 M GdmCl is a monophasic reaction with a time constant of 35 s, and the entire changes in fluorescence and in CD as expected from the respective equilibrium transitions occur in this

Table 2: Unfolding Assays To Determine the Fraction of Native Protein in the GdmCl-Induced Unfolding Transition of Apo-α-lactalbumin: Analyses of Unfolding Kinetics in Assays[a]

unfolding measured by fluorescence				unfolding measured by CD			
[GdmCl][b] (M)	A^c	τ^d (s)	A_{rel}^e	[GdmCl][b] (M)	A^c	τ^d (s)	A_{rel}^e
0	36.6	35	1.00	0	19.2	33	1.00
0.37	36.0	35	0.99	0.40	19.9	34	1.04
0.57	35.4	35	0.97	0.80	19.3	33	1.01
0.79	34.8	35	0.95	1.19	16.3	36	0.85
0.95	33.4	35	0.91	1.40	13.0	37	0.68
1.06	32.5	37	0.89	1.57	10.6	44	0.55
1.15	30.5	36	0.83	1.81	5.9	38	0.31
1.30	26.6	36	0.73	2.01	2.7	30	0.14
1.42	23.1	36	0.63	2.22	0.9	108	0.05
1.45	20.7	38	0.57	2.39	0.3	72	0.01
1.57	16.6	37	0.45				
1.68	12.2	39	0.33				
1.76	8.6	37	0.23				
1.87	5.5	38	0.15				
2.03	3.5	37	0.09				
2.16	1.8	16	0.05				

[a] Samples of apo-α-lactalbumin were equilibrated in 0.1 M Hepes-Na^+/1 mM EGTA, pH 7.0, 5 °C, and the indicated concentrations of GdmCl, and then unfolding was inititated by a 40-fold dilution (fluorescence) or a 20-fold dilution (amide CD) to give a common final concentration of 3.0 M GdmCl. The resulting unfolding kinetics were followed by the change in fluorescence at 350 nm and by the change in amide CD at 222 nm. The final protein concentrations were 1 and 5 μM, respectively. [b] Concentration of GdmCl in the incubation step. [c] Measured amplitude of unfolding. [d] Time constant of the unfolding reaction. [e] Normalized change in fluorescence or CD in the unfolding assay relative to the value observed at 0 M GdmCl.

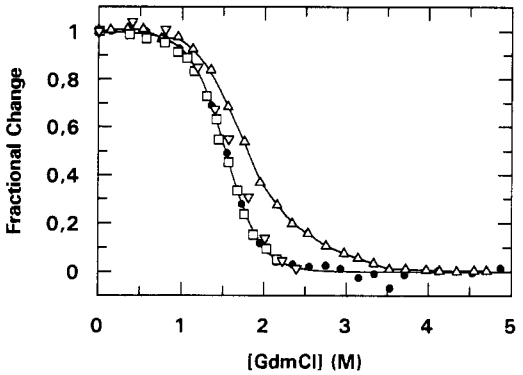

FIGURE 3: Comparison of the normalized unfolding transitions of apo-α-Lactalbumin measured by fluorescence (●), by amide CD (△), and by the kinetic unfolding assays for N molecules followed by the increase in fluorescence (□) and by the decrease in amide CD (▽). The transitions were measured in 0.1 M Hepes-Na^+/1 mM EGTA, pH 7.0, 5 °C, as a function of the GdmCl concentration by measuring the fluorescence at 350 nm after excitation at 278 nm (●) and the CD at 222 nm (△). The fluorescence-detected transition was analyzed on the basis of a two-state model, and a linear dependence of the base lines in the pre- and posttransitional regions was assumed to determine the fractional change in signal. The line represents the best fit of the data by using the formalism of Santoro and Bolen (1988). The observed transition measured by amide CD was modeled by a spline function. The protein (1 μM for the fluorescence measurements, 10 μM for CD measurements) was incubated for at least 2 h in 0.1 M Hepes-Na^+/1 mM EGTA, pH 7.0, 5 °C, and the indicated concentration of GdmCl. Unfolding of apo-α-lactalbumin in the kinetic assays was initiated by a 40-fold dilution (fluorescence) or a 20-fold dilution (near-UV CD) of samples that were equilibrated under the conditions of the respective equilibrium transition to common final conditions of 0.1 M Hepes-Na^+, 1 mM EGTA, pH 7.0, 5 °C, and 3 M GdmCl. The unfolding kinetics were followed by the change in fluorescence at 350 nm (□) and by the change in amide CD at 222 nm (▽). The final protein concentrations were 1 and 5 μM, respectively. The resulting amplitudes were normalized by setting the amplitude of unfolding of the folded protein (at 0 M GdmCl) as 1.0. The respective time constants and amplitudes are shown in Table 2.

reaction. Apo-α-lactalbumin does not contain cis prolines, and therefore prolyl isomerizations do not contribute significantly to the unfolding kinetics.

Again, the amplitudes of unfolding decrease with increasing concentration of GdmCl, but the time constants of unfolding are independent of the initial conditions (Table 2). This suggests that the native protein which occurs in equilibrium with I and U in the transition region does not differ from the folded protein as it exists in the absence of GdmCl. The amplitudes of unfolding observed after incubation without GdmCl were used as reference values to calculate the fraction of native molecules as a function of the GdmCl concentration. Identical results were obtained when unfolding in the assays was followed either by fluorescence or by CD (Figure 3). In both cases, the concentration of N molecules follows closely the first transition of apo-α-lactalbumin, which is observed by the spectroscopic properties of the aromatic amino acids. It indicates that this transition is indeed the N ⇌ I transition and that there are no additional intermediates detectable between N and I. The noncoincidence in Figure 3 between the equilibrium transition monitored by amide CD and the transition derived from the CD-monitored unfolding assays for N molecules provides decisive evidence that the unfolding of apo-α-lactalbumin is not a N ⇌ U two-state reaction. It shows that a large fraction of the amide CD in the transition region does indeed not originate from native molecules, but from a species that unfolds very rapidly in the assays, i.e., from the molten globule intermediate, which has been found before in this transition (Kuwajima, 1989).

DISCUSSION

We have developed a method to measure the fraction of native molecules (N) which coexist in equilibrium with other partially (I) or fully unfolded (U) forms of a protein in a solvent-induced unfolding transition. The method consists of two steps. In the first step, aliquots of the protein are incubated at varying concentrations of denaturant in the transition region, and in the second step, unfolding assays are performed to determine the concentration of N molecules in these samples from the kinetic amplitude of unfolding. This procedure has major advantages when compared with the use of changes in physical properties of the system to measure unfolding transitions. Usually these changes comprise contributions from the native and from the unfolded molecules, as well as from potential folding intermediates. All these contributions may depend on the denaturant concentration, and, as a consequence, often an analysis is only possible when a two-

state mechanism is assumed before analysis. In contrast, the unfolding assays for N monitor selectively the concentration of a single species, i.e., the native protein. The reference value for 100% native molecules is readily obtained from the unfolding kinetics of the native protein. An extrapolation of base lines is not required.

Ideally, a related kinetic procedure would be desirable for measuring the concentration of the unfolded protein (U) by a refolding assay and to examine whether the sum of the concentrations of N and U remains constant and equal to the total protein concentration throughout a folding transition. Unfortunately, the refolding of proteins is usually a complex process that involves parallel and sequential steps with strongly different rates, and a simple and accurate determination of the total change in signal during refolding is very difficult. In addition, the unfolded molecules (U) and the folding intermediates (I) are often in rapid exchange and therefore cannot be discriminated in refolding experiments. In some cases, folded and unfolded molecules in an unfolding transition can also be detected by gel filtration (Kelley et al., 1987; Ptitsyn et al., 1990; Uversky, 1993). This method differs from the kinetic unfolding assays in an important aspect. In gel filtration, usually the native protein and compact folding intermediates cannot be discriminated. They coelute, but are distinct from the unfolded protein. The unfolding assays discriminate between native and partially structured molecules, but do not distinguish between unfolded and partially folded molecules that unfold rapidly.

The two-state character of a solvent-induced unfolding transition can now be evaluated. When the fractional change of the spectral properties in a transition follows the decrease in the concentration of the native molecules, as measured by the unfolding assays, then intermediates with spectral properties other than those of the unfolded protein can be excluded definitely. Such a coincidence and a constant rate of unfolding in the assay also rule out that the transition is second rather than first order. A noncoincidence, as observed for apo-α-lactalbumin, provides strong evidence for an intermediate with partially native properties. Our test would not detect intermediates which do not differ from the unfolded state in spectral properties, or which unfold as slowly as the native protein. Such slowly unfolding species are not likely to exist. Even minor changes in the stability of a native protein often lead to strong changes in the unfolding kinetics, and nativelike kinetic intermediates that accumulate transiently in the course refolding unfold much more rapidly than the native protein (Schmid, 1983; Kiefhaber et al., 1990a).

This test is similar to the calorimetric test for intermediates in thermal unfolding transitions (Privalov, 1979, 1992; Privalov & Potekhin, 1986). In this case, the coincidence of the calorimetric (ΔH_{cal}) and van't Hoff enthalpies (ΔH_{vH}) of denaturation indicates that intermediates with an enthalpy other than that of the unfolded protein are not populated at equilibrium. These two tests of the two-state approximation complement each other. The assay for N molecules is best applied to evaluate isothermal solvent-induced unfolding transitions, and the calorimetric method is best suited to analyze thermal unfolding transitions.

ACKNOWLEDGMENT

We thank U. Hahn for providing the strain producing the Ser54Gly,Pro55Asn variant of RNase T1 and C. Frech, C. Odefey, T. Schindler, and S. Walter for many stimulating discussions.

REFERENCES

Griko, Y. V., Privalov, P. L., & Kutyshenko, V. P. (1988) *J. Mol. Biol. 202*, 127–138.
Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986a) *J. Biochem. 99*, 1191–1201.
Ikeguchi, M., Kuwajima, K., Mitani, M., & Sugai, S. (1986b) *Biochemistry 25*, 6965–6972.
Kelley, R. F., Shalongo, W., Jagannadham, M. V., & Stellwagen, E. (1987) *Biochemistry 26*, 1406–1411.
Kiefhaber, T., & Schmid, F. X. (1992) *J. Mol. Biol. 224*, 231–240.
Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990a) *Biochemistry 29*, 3061–3070.
Kiefhaber, T., Grunert, H. P., Hahn, U., & Schmid, F. X. (1990b) *Biochemistry 29*, 6475–6480.
Kiefhaber, T., Kohler, H. H., & Schmid, F. X. (1992) *J. Mol. Biol. 224*, 217–229.
Kronman, M. J., & Andreotti, R. E. (1964) *Biochemistry 3*, 1145–1151.
Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.
Martinez-Oyanedel, J., Choe, H.-W., Heinemann, U., & Saenger, W. (1991) *J. Mol. Biol. 222*, 335–352.
Mayr, L. M., & Schmid, F. X. (1993a) *Protein Expression Purif. 4*, 52–58.
Mayr, L. M., & Schmid, F. X. (1993b) *J. Mol. Biol. 231*, 913–926.
Mücke, M., & Schmid, F. X. (1992) *Biochemistry 31*, 7848–7854.
Mücke, M., & Schmid, F. X. (1994) *J. Mol. Biol. 239*, 713–725.
Oobatake, M., Takahashi, S., & Ooi, T. (1979) *J. Biochem. 86*, 55–63.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Pace, C. N., Grimsley, G. R., Thomson, J. A., & Barnett, B. J. (1988) *J. Biol. Chem. 263*, 11820–11825.
Pace, C. N., Shirley, B. A., & Thomson, J. A. (1989) in *Protein structure: a practical approach* (Creighton, T. E., Ed.) pp 311–330, IRL Press, Oxford.
Pace, C. N., Heinemann, U., Hahn, U., & Saenger, W. (1991) *Angew. Chem., Int. Ed. Engl. 30*, 343–360.
Permyakov, E. A., Morozova, L. A., & Burstein, E. A. (1985) *Biophys. Chem. 21*, 21–31.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Privalov, P. L. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 83–126, Freeman, New York,
Privalov, P. L., & Potekhin, S. A. (1986) *Methods Enzymol. 131*, 4–51.
Privalov, P. L., Griko, Y. V., & Venyaminov, S. Y. (1986) *J. Mol. Biol. 190*, 487–498.
Ptitsyn, O. B. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 243–300, Freeman, New York.
Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990) *FEBS Lett. 262*, 20–24.
Quaas, R., McKeown, Y., Stanssens, P., Frank, R., Bloecker, H., & Hahn, U. (1988) *Eur. J. Biochem. 173*, 617–623.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063–8068.
Schmid, F. X. (1983) *Biochemistry 22*, 4690–4696.
Schmid, F. X. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 197–241, Freeman, New York.
Schmid, R. W., & Reilley, C. N. (1957) *Anal. Chem. 29*, 264–268.
Takahashi, K., Uchida, T., & Egami, F. (1970) *Adv. Biophys. 1*, 53–98.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Uversky, V. N. (1993) *Biochemistry 32*, 13288–13298.
Yu, Y., Makhatadze, G. I., Pace, C. N., & Privalov, C. N. (1994) *Biochemistry 33*, 3312–3319.