Cold Denaturation of the Molten Globule States of Apomyoglobin and a Profile for Protein Folding†

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Received November 24, 1993; Revised Manuscript Received February 14, 1994®

ABSTRACT: Protein folding is a process in which an extended polypeptide chain acquires compact packing through the formation of specific secondary and tertiary structures and hydrophobic interactions. Although much attention has been paid to secondary and tertiary structures, there is no definitive view about the relationship between these structures, compactness, and hydrophobic interactions during the process of protein folding. We show here that the molten globule intermediates of horse apomyoglobin exhibit cold denaturation in addition to heat denaturation, which indicates that the heat capacity change upon unfolding is positive and significant. This demonstrates a small but distinct contribution of hydrophobic interactions to the stability of the molten globule state. We determined the radius of gyration of the various conformational states of horse apomyoglobin and holomyoglobin by measuring small angle X-ray scattering. By comparing the conformational states in terms of secondary structure, radius of gyration, and change in heat capacity upon unfolding, we constructed a folding profile. The profile shows that the protein becomes more compact with formation of the secondary structure, but does not form substantial hydrophobic interactions until a later rate-limiting stage when tight packing of the protein side chains occurs. A very similar profile was also obtained with horse cytochrome *c*. We propose that the folding profile obtained with these proteins will be common to many globular proteins.

The molten globule state, a compact denatured state with a significantly native-like secondary structure but a largely flexible and disordered tertiary structure, has been proposed to be a major intermediate of protein folding (Kuwajima, 1989, 1992; Ptitsyn, 1992; Dobson, 1992; Barrick & Baldwin, 1993). The molten globule state has also been suggested to be involved in various processes in the cell, such as the interaction of nascent proteins with molecular chaperones (Martin et al., 1991; Hartl et al., 1994) or the interaction of proteins with the membrane (van der Goot et al., 1991). Structural characterizations of the equilibrium and kinetic intermediates of several proteins, including apomyoglobin, have been carried out extensively, and the results are consistent with a view that the molten globule is a major intermediate of protein folding (Kuwajima, 1989; Hughson et al., 1990; Ptitsyn, 1992; Dobson, 1992; Barrick & Baldwin, 1993; Baldwin, 1993; Jennings & Wright, 1993; Dobson et al., 1994).

However, we do not know how the thermodynamic factors that are important for the stability of the native state apply to the molten globule state. In particular, the role of hydrophobic interactions in the molten globule state is controversial. It should be noted that the definition of hydrophobic interactions and their role in the stability of the native structure is also controversial (Dill, 1990a,b; Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). By hydrophobic interactions, we mean the total interactions associated with the transfer of hydrophobic substances from water to a nonpolar environment, thus including both the terms of the van der Waals interactions between the nonpolar substances and their hydration.

With the exceptions of cytochrome *c* (Potekhin & Pfeil, 1989; Kuroda et al., 1992; Hagihara et al., 1994) and retinol-binding protein (Bychkova et al., 1992), molten globule states do not seem to exhibit a cooperative thermal unfolding transition, suggesting that the enthalpy change of unfolding (ΔH_U)[1] is small (Kuwajima, 1989; Ptitsyn, 1992). The heat capacity (C_p) of the intermediate conformations is reported to be essentially the same as that of the fully unfolded state (Privalov & Makhatadze, 1990; Yutani et al., 1992). This means that the increase in C_p upon unfolding ($\Delta C_{p,U}$), which reflects the contribution of hydrophobic interactions in protein folding (Privalov & Gill, 1988; Privalov & Makhatadze, 1990; Oobatake & Ooi, 1992), is negligible. Although large ΔH_U and $\Delta C_{p,U}$ values have been reported for the molten globule state of α-lactalbumin (Xie et al., 1991, 1993; Griko et al., 1994), the validity of this observation was questioned (Yutani et al., 1992).

On the other hand, there have been several experiments showing the importance of hydrophobic interactions in the molten globule state. These include the presence of hydrophobic cores detected by NMR (Dobson, 1992; Ptitsyn, 1992; Shimizu et al., 1993) and stabilization by site-directed mutagenesis introducing a nonpolar amino acid (Hughson et al., 1991; Barrick & Baldwin, 1993). In addition, the theoretical calculations predict a relatively large value of $\Delta C_{p,U}$

† Contribution No. 77 from the Microcalorimetry Research Center. This work was supported by the Ministry of Education, Science and Culture of Japan and by the Hyogo Science Association.

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

[1] Abbreviations: CD, circular dichroism; $\Delta C_{p,U}$, heat capacity change of unfolding; ΔG_U, free energy change of unfolding; ΔH_U, enthalpy change of unfolding; ΔS_U, entropy change of unfolding; DSC, differential scanning calorimetry; Gdn-HCl, guanidine hydrochloride; Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MG(Cl^-), molten globule state stabilized by chloride; MG(TCA^-), molten globule state stabilized by trichloroacetate; NaTCA, sodium trichloroacetate; NMR, nuclear magnetic resonance; R_g, radius of gyration; SAXS, small angle X-ray scattering.

0006-2960/94/0433-4903$04.50/0

for the unfolding of the molten globule state (Haynie & Freire, 1993; Griko et al., 1994). Therefore, to understand the molten globule state, it is essential to clarify the role of hydrophobic interactions in its stability.

In the present study, we have shown that the molten globule state of apomyoglobin exhibits cold-denaturation in addition to heat-denaturation. Cold-denaturation arises from the negative enthalpy of unfolding and is evidence that $\Delta C_{p,U}$ is positive and significant (Griko et al., 1988; Privalov & Gill, 1988; Oobatake & Ooi, 1993). The results demonstrate a small but distinct contribution of hydrophobic interactions to the stability of the molten globule state.

Protein folding is a process in which an extended polypeptide chain acquires a compact structure through the formation of specific secondary and tertiary architectures (Dill, 1990b; Dill & Shortle, 1991). However, there is no definitive view about the relationship between compactness and secondary and tertiary structures during the process of protein folding. Therefore we have characterized the various conformational states of myoglobin and apomyoglobin in terms of secondary structure, radius of gyration (R_g), and $\Delta C_{p,U}$ upon unfolding. On the basis of the comparison of these parameters, we propose a common profile of protein folding.

MATERIALS AND METHODS

Materials. Horse myoglobin was purchased from Sigma. Apomyoglobin was prepared from myoglobin as described previously (Goto et al., 1990a,b) and purified by Sephadex G-50 filtration in order to remove aggregates. Intact and acetylated horse ferricytochrome *c* were obtained as described previously (Goto & Nishikiori, 1991, 1992).

Methods. CD spectra were measured by use of a Jasco spectropolarimeter, Model J-500A, equipped with a personal computer. The instrument was calibrated using ammonium *d*-10-camphorsulfonate. The results are expressed as the mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ is the observed ellipticity in degrees, c is the concentration of the residue in moles per liter, and l is the length of the light path in centimeters. Far-UV CD spectra were measured with a 1-mm cell at a protein concentration of 0.2 mg mL^{-1}. Near-UV CD spectra were measured with a 1-cm cell at a protein concentration of 0.5 mg mL^{-1}. The heat- and cold-denaturation processes were measured separately using the ellipticity at 222 nm. Temperatures were increased from 10 °C at a rate of 1 °C min^{-1} for the heat-denaturation measurements and decreased from 20 °C at 0.5 °C min^{-1} for the cold-denaturation measurements. Cold denaturation was followed to temperatures as low as −9 °C, taking advantage of the supercooling effect. The temperature changes were monitored with a thermocouple (Senserteck, BAT-12, and a flexible probe) inserted directly into a 1-mm cell. The ellipticities in the overlapped temperatures (i.e., 10–20 °C) agreed well, and the combined results are presented. The reversibility of the unfolding transitions was checked by examining the ellipticities after returning the samples to the starting temperatures.

Small angle X-ray scattering (SAXS) data were collected from the solution scattering station installed at BL-10C, the Photon Factory, Tsukuba, Japan (Ueki et al., 1985; Kataoka et al., 1991), with approval from the Program Advisory Committee of the Photon Factory (Proposal No. 92-067). The sample cell was 50 μL in volume and had a 1-mm path length. The measurements were carried out at 20 °C with a thermostatically controlled cell holder. Protein concentrations were varied within the range of 2–10 mg mL^{-1}, and correction

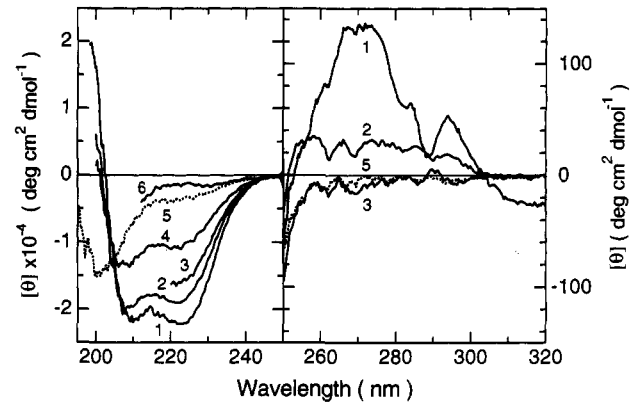

FIGURE 1: CD spectra of the various conformational states for myoglobin and apomyoglobin at 20 °C: (1) native-state myoglobin at pH 6.0 (10 mM Hepes buffer); (2) native-state apomyoglobin at pH 6.0 (10 mM Hepes buffer); (3) molten globule state stabilized by 20 mM NaTCA at pH 2.0 (10 mM HCl); (4) molten globule state stabilized by 0.4 M NaCl at pH 2.0 (10 mM HCl); (5) acid-unfolded state at pH 2.0 (10 mM HCl); (6) unfolded state in 4.0 M Gdn-HCl at pH 6.0.

was made for the concentration dependence of the scattered intensity to obtain the scattering curves at infinite dilution (Kataoka et al., 1989). X-ray scattering intensities in the small-angle region are given as $I(Q) = I(0)\exp(-R_g^2Q^2/3)$, where Q and $I(0)$ are momentum transfer and intensity at 0 scattering angle, respectively (Glatter & Kratky, 1982). Q is defined by $Q = (4\pi \sin\theta)/\lambda$, where 2θ and λ are the scattering angle and the wavelength of the X-rays, respectively. The R_g value is obtained from the slope of the Guinier plot, ln $I(Q)$ vs Q^2. There was no aggregation problem except in the molten globule induced by NaCl, for which we could not determine R_g.

Differential scanning calorimetry (DSC) for determining $\Delta C_{p,U}$ of the native states of myoglobin and apomyoglobin was carried out with a DASM4 microcalorimeter equipped with a personal computer. The scan rate was 1.0° min^{-1}. Protein concentrations were 3 mg mL^{-1}.

The buffers used were 10 mM sodium acetate between pH 4 and 6 and 10 mM Hepes buffer at pH 6. The solutions at pH 2 were prepared with 10 mM HCl. The pH was measured using a Radiometer PHM83 meter at 20 °C.

RESULTS AND DISCUSSION

Conformational States of Apomyoglobin. In the present study, we characterized the molten globule states of apomyoglobin. Since apomyoglobin assumes various conformational states (Hughson et al., 1990; Griko et al., 1988; Goto & Fink, 1990; Goto et al., 1990a,b), it is useful to describe them first. Figure 1 summarizes the conformational states of horse myoglobin and apomyoglobin on the basis of their CD spectra. Myoglobin consists of eight α-helices (A–H) [see Hughson et al. (1990); Jennings and Wright (1993)], and the far-UV CD spectrum identifies a high α-helical content. Apomyoglobin, which is prepared from myoglobin by removal of the noncovalently bound heme group, is one of the most extensively studied proteins with respect to protein folding. Whereas the α-helical content at neutral pH is decreased by 20% upon removal of the heme, it shows several native-like characteristics, including an NMR spectrum with specific chemical shifts suggesting unique environments surrounding the amino acid residues (Griko et al., 1988; Cocco et al., 1992). The large decrease in aromatic CD (Figure 1) arises mainly from the disappearance of the heme contribution.

Table 1: Structural and Thermodynamic Parameters of the Various Conformational States of Horse Myoglobin and Apomyoglobin at 20 °C

	$-[\theta]_{222}$ (deg cm^2 dmol^{-1})	f_H[a] (%)	R_g (Å)	$\Delta C_{p,U}$ (kJ mol^{-1} K^{-1})
holoN	22 000	66	17.5 ± 0.1	8.7[b]
apoN	19 000	55	20.1 ± 0.1	4.0[b]
				4.12 ± 0.02[c]
MG(TCA$^-$)	16 000	45	22.2 ± 0.3	1.80 ± 0.02[c]
MG(Cl$^-$)	11 000	28		1.50 ± 0.02[c]
U_A	3900	5	29.3 ± 1.0	
U_{urea}	1300	~0	34.2 ± 1.5	
U_{GuHCl}	500	~0	35.8 ± 1.0	

[a] f_H values were calculated by the method of Chen et al. (1972), from $[\theta]_{222}$. [b] The value for holoN was estimated at T_m (74 °C at pH 4.3) of the heat-denaturation using the DSC curve. The value for apoN was estimated at 30 °C using the DSC curve at pH 5.3. Errors in estimation at ±10%. [c] Estimated from the thermal transitions measured by CD.

Apomyoglobin is largely unfolded at pH 2 in the absence of salt (i.e., the acid-unfolded state, U_A), but in the presence of 0.4 M NaCl at the same pH, it assumes an intermediate conformational state which we call the chloride-stabilized molten globule state [MG(Cl$^-$); Goto & Fink, 1990]. The structural characterization of the molten globule state using hydrogen/deuterium exchange and two-dimensional NMR techniques indicated the presence of stable A, G, and H helices [Hughson et al., 1990; see also Jennings and Wright (1993)]. A similar molten globule state, but one with a higher α-helical content, which we call the trichloroacetate-stabilized molten globule state [MG(TCA$^-$)], can be induced by the addition of sodium trichloroacetate (NaTCA) at pH 2 (Goto et al., 1990b). MG(TCA$^-$) is a state distinct from MG(Cl$^-$) since we can observe a cooperative conformational transition from the former to the latter induced by Gdn-HCl, suggesting that the additional α-helical segments are stabilized in the former (I. Nishii and Y. Goto, manuscript in preparation). Recently, on the basis of a hydrogen exchange pulse labeling study, Jennings and Wright (1993) proposed that the dominant pathway of folding of apomyoglobin is U → A·G·H → A·B·G·H → A·B·C·CD·E·G·H → N. In this context, it is intriguing whether the additional α-helical segments present in the equilibrium MG(TCA$^-$) state are consistent with the proposed kinetic pathway.

In addition, apomyoglobin is maximally unfolded in the presence of high concentrations of urea (U_{urea}) or Gdn-HCl (U_{GdnHCl}). According to the α-helical content of these states (Table 1), we can consider the following equation to model the folding process:

$$(U_{urea} \approx U_{GdnHCl}) \rightarrow U_A \rightarrow MG(Cl^-) \rightarrow MG(TCA^-) \rightarrow apoN \rightarrow holoN \quad (1)$$

where holoN and apoN are the native states of myoglobin and apomyoglobin, respectively, and the other states are of apomyoglobin.

Cold Denaturation of the Molten Globule States. To examine the validity of the assumption that the thermal transition of the molten globule states is not cooperative, we measured the effects of heat on the molten globule states of apomyoglobin by far-UV CD. Figure 2 shows the temperature-dependent change in ellipticity at 222 nm in the presence of various concentrations of NaTCA at pH 2. In the absence of salt, the protein was unfolded (i.e., U_A) at any temperature. With an increase in NaTCA concentration, the CD intensity at 20 °C increased, indicating formation of the molten globule state. The salt-induced stabilization of the molten globule

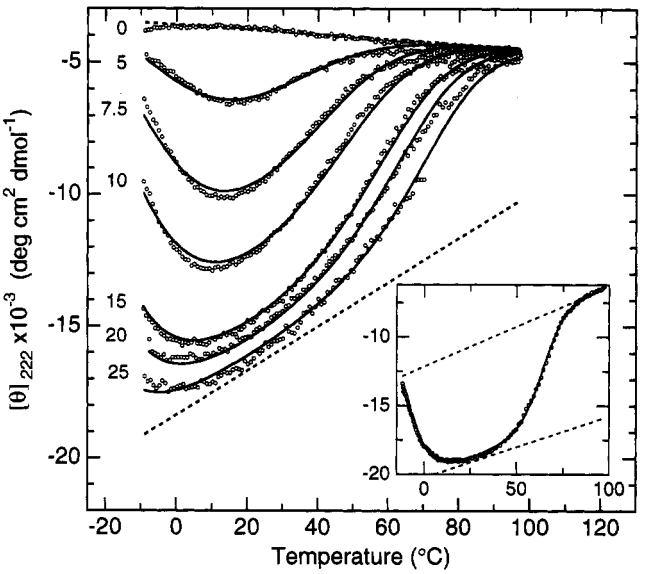

FIGURE 2: The heat- and cold-denaturation processes of the molten globule state of apomyoglobin. The dependence on temperature of the ellipticity at 222 nm was measured in the presence of various concentrations of NaTCA at pH 2.0 (10 mM HCl). The numbers refer to the NaTCA concentration in millimolar units. The small circles are the observed signals, and the solid lines are theoretical lines calculated with the base lines shown by the dashed lines and eqs 3–6. The parameters used for the theoretical lines and their fitting errors were T_0 = 20 °C, $\Delta H_U(T_0)$ = 5.4 ± 0.1 kJ mol^{-1}, $\Delta C_{p,U}$ = 1.80 ± 0.02 kJ mol^{-1}K^{-1}, and $\Delta S_U(T_0)$ = 30.0 ± 0.4, 19.9 ± 0.4, 13.1 ± 0.4, 3.5 ± 0.4, –0.6 ± 0.4, and –7.2 ± 0.5 J mol^{-1} K^{-1} with increasing concentrations of NaTCA. The inset shows the heat and cold denaturation of the native state of apomyoglobin in 10 mM sodium acetate buffer at pH 5.9. The parameters used for the theoretical line were T_0 = 65 °C, $\Delta H_U(T_0)$ = 159 ± 1 kJ mol^{-1}, $\Delta C_{p,U}$ = 4.12 ± 0.02 kJ mol^{-1} K^{-1}, and $\Delta S_U(T_0)$ = 473 ± 2 J mol^{-1} K^{-1}.

state is a highly cooperative process with a midpoint concentration of 7.7 mM NaTCA (Goto et al., 1990b), and in the presence of 15 mM NaTCA at pH 2, 90% of the molecules were transformed to the molten globule state.

Remarkably, the MG(TCA$^-$) was unfolded in the lower temperature region below 5 °C (i.e., cold denaturation) in addition to the higher temperature region (i.e., heat denaturation). As can be seen, both the heat- and cold-denaturation processes were relatively cooperative. Whereas the cold denaturation was fully reversible, the heat denaturation shown here was about 80% reversible. A similar pattern was obtained with the NaCl-stabilized molten globule state at pH 2 (data not shown).

Cold denaturation arises from the negative enthalpy of unfolding and is evidence that $\Delta C_{p,U}$ is positive and significant (Privalov & Gill, 1988; Oobatake & Ooi, 1993). In fact, the exact thermodynamics of cold denaturation were first reported for the native state of sperm whale myoglobin (Privalov et al., 1986) and later for the native state of sperm whale apomyoglobin (Griko et al., 1988). For comparison, the inset of Figure 2 shows the heat- and cold-denaturation processes of the native state of apomyoglobin in 10 mM sodium acetate buffer at pH 5.9.

It is important to emphasize that the cold denaturation we observed is not of the native state, but of the molten globule state. The pH- and salt-dependent phase diagram for acidic conformational states of apomyoglobin indicates that, at pH 2, the salts stabilize the molten globule state [see Figure 2 of Goto and Fink (1990)]. The native state is stable above pH 5. Thus, it is clear that the cold denaturation at pH 2 in the presence of salt represents the unfolding of the molten globule state. In addition, by a comparison of the transitions measured

by CD at pH 2 and that at pH 5.9 (Figure 2, inset), it is seen that the heat- and cold-denaturation processes at pH 2 are less cooperative than that at pH 5.9, indicating that the conformational transition at pH 2 is distinct from that at pH 5.9.

Mechanism of the Conformational Transition. The mechanism responsible for the conformational change between the molten globule state and the more substantially unfolded state is controversial (Ptitsyn, 1992; Yutani et al., 1992; Goto et al., 1993; Shimizu et al., 1993; Griko et al., 1994). The thermal- and denaturant-induced unfolding transitions of the molten globule state of cytochrome *c* are well approximated by a two-state transition between the molten globule state and the unfolded state (Goto et al., 1993; Hagihara et al., 1994). On the other hand, the thermal transitions of other molten globule states, including that of α-lactalbumin, suggest that the molten globule state, within its macroscopic state, disorders gradually with an increase in temperature (Kuwajima, 1989; Yutani et al., 1992; Griko et al., 1994). It is important to notice that the denaturant-induced unfolding of the molten globule state of α-lactalbumin is cooperative (Kuwajima, 1989), although Shimizu et al. (1993) reported that it is not a cooperative two-state process.

The statistical mechanical theories predicted that the conformational transition of the denatured proteins involves properties of both the first-order phase transition (i.e., a two-state transition) and the gradual structural disordering (Ikegami, 1977; Alonso et al., 1991). The theories suggested that the apparent transition may change from the first-order phase transition to the gradual structural change depending on the solvent conditions.

The apparent cooperativity for the heat denaturation of the molten globule state was less than that for the native state of apomyoglobin (Figure 2, inset) or that for the molten globule state of cytochrome *c* (Potekhin & Pfeil, 1989; Kuroda et al., 1992; Hagihara et al., 1994). However, the cooperativity is evidently higher than that for the molten globule state of α-lactalbumin measured by CD (Kuwajima et al., 1985). Therefore, we analyzed the heat- and cold-denaturation processes of the molten globule state measured by CD in terms of a minimal two-state transition mechanism between the molten globule (MG) and acid-unfolded (U_A) states:

$$MG \rightleftharpoons U_A \tag{2}$$

The $\Delta G_U(T)$, $\Delta H_U(T)$, and $\Delta S_U(T)$ values at temperature T can be represented by the respective standard thermodynamic equations (Privalov & Gill, 1988; Pace, 1990):

$$\Delta G_U(T) = \Delta H_U(T) - T\Delta S_U(T) \tag{3}$$

$$\Delta H_U(T) = \Delta H_U(T_0) + \Delta C_{p,U}(T - T_0) \tag{4}$$

and

$$\Delta S_U(T) = \Delta S_U(T_0) + \Delta C_{p,U} \ln(T/T_0) \tag{5}$$

where T_0 is a reference temperature. $\Delta G_U(T)$ is related to the observed ellipticity, $[\theta]$, by

$$\Delta G_U(T) = -RT \ln K_U = -RT \ln[([\theta] - [\theta]_M)/([\theta]_U - [\theta])] \tag{6}$$

where K_U is the equilibrium constant for the unfolding, R is the gas constant, and $[\theta]_M$ and $[\theta]_U$ are the base-line ellipticities for the molten globule and unfolded states, respectively. We assumed that the enthalpy function is common to the transition curves in the presence of various concentrations of salt and, therefore, that the difference in the transition curves arises from the difference in $\Delta S_U(T_0)$ (Privalov et al., 1986).

Since each transition curve was broad, the curve fitting to each observed transition can produce significant errors of the respective fitting parameters. In addition, whereas we could define the base line for the unfolded state ($[\theta]_U$), which was assumed to be equal to the CD intensity in the absence of salt at pH 2, the base line for the molten globule state ($[\theta]_M$) was not explicit. Therefore, the molten globule base line, which was assumed to be linear, was also included in the fitting parameters. Then, all of the observed curves were fitted with eqs 3–6 simultaneously to obtain a set of molten globule base line, $\Delta H_U(T_0)$, $\Delta C_{p,U}$, and various $\Delta S_U(T_0)$ values. With a least-squares curve-fitting program, we determined a common base line for the molten globule state as shown in Figure 2A and the $\Delta H_U(T_0)$ and $\Delta C_{p,U}$ values to be 5.4 ± 0.1 kJ mol^{-1} at 20 °C and 1.80 ± 0.02 kJ mol^{-1} K^{-1}, respectively. The $\Delta S_U(T_0)$ values are shown in the caption of Figure 2. As can be seen, a set of parameters quantitatively reproduces the CD signals observed over the wide ranges of temperature and salt concentration, confirming the validity of the two-state approximation. A similar $\Delta C_{p,U}$ value (1.50 ± 0.02 kJ mol^{-1} K^{-1}) was also obtained from the analysis of the heat- and cold-denaturation processes of the molten globule state stabilized by NaCl at pH 2 (Table 1).

Heat Capacity Change of Other Conformational States. To compare the $\Delta C_{p,U}$ value of the molten globule states with those of the native state, we analyzed the heat- and cold-denaturation processes of the native state of apomyoglobin at pH 5.9 measured by CD (Figure 2, inset). We again assumed the two-state mechanism. In this case, we used the base lines for the native and unfolded states as shown in Figure 2. With a least-squares fitting program and T_0 = 65 °C, we determined the $\Delta H_U(T_0)$, $\Delta S_U(T_0)$, and $\Delta C_{p,U}$ values to be 159 ± 1 kJ mol^{-1}, 473 ± 2 J mol^{-1} K^{-1}, and 4.12 ± 0.02 kJ mol^{-1} K^{-1}, respectively.

We estimated the $\Delta C_{p,U}$ values for the unfolding of the native state of holomyoglobin in 10 mM sodium acetate buffer at pH 4.3 and that of the native state of apomyoglobin in 10 mM sodium acetate buffer at pH 5.3 on the basis of the DSC curves. The observed DSC curves (data not shown) were similar to those reported for sperm whale myoglobin (Privalov et al., 1986) and apomyoglobin (Griko et al., 1988) under the corresponding conditions. We also measured the DSC curve for the acid-unfolded state in 10 mM HCl at pH 2, which showed no heat absorption peak (data not shown) since the protein was unfolded at any temperature. We assumed that the heat capacities of the thermally unfolded states agree with that of the acid-unfolded state in the absence of salt at pH 2. Then, the $\Delta C_{p,U}$ values were estimated from the difference in heat capacities between the acid-unfolded state and the native states (Table 1). The $\Delta C_{p,U}$ value for the native state of myoglobin (8.7 kJ mol^{-1} K^{-1}) was similar to that reported for horse myoglobin (7.8 ± 0.5 kJ mol^{-1} K^{-1}; Kelly & Holladay, 1990) and was slightly smaller than that of sperm whale myoglobin (10.4 ± 0.7 kJ mol^{-1} K^{-1}; Privalov et al., 1986). The $\Delta C_{p,U}$ value for the native state of apomyoglobin (4.0 kJ mol^{-1} K^{-1}) was consistent with that estimated from the CD transition curve as described (4.12 kJ mol^{-1} K^{-1}) and was slightly smaller than that of sperm whale apomyoglobin (6.5 kJ mol^{-1} K^{-1}; Griko et al., 1988). We also tried to characterize the molten globule state of apomyoglobin with DSC, which will be described elsewhere (I. Nishii and Y. Goto, manuscript in preparation).

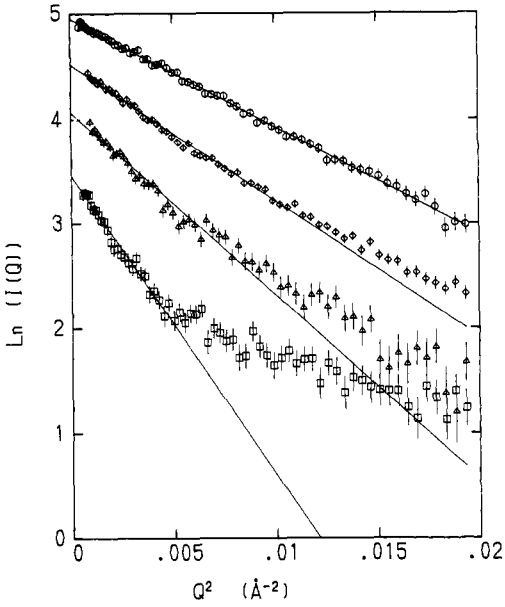

FIGURE 3: Guinier plots of the various conformational states of horse myoglobin and apomyoglobin at 20 °C: (○) native-state myoglobin at pH 6.0 (10 mM Hepes buffer); (◇) native-state apomyoglobin at pH 6.0 (10 mM Hepes buffer); (△) molten globule state stabilized by 20 mM NaTCA at pH 2.0 (10 mM HCl); (□) acid-unfolded state at pH 2.0 (10 mM HCl). For clarity, the values of each plot are shifted on the ln I axis. Protein concentrations were varied within the range of 2–10 mg mL^{-1}, and the scattering curves at infinite dilution are presented.

Comparison of the $\Delta C_{p,U}$ values for the different conformational states demonstrates quantitatively the contribution of hydrophobic interactions in stabilizing the molten globule states of apomyoglobin (Table 1). The $\Delta C_{p,U}$ values obtained for the molten globule states are substantial. However, they are much smaller than the values for the native states of holomyoglobin and apomyoglobin.

Small Angle X-ray Scattering. Comparison of the estimated $\Delta C_{p,U}$ value with other structural properties should provide a clearer image of the molten globule state and protein folding. Although the compactness of the protein molecule is an important property characterizing the degree of protein folding (Dill, 1990b; Dill & Shortle, 1991), direct measurement of the compactness during the process of protein folding has not been done. At present, solution X-ray scattering is probably the best technique for estimating the size and shape of a protein molecule in solution (Glatter & Kratky, 1982). We reported previously that SAXS analysis of horse cytochrome *c* distinguishes the native, molten globule, and unfolded states in terms of their R_g values and shapes (Kataoka et al., 1993).

Figure 3 shows the Guinier plot of the different conformational states of myoglobin and apomyoglobin, and Table 1 shows their R_g values. The R_g value of the native state of myoglobin is consistent with that (15.5 Å) predicted from the X-ray crystallographic structure. The native state of apomyoglobin is expanded by about 15% in comparison with holomyoglobin. The molten globule state of apomyoglobin is further expanded, yet it is compact when compared to that of the U_A state. The U_A state is more compact in comparison with the denaturant-unfolded states. Thus, the order of the sizes is consistent with eq 1.

A Profile for Protein Folding. On the basis of the structural and thermodynamic parameters shown in Table 1, we constructed a folding profile (Figure 4A). Folding transitions measured by the α-helical content, R_g, and $\Delta C_{p,U}$ were

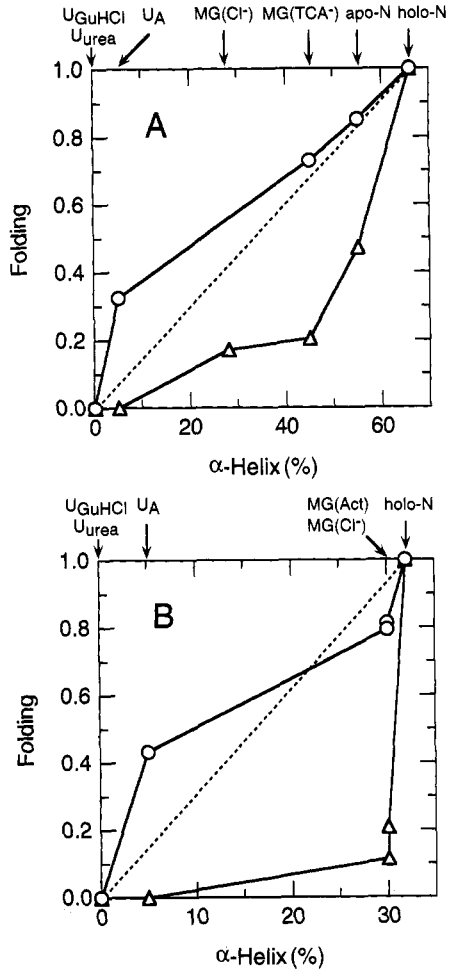

FIGURE 4: Correlation between the folding followed by R_g (○) and $\Delta C_{p,U}$ (△) and the α-helical content of horse myoglobin (A) and horse ferricytochrome *c* (B). The dashed straight line in each panel represents the formation of α-helices. On the abscissa, the conformational states corresponding to the respective points are indicated.

normalized from 0 to 1 by using the average values of U_{urea} and U_{GdnHCl} for the fully unfolded state and assuming that the values for the native state of myoglobin are for the fully folded state. The $\Delta C_{p,U}$ values of the U_A, U_{urea}, and U_{GdnHCl} states were assumed to be 0. Because the abscissa is scaled by the α-helical content, the dashed straight line represents the formation of α-helices. Whereas the decrease in R_g proceeds simultaneously with the formation of α-helices, a retardation of $\Delta C_{p,U}$ is evident.

We regard this retardation of $\Delta C_{p,U}$ as the most important point when considering the mechanism of protein folding. Protein folding is a process in which an expanded polypeptide chain attains maximal packing along with the formation of unique secondary and tertiary structures and hydrophobic interactions. The profile indicates that compaction proceeds almost in parallel with the acquisition of the secondary structure. The small tendency for the compaction to precede the formation of secondary structure in the early stages of protein folding may be consistent with a previous prediction (Chan & Dill, 1990). In contrast, the folding monitored by $\Delta C_{p,U}$ indicates that only a small fraction (10–20% of holoN) of the native-like hydrophobic interactions occur in the molten globule states.

It is important to emphasize that $\Delta C_{p,U}$ of the molten globule states is small but not zero. At the later stages of folding, expulsion of solvent occurs with concomitant tight packing of the side chains, and this results in the substantial increase of

Table 2: Structural and Thermodynamic Parameters of the Various Conformational States of Horse Ferricytochrome *c* at 20 °C[a]

	$-[\theta]_{222}$ (deg cm^2 dmol^{-1})	f_H (%)	R_g (Å)	$\Delta C_{p,U}$ (kJ mol^{-1} K^{-1})
N	12 000	32	13.5 ± 0.1	5.27 ± 0.91[b]
MG(Cl$^-$)	11 500	30	17.4 ± 0.1	0.56[c]
MG(Act)	11 500	30	17.0 ± 0.1	1.57 ± 0.14[b]
U_A	3900	5	24.2 ± 0.3	
U_{urea}	1000	~0		
U_{GuHCl}	1000	~0	32.4 ± 1.6	

[a] Data for $[\theta]_{222}$, f_H, and R_g were taken from our previous studies (Goto et al., 1990b; Goto & Nishikiori, 1991; Kataoka et al., 1993). [b] Determined from the dependence on temperature of ΔH_U measured by CD under various pH conditions (Hagihara et al., 1994). [c] Potekhin and Pfeil (1989).

$\Delta C_{p,U}$ to the level of that of the native state of myoglobin. The folding profile indicates that the compact molten globule state is composed of many units of secondary structure which are largely exposed to solvent. A small increase in R_g compared to the native state results in a large increase in volume, which provides room for the internal water molecules. Whereas the formation of the hydrophobic cores (Dobson, 1992; Ptitsyn, 1992) is one of the important characteristics of the molten globule state, the present results suggest that their size and stability would be much less than that of the native state.

If the structure of the molten globule state is known or modeled, then $\Delta C_{p,U}$ can be predicted. Haynie and Freire (1993) estimated $\Delta C_{p,U}$ of the molten globule state of sperm whale apomyoglobin to be 3.64 ± 0.54 kJ mol^{-1} K^{-1} under the assumptions that the folded regions (helices A, G, and H) are in a structural arrangement identical to that of the native state, that the helices are tightly packed, and that the remainder of the molecule is unfolded. They also suggested, however, that because the interactions of this subdomain are much less specific in the molten globule state than in the native state, a somewhat smaller $\Delta C_{p,U}$ should be expected experimentally. The present experimental results indicate that the hydrophobic interactions in the molten globule state are much less than those expected from the rigid structure consisting of the native-like A, G, and H helices.

Folding Profile of Cytochrome c. The generality of the profile of protein folding obtained with myoglobin can be validated with cytochrome *c*. Horse cytochrome *c* contains 30% α-helices and has no β-sheet structure. Whereas it is unfolded at pH 2 in the absence of salt, it assumes a molten globule state in the presence of 0.4 M NaCl (Ohgushi & Wada, 1983; Goto et al., 1990a,b). Goto and Nishikiori (1991) have shown that the molten globule state is also stabilized at pH 2 by the removal of charge repulsion through the acetylation of amino groups. Table 2 shows structural and thermodynamic parameters for the various conformational states of cytochrome *c* taken from previous reports. Figure 4B shows a folding profile of horse cytochrome *c* constructed by the same normalization procedure as that used for myoglobin and apomyoglobin.

The molten globule state of cytochrome *c* is different from that of apomyoglobin in that its α-helical content is essentially the same as that of the native state. It is clear that the folding profile of cytochrome *c* is very similar to that of myoglobin. The folding measured by $\Delta C_{p,U}$ is again retarded significantly with respect to that measured by other parameters, and increases sharply at the final stage of protein folding, i.e., at the conversion from the molten globule to the native state.

Conclusion. Although the compact molten globule state with significant secondary structures has been identified as a major intermediate of protein folding, we have no definitive view about the relationship between the compactness, the secondary structure, and the hydrophobic interactions during the process of protein folding. We propose that the folding profile obtained with myoglobin and cytochrome *c* will be common to many globular proteins. Although the $\Delta C_{p,U}$ of the molten globule states is *not zero*, its amplitude is much less than that of the native states. In protein folding, the protein molecule becomes more compact with formation of secondary structure, but does not form substantial hydrophobic interactions until the final stages of folding. A slightly larger R_g for the molten globule state than for the native state provides a space for the internal water molecules. The highest energy barrier for protein folding, or in other words the rate-limiting step, is located between the molten globule and native states. During the rate-limiting transition from the molten globule to the native state, expulsion of the internal water molecules occurs with concomitant tight packing of the protein interior. This process would be largely responsible for acquiring the hydrophobic interactions and establishing the cooperativity of protein folding.

ACKNOWLEDGMENT

We thank A. L. Fink, University of California, for the valuable suggestion of using NaTCA and Y. Hagihara and K. Shiraki, Osaka University, for performing some of the experiments.

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