Conformational Heterogeneity and Stability of Apomyoglobin Studied by Hydrogen/Deuterium Exchange and Electrospray Ionization Mass Spectrometry†

Fang Wang and Xue-jun Tang*

Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: The solution conformations and stability of apomyoglobin (apo-Mb), at both neutral and acidic pH, have been investigated by analyzing charge state distributions observed in the mass spectra, and by on-line monitoring of the hydrogen/deuterium (H/D) exchange using electrospray ionization mass spectrometry (ESI-MS) in combination with circular dichroism (CD). The results demonstrate that the conformation of apo-Mb, which lacks the heme group, is considerably less stable than that of holomyoglobin in identical solution conditions at neutral pH. ESI-MS shows that apo-Mb in the buffered solution at pH 7 (native state) and at pH 4.3 (intermediate state) yields two distinct charge state distributions that presumably correspond to different protein conformations. Both conformations have the same H/D exchange rate. This provides strong evidence that both the native and the intermediate states of apo-Mb have highly dynamic structures, consisting of two or more rapidly interconverting conformations rather than single fixed conformations. However, the H/D exchange rate of the acid-induced compact state of apo-Mb at pH ∼2 [Goto, Y., Calciano, L. J., & Fink, A. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573−577] indicates that it has a stable, partially folded conformation. Although CD data suggest that apo-Mb in H_2O at pH 6 and in the buffered solution at pH 7 has a native-like secondary structure, the charge state distribution and the H/D exchange rate measurements indicate that a large portion of the apo-Mb molecules are unfolded or partially unfolded under these conditions. Thus, conformational information obtained from ESI-MS measurements of the charge state distributions and H/D exchange rates is complementary to that obtained from CD measurements. The combination of these three measurements can be used to assess the conformational stability and conformational heterogeneity of a protein.

The process by which a protein folds from a linear chain of amino acid residues into its biologically active three-dimensional structure is still not well understood. A major experimental approach to understand the folding process has been the structural characterization of partially folded intermediates in the folding pathway. Detection and characterization of the partially folded intermediates have been difficult due to the cooperative nature of a two-state transition between native and denatured states, such that these intermediates exist only transitorily in the course of folding. Recently, however, it has been shown that several globular proteins including myoglobin and apomyoglobin, when placed under certain denaturing conditions (e.g., extreme acidic pH or moderate denaturant concentration), can exist in partially folded equilibrium states with properties similar to those of a molten globule, a protein state that is compact with native-like secondary structure but a largely unfolded tertiary structure (Ohgushi & Wada, 1983; Ptitsyn, 1987; Kuwajima, 1989; Kim & Baldwin, 1990; Goto et al., 1990a,b, 1994). The partly folded equilibrium states are currently the subject of considerable interest because of their possible roles as intermediate states in protein folding (Ptitsyn, 1987, 1990; Kuwajima, 1989; Kim & Baldwin, 1990; Dobson, 1992; Jennings & Wright, 1993; Barrick & Baldwin, 1993a). Various experimental techniques, including circular dichroism (CD),[1] nuclear magnetic resonance (NMR) monitoring of hydrogen/deuterium (H/D) exchange, fluorescence, viscosimetry, and calorimetry, have been employed to study protein conformational changes and partially folded molten globular states.

The recent advent of electrospray ionization mass spectrometry (ESI-MS) (Fenn et al., 1989; Smith et al., 1991) has made it possible not only to measure accurate molecular weights of proteins but also to study protein conformations in solution. The main feature of ESI of proteins and peptides is the production of a coherent series of multiply charged gas-phase ions from a protein solution (Fenn et al., 1989; Smith et al., 1991). A typical positive ESI mass spectrum of a protein is characterized by a distribution of peaks that correspond to different charge states of the same intact protein. The net positive charge on proteins observed in a given solution is determined by several factors including the number, distribution, and pK_a's of ionizable amino acid residues as well as the conformational state of the protein, which affects ionization of these residues. The conformation of a protein has a significant effect on the charge state distribution observed in the positive ESI mass spectrum. Denatured proteins exhibit considerably higher average

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* To whom correspondence should be addressed.

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[1] Abbreviations: ESI, electrospray ionization; MS, mass spectrometry; CD, circular dichroism; NMR, nuclear magnetic resonance; H/D, hydrogen/deuterium; apo-Mb, apomyoglobin; holo-Mb, holomyoglobin; TCA, trichloroacetate.

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charge states than the corresponding native proteins. This may be because some of the basic groups (especially histidine) buried or involved in interactions, such as those involved in salt bridges with acidic residues (Guevremont et al., 1992; Creighton, 1993) in the native protein, become accessible for protonation in the denatured form, leading to a greater number of charged residues. In addition, the extended conformation of a denatured protein has a larger surface area interacting with the surface of a charged droplet than the compact native conformation, resulting in an increase of the total number of net charge in the ESI process (Fenn, 1993). It has been shown that the charge state distributions observed in the positive ESI mass spectra of proteins reflect, to some extent, the degrees of protonation of proteins in solution, and hence can be used to probe protein conformational changes in solution (Katta & Chait, 1991; Loo et al., 1991; LeBlanc et al., 1991; Guevremont et al., 1992; Mirza et al., 1993; Feng & Konishi, 1993; Smith & Zhang, 1994).

H/D exchange in combination with NMR has proven to be a useful technique in monitoring protein conformational changes (Englander & Mayne, 1992). It is well established that the H/D exchange rates of labile hydrogens in a protein can vary dramatically, and depend on the protein conformational state (Woodward et al., 1982; Englander & Kallenbach, 1984; Englander & Mayne, 1992). For a protein in completely unfolded states, the exchange rates are similar to those in small peptides, and the rates depend mainly on chemical and physical parameters of the environment, such as solution pH, temperature, and content of organic solvents (Englander & Kallenbach, 1984; Englander & Mayne, 1992; Englander & Poulsen, 1969; Englander et al., 1979, 1985; Creighton, 1990). However, the H/D exchange rates are much slower, by many orders of magnitude, in the tightly folded native state. This has been explained in terms of restricted solvent accessibility to the slowly exchanging hydrogens (Woodward et al., 1982). Intrinsically labile hydrogens buried in a native protein are shielded from solvent and are not available for exchange. As the protein structure fluctuates, slow exchange takes place when the buried sites are occasionally exposed to solvent through transient channels. An alternative explanation is that the slowly exchanging hydrogens are involved in stable hydrogen bonds. In the native states of a protein, amide hydrogens involved in hydrogen bonds that form the secondary and tertiary structures are protected from exchange. Exchange of the protected hydrogens occurs via transient hydrogen-bond breakage, referred to as local unfolding (Englander & Kallenbach, 1984; Englander & Mayne, 1992), and exhibits far slower rates than those for hydrogens freely exposed to solvent. The difference in H/D exchange rates between structurally involved and freely exposed amide hydrogens has been explored in structural characterization of partially folded proteins using NMR (Roder et al., 1988; Baum et al., 1989; Hughson et al., 1990; Jeng et al., 1990; Englander & Mayne, 1992; Baldwin, 1993; Jennings & Wright, 1993).

ESI-MS permits the accurate and precise measurement of protein molecular mass (uncertainties $\leq 0.01\%$) using picomolar quantities (Smith et al., 1991; Chait & Kent, 1992), which has made H/D exchange in combination with ESI-MS an attractive choice for protein conformational studies because the ESI mass spectra exhibit both charge state distributions and the extent of H/D exchange. Recently, several reports have shown the utility of H/D exchange followed by ESI-MS measurements in probing protein conformational changes (Katta & Chait, 1993; Wagner & Anderegg, 1994; Johnson & Walsh, 1994), in detecting transient protein folding intermediates (Miranker et al., 1993), and in detecting protein conformational changes upon ligand binding (Anderegg & Wagner, 1995). These studies have demonstrated that the H/D exchange and ESI-MS measurements of whole proteins can be a sensitive probe of protein global conformation, complementary to that obtained by CD (Wagner & Anderegg, 1994) and NMR (Miranker et al., 1993; Anderegg & Wagner, 1995). The speed of several seconds per spectrum, the micromolar sensitivity, and its ability to continuously monitor the exchange behavior are additional advantages of the ESI-MS analysis.

Apo-Mb at pH 4−5 has been shown to exist as a stable partly folded intermediate, detected in the acid-induced unfolding pathway of this protein (Griko et al., 1988; Hughson et al., 1990; Griko & Privalov, 1994). The intermediate was described as having characteristics of a "molten globule", based on the H/D exchange and NMR measurements (Hughson et al., 1990). The structure was later found to be similar to the earliest detectable kinetic folding intermediate investigated by pulsed H/D exchange and NMR measurements (Jennings & Wright, 1993). Apo-Mb was also found to adopt a partially folded conformation with characteristics of a "molten globular" state in strongly acidic solutions that contain specific concentrations of anions (Goto et al., 1990a,b; Goto & Fink, 1994). These findings provide an opportunity to study the structures of partially folded proteins and the equilibrium molten globule state. Considerable efforts have been made to characterize the partly folded and native states of apo-Mb using a variety of techniques including CD, NMR, fluorescence, viscosimetry, and calorimetry (Griko et al., 1988; Griko & Privalov, 1994; Goto et al., 1990a,b; Goto & Fink, 1994; Hughson et al., 1990; Barrick & Baldwin, 1993a,b; Lin et al., 1994; Sirangelo et al., 1994) and recently ESI-MS (Johnson & Walsh, 1994). Despite these many studies, there are conflicting reports in the literature regarding the partially folded and the native states of apo-Mb. Relative to holo-Mb, apo-Mb has been shown to retain an extended hydrophobic core (Griko et al., 1988; Cocco & Lecomte, 1994), to contain ~20% less helical structure (Goto & Fink, 1994; Lin et al., 1994), to be less compact (Griko et al., 1988) and less stable (Griko et al., 1988; Goto & Fink, 1994), with lower heat capacity (Griko & Privalov, 1994), and to unfold less cooperatively (Goto & Fink, 1994). The native state of apo-Mb was proposed to have molten globular characteristics based on CD and fluorescence studies of mutant apo-Mbs (Lin et al., 1994). The NMR study of H/D exchange behavior of the native apo-Mb suggested, however, that the structure of native apo-Mb is similar to that of holo-Mb, based on the pattern of protection of a selected set of amide hydrogens (Hughson et al., 1990). Therefore, the structure of the native state of apo-Mb, and its relationship to the molten globular states of the partly folded intermediate at pH ~4.2 (Hughson et al., 1990; Griko & Privalov, 1994) and the acid-induced compact state at pH ≤ 2 (Goto et al., 1990a,b; Goto & Fink, 1994), remains unclear.

In this investigation, we use H/D exchange and ESI-MS in combination with CD to probe the conformations of apo-Mb and holo-Mb. Our results suggest that apo-Mb is

considerably less stable than holo-Mb and, depending on buffer conditions, consists of a set of different conformation states which undergo rapid interconversion. Similar characteristics were found for the partially folded intermediate in the acid unfolding pathway of the native state, which is different from the acid-induced compact state (Goto et al., 1990a,b, 1994) that appears to be a stable, partially folded state. The conformational information from ESI-MS measurements of charge state distributions and H/D exchange rates is complementary to that obtained from CD measurements. A combination of these three measurements permits detection of not only the conformational changes but also the conformational stability and heterogeneity of protein states.

MATERIALS AND METHODS

Materials. Horse heart myoglobin was purchased from Sigma Chemical (St. Louis, MO). Purity was confirmed by HPLC with UV and ESI mass spectrometry detection, and the protein was used without further purification. Apo-Mb was prepared by 2-butanone extraction of heme (Hapner et al., 1968). The deuterated solvents D_2O (99.9%) and acetic acid-d_4 (99.5%) were obtained from Sigma. DCl, CCl_3COOND_4, and CH_3COOND_4 were prepared by deuterium exchange of the nondeuterated chemicals in D_2O solvent. All other chemicals were of reagent grade.

Electrospray Ionization Mass Spectrometry. All mass spectra were acquired using an API-III triple quadrupole mass spectrometer equipped with an atmospheric pressure ionization (API) source and a SCIEX ionspray (pneumatically assisted electrospray) interface (PE-SCIEX, Thornhill, Ontario, Canada). A Macintosh Quadra 950 computer was used for instrument control, data acquisition, and data processing. The ESI-MS samples were prepared by first lyophilizing prequantified aliquots of proteins to dryness in an Eppendorf tube and then adding appropriate solvents to give a final concentration of 5 μM. For ESI-MS measurements of H/D exchange rates, the lyophilized proteins were first dissolved in deionized H_2O with or without ammonium acetate buffer, and the H/D exchange was then initiated by diluting the 500 μM solution 100-fold into D_2O with or without buffers. The sample solutions were immediately introduced into the spectrometer at flow rates of 4−10 μL/min using a Harvard Model 22 syringe pump (Harvard Apparatus, South Natick, MA). In the low-temperature (0−4 °C) H/D exchange experiments, the infusion syringe was kept cold by wrapping it with an ice bag. The enclosed ionization chamber was maintained at room temperature (∼21 °C) and atmospheric pressure. High-purity nitrogen was used as the nebulizing gas to prevent back-exchange of deuterium by hydrogen in laboratory air during the ionspray process.

The mass scale of the spectrometer was first calibrated with polypropylene glycol and then tuned and recalibrated using peaks from multiply protonated myoglobin. For most of the experiments, the mass resolution of the spectrometer was tuned to give a constant peak width of 1 Da (full width at half-maximum) across the mass range of interest. For full scan (m/z 600−2400) mass spectra of the proteins, typically 10 scans of 10−20 s duration were acquired and added to yield a mass spectrum. For H/D exchange rate measurements, the time course of mass shift of protein molecular ions of several different charge states was selected for monitoring. Several mass windows 25−30 Da wide were scanned repetitively, each using a step size of 0.1 Da and a total scan time of 3−4 s. The molecular mass at each time point was calculated from the measured m/z (mass-to-charge) ratios and the predetermined charge states. At each time point, the mass difference between the deuterated protein and the nondeuterated protein gives the average number of deuterium atoms incorporated into the protein. These average numbers of incorporated deuterium atoms were plotted against time to give the exchange curves.

Circular Dichroism Spectroscopy. All CD spectra were recorded using a Jasco Model J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), at protein concentrations of 5 μM, using quartz cells with a 1 mm path length. The measured ellipticity data were averaged and converted into mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = \theta/10nCl$, where θ is the measured ellipticity in millidegrees, l is the path length of the cell in centimeters, C is the concentration (moles per liter) of the protein, and n is the number of its residues. The CD data were plotted as mean residue ellipticity $[\theta]$ (in degrees centimeter squared per decimole) versus wavelength, in 0.5 nm steps.

pH was measured using a Corning 340 pH meter at room temperature.

Unless indicated otherwise, the CD spectra, the ESI mass spectra, and the H/D exchange curves presented below were measured at room temperature (∼21 °C).

RESULTS AND DISCUSSION

CD Spectra of Holo- and Apo-Mb. CD spectroscopy is an established method for probing the conformational changes of proteins in solution. The technique is sensitive to the secondary structure of proteins and is often used to quantify the relative proportions of α-helicity, β-sheet, and random coil (Chen et al., 1974; Johnson, 1988). Myoglobin mainly consists of eight α-helices that assemble to form a hydrophobic pocket for the heme group. Experiments were designed to compare the results obtained from H/D exchange and ESI-MS measurements with those from CD spectra measured at the same solution conditions. Figure 1 shows the CD spectra of holo-Mb and apo-Mb in deionized water at pH 6.0, apo-Mb in 10 mM HCl at pH 2, and with the addition of ammonium trichloroacetate (NH_4TCA) in five concentrations, respectively. Analysis of the CD spectrum of holo-Mb in H_2O shows a significant amount of secondary structure, with a mean residue ellipticity at 222 nm of −22 354, indicating that a substantial proportion of the residues are in the α-helical conformation. The CD spectrum of apo-Mb in H_2O is similar to that of holo-Mb in H_2O, with a mean residue ellipticity at 222 nm of −16 275, indicating that ∼73% of the helical structure remains in apo-Mb. This finding is consistent with the CD data for holo- and apo-Mb reported by others (Goto & Fink, 1994, Lin et al., 1994). The CD spectrum of apo-Mb in 10 mM HCl at pH 2 indicates a complete loss of α-helicity, suggesting that the protein was in a maximally unfolded state in agreement with earlier CD measurements of apo-Mb (Goto et al., 1990a). The addition of NH_4TCA salt to the acid-unfolded protein solution induced a refolding transition. The conformational transition is probably caused by the preferential binding of anions to the acid-unfolded protein. The NH_4TCA salt was used here because the TCA anion was found to be much more effective

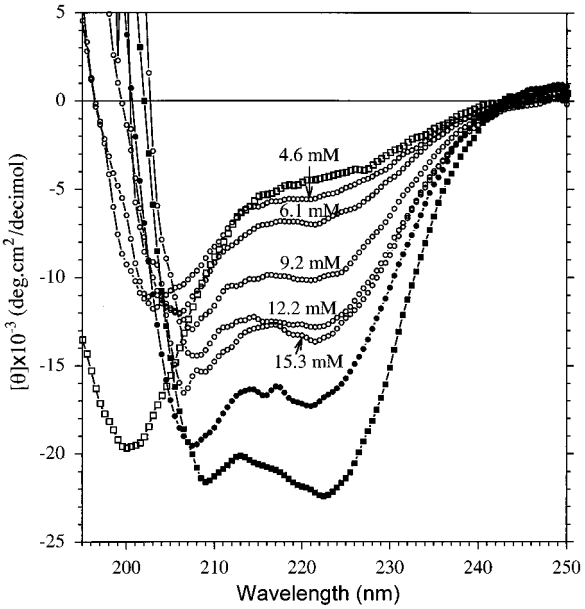

FIGURE 1: The CD spectra of holo-Mb (■) and apo-Mb (●) in deionized H_2O at pH 6, and apo-Mb in 10 mM HCl at pH 2 (□), and in 10 mM HCl with addition of 4.6, 6.1, 9.2, 12.2, and 15.3 mM CCl_3COONH_4 at pH ~2 (○), respectively. The spectra were acquired at room temperature (~21 °C), for protein concentrations of 5 μM.

in inducing the refolding transition than some of the simpler anions such as chloride (Goto et al., 1990b; Tang & Wang, 1995). The CD spectra of apo-Mb at pH 2 with NH_4TCA indicate considerable amounts of secondary structure. The α-helical content increased with the concentration of NH_4-TCA, and reached a plateau at about 15.3 mM NH_4TCA, where the ellipticity at 222 nm of −13 553 indicates a helical content of 83% of apo-Mb in H_2O. This result is qualitatively consistent with the findings of Goto et al. (1990b).

ESI-MS Measurements of Charge State Distributions of Holo- and Apo-Mb. ESI mass spectra of holo- and apo-Mb were recorded under various solution conditions in order to obtain charge state distributions of the proteins. Figure 2 shows the ESI mass spectra of holo-Mb recorded using the same instrumental conditions but with different solution conditions. Holo-Mb dissolved in deionized water at pH 6.0 (Figure 2a) produces ions corresponding to the intact holo-Mb, a heme plus globin noncovalent complex (MW = 17 567), with the majority of ions observed at charge states of +8 and +9 consistent with similar observations for native holo-Mb (Feng & Konishi, 1993). The spectrum obtained from a 10 mM HCl solution at pH 2 (Figure 2b) gave a wide charge distribution with the mean charge[2] increasing substantially to +19.5 ± 0.3 (mass-to-charge *m*/*z* ratio decreasing). The protein ions recorded in this spectrum correspond to the apo-Mb (MW = 16 951), as expected for an unfolded protein in denaturing solution conditions. Figure 2c shows the spectrum of holo-Mb in 10 mM HCl with addition of 15 mM NH_4TCA, at pH 2. The protein ions correspond to apo-Mb with a mean charge of +17.0 ± 0.2, lower by 2.5 charge units than that measured in 10 mM HCl. The charge reduction in acidic solutions containing TCA anions is likely due to strong charge neutralization caused by the TCA

[2] The mean charge was calculated from the observed charge state distribution in the ESI mass spectrum by Gaussian curve-fitting a plot of ion abundance versus charge.

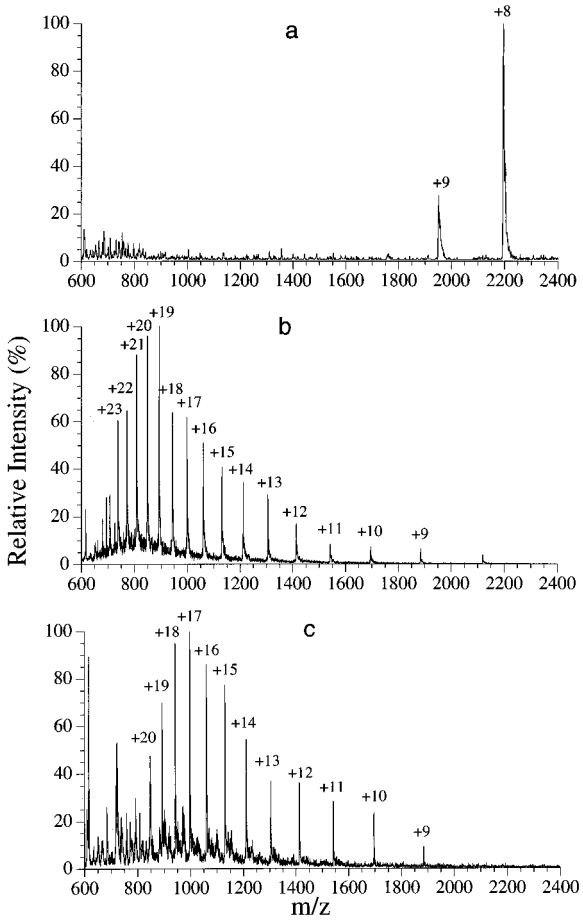

FIGURE 2: Positive ion ESI mass spectra of holo-Mb in deionized H_2O at pH 6 (a), in 10 mM HCl at pH 2 (b), and in 10 mM HCl with addition of 15 mM CCl_3COONH_4 at pH ~2 (c). Protein concentration was 5 μM. The labels on the peaks indicate the protonation (charge) states of the protein.

anions, rather than to conformational changes of the protein. Similar effects have been observed for small peptides that are not expected to have high-order structure (Mirza & Chait, 1994).

At strong acidic pH, ESI mass spectra of apo-Mb are essentially the same as those of holo-Mb. At neutral pH, however, the spectra are quite different. The ESI mass spectrum obtained from apo-Mb in H_2O at pH 6.0 (Figure 3a) gave a broad charge distribution with a mean charge of +19.2 ± 0.2, resembling closely the charge distribution of holo-Mb in 10 mM HCl solution (Figure 2b) where the protein is essentially unfolded. The large difference between the charge state distributions observed for apo-Mb and holo-Mb, in H_2O at pH 6, suggests that their conformational states are different and that apo-Mb in H_2O is not as tightly folded as holo-Mb. The high charge state distribution observed for apo-Mb in H_2O suggests that more basic side chains, originally buried or involved in intramolecular interactions in holo-Mb, become accessible for protonation in apo-Mb, or possibly that the less compact conformation of apo-Mb allows higher charge states than holo-Mb in the ESI process (Fenn, 1993). Since the CD spectrum indicates that apo-Mb in H_2O still has ~80% helical structure, the high charge state observed in the ESI spectrum may reflect its unfolded tertiary structure and an extended conformation.

One way to increase the degree of folding of a protein is to increase the ionic strength of the solution by adding

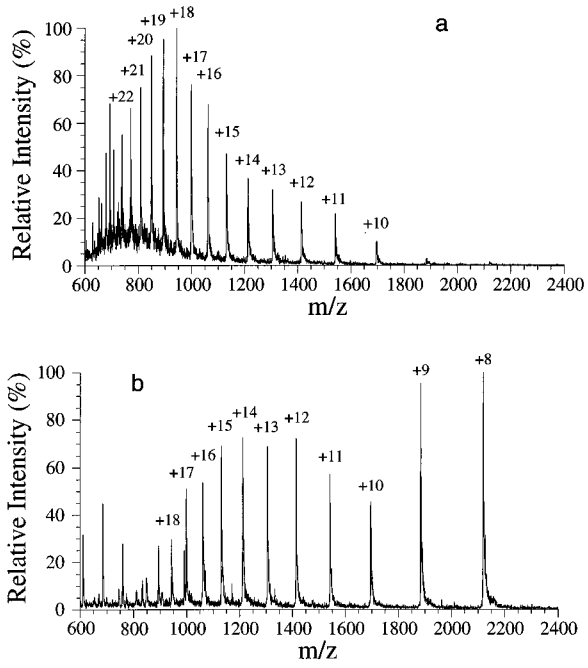

FIGURE 3: Positive ion ESI mass spectra of apo-Mb in deionized H_2O at pH 6 (a) and in 10 mM CH_3COONH_4 at pH 7 (b). Protein concentrations were 5 μM. The labels on the peaks indicate the protonation (charge) states of the protein.

buffers. We have measured the ESI mass spectra of holo- and apo-Mb in 10 mM ammonium acetate at pH 7. The charge state distribution of holo-Mb observed using the buffered solution (spectrum not shown) is basically the same as that from H_2O (Figure 2a), with the majority of ions at charge states of +8 and +9 reflecting a tightly folded structure in both conditions. The ESI mass spectrum of apo-Mb obtained from the buffered solution (Figure 3b) is significantly different from that obtained from the H_2O solution (Figure 3a). It shows two distinct charge state distributions (a bimodal distribution) with a lower average charge state, consistent with a higher pH of the buffered solution. One distribution ranges from +10 to +20 with a mean charge of $+13.5 \pm 0.2$, and the other consists of the +8 and +9 charge states similar to the distribution observed for holo-Mb (Figure 2a). The observation of two distinct charge state distributions suggests that there may be two coexisting conformations of apo-Mb in the buffered solution, with the more compact native states appearing at the low charge states (high *m*/*z*) and a loose denatured state represented by the distribution at the higher charge states (low *m*/*z*).

ESI-MS Measurements of H/D Exchange Rates of Holo-Mb and Apo-Mb at Acidic and Neutral pH. Measurements of ESI mass spectra of holo-Mb and apo-Mb under different solution conditions provided information on masses and charge state distributions of the proteins, which revealed information regarding protein conformational states complementary to that obtained from the CD spectra. Deuterium exchange rates are expected to be sensitive to both secondary and tertiary structures. It should be pointed out that, although H/D exchange can be measured by both NMR and ESI-MS, the information produced by these two techniques is complementary (Miranker et al., 1993). The NMR method monitors the average exchange at individual amide groups, which provides information on the detailed folding structure of a protein. However, it is not possible using the NMR method

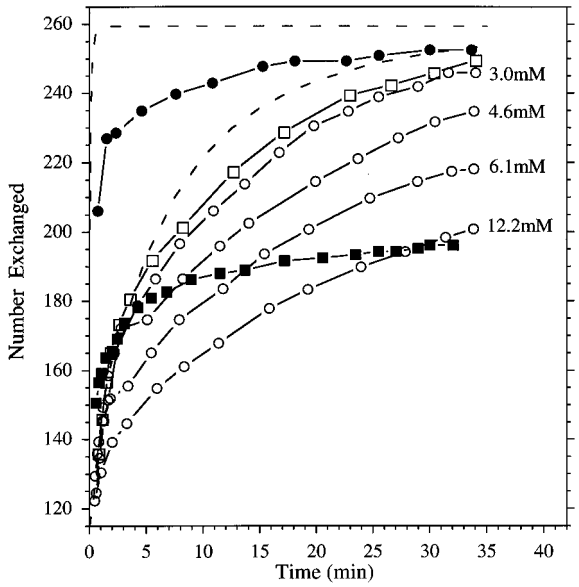

FIGURE 4: Plot of the number of H/D exchanges as a function of time for holo-Mb (■) and apo-Mb (●) in D_2O at pH 6, and for apo-Mb in D_2O containing 10 mM DCl at pH 2 (□), and in D_2O containing 10 mM DCl with addition of 3.0, 4.6, 6.1, and 12.2 mM CCl_3COOND_4 at pH ~2 (○). The dashed lines with no symbols are the predicted exchange curves for the random coil apo-Mb at pH 2 (lower) and pH 6 (upper), respectively. The exchanges were carried out at room temperature (~21 °C), and the protein concentrations were 5 μM. The number of deuterium atoms incorporated was computed from the change in *m*/*z* of each representative charge state of the proteins observed in the ESI mass spectra (+8 and +9 for holo-Mb in D_2O; +16 and +18 for apo-Mb in D_2O; +16, +18, and +20 for apo-Mb in 10 mM DCl; and +14, +15, and +16 for apo-Mb in 10 mM DCl with different concentrations of CCl_3COOND_4).

to analyze a conformational mixture such as that of apo-Mb, since the NMR method measures only the average conformation. In contrast, it is possible using ESI-MS to distinguish coexisting conformations with distinct charge state distributions, and to distinguish those different conformations that have similar charge state distributions but distinct masses acquired in the H/D exchange process. Apo-Mb contains 153 amino acid residues and a total of 262 exchangeable hydrogens, of which 148 are on the amide backbone, 111 are on the side chains, and 3 on the 2 termini. Holo-Mb has two additional exchangeable hydrogens on the heme group. Thus, if all the exchangeable hydrogens were replaced by deuterium, the molecular mass increment would be 262 Da for apo-Mb and 264 Da for holo-Mb.

H/D exchange rates were measured by ESI-MS using solution conditions similar to those used to obtain the CD spectra in Figure 1. Rates of deuterium incorporation were plotted as molecular mass increments as a function of time (Figure 4). The H/D exchange rates are consistent with the CD spectra in most cases. Holo-Mb underwent the least exchange, 68 labile hydrogens remaining unexchanged even after 2 h. It has been shown that, for simple peptides dissolved in D_2O at pH 5−9 and at temperatures of 0−40 °C, the exchange of unstructured amide hydrogens with solvent proceeds with half-times between 0.1 ms and 100 s. The exchange can be essentially complete within minutes even for the slowly exchanging amide hydrogens (Englander & Mayne, 1992; Wuthrich, 1986). Therefore, the unexchanged hydrogens in holo-Mb are expected to be those located on the backbone that are involved in hydrogen

bonding in an α-helix, or those on side chains buried inside the hydrophobic core.

It is interesting to compare some of the H/D exchange data in Figure 4 with the known structures and folding data obtained from X-ray crystallographic studies. The crystal structure of myoglobin shows that it consists of 8 α-helices that assemble to form a hydrophobic pocket for the heme group; 130 residues (including 3 proline residues that have no amide hydrogens) out of a total of 153 are in an α-helical conformation (Evans & Brayer, 1990). With its tightly bound heme group, holo-Mb is expected to be stable, and its hydrogen atoms involved in H-bonding in the α-helices should be protected from exchange. As the protein denatured in 10 mM DCl, the number of exchangeable hydrogens increased considerably with only 12 (or 4.6%) backbone hydrogens remaining after 34 min, consistent with the unfolded structure suggested by the CD measurements (Figure 1). The exchange rates for the solutions containing ND_4TCA decreased with increase of salt concentration. For the solution containing 12.2 mM ND_4TCA, the 62 unexchanged hydrogens remaining after 34 min represent ∼49% of the total exchangeable backbone hydrogens involved in the α-helical conformation in native holo-Mb. This finding is consistent with the CD data that suggest the partially folded molten globular state of apo-Mb at pH 2 has ∼57% of the α-helical content of the native state of holo-Mb. To ensure that the reduction of the exchange rate in the presence of ND_4TCA is due to the more compact protein conformation rather than to the effect of ND_4TCA on exchange reaction chemistry, we measured the H/D exchange rates of a 10 residue peptide (bradykinin) that is not expected to have much high-order structure. The two exchange curves (not shown) of the peptide in 10 mM DCI, with and without 15 mM ND_4TCA, were indistinguishable, indicating that the addition of ND_4TCA does not change the H/D exchange reaction rate. This provides strong supporting evidence for the conclusion that the slower H/D exchange rates observed for apo-Mb in DCI solution containing ND_4TCA salt must be due to the more compact conformations of apo-Mb induced by the TCA anions at the acidic pH.

Apo-Mb in H_2O Has an Unstable and Less Folded Conformation than Holo-Mb. Surprisingly, the exchange curve of apo-Mb in D_2O (Figure 4) shows that within 44 s apo-Mb incorporates 52 more deuterons than holo-Mb. This is in agreement with the recent results from the ESI-MS measurement of the quenched H/D exchange of apo-Mb and holo-Mb in buffered solution (Johnson & Walsh 1994), and corresponds to exchange of an additional 35% of backbone amide hydrogens. This difference in the extent of deuterium incorporation between apo- and holo-Mb then remained about the same throughout the course of the exchange (difference of 56 at 5 min and at 32 min). Since the exchange of hydrogens on the exposed side chains and unstructured backbone amides can be complete within minutes in D_2O (pH >5) at room temperature (Wuthrich, 1986), the exchangeable hydrogens remaining after 5 min must have been protected from solvent either because of their involvement in hydrogen bonds in the α-helices or by protection in the hydrophobic core of holo-Mb. The large and fast increase in deuterium incorporation in apo-Mb suggests that the removal of the heme group in holo-Mb causes exposure of many amide groups to the solvent, or possibly the breakage of many hydrogen bonds. The latter interpretation is in agreement with the CD results indicating that apo-Mb in H_2O has 27% less α-helical structure than holo-Mb. Deuterium incorporation of apo-Mb in D_2O is also higher than that of apo-Mb in 10 mM DCl for the first 30 min of exchange, after which the two curves approach one another. The fast exchange rate for apo-Mb in D_2O would be expected if it were unfolded as in DCl. This is because the pH dependence of H/D exchange for exposed amide hydrogens is V-shaped, with a minimum around pH 3 where the acid- and base-catalyzed processes are equal in rate, and each unit change in pH (in either direction) produces a 10-fold increase in the exchange rate (Englander & Mayne, 1992). Thus, the exchange of freely exposed amide hydrogens at pH 2 is expected to be about 100 times slower than that at pH 6. As indicated in Figure 4, within 44 s, 206 (or 79%) of the labile hydrogens are exchanged, and within 5 min 235 (or 90%) of the hydrogens are exchanged. Only 10 (or 4%) of the labile hydrogens remain unexchanged after 30 min. Based on the rapid exchange behavior and the charge state distribution, one might conclude that apo-Mb in H_2O (pH ∼6) is in a much less folded conformation than holo-Mb. Such a conclusion, however, would apparently contradict the CD data (Figure 1), which suggest that apo-Mb in H_2O has an α-helix content of ∼73% of holo-Mb.

A more plausible interpretation is that the apo-Mb in H_2O has a substantial amount of secondary structure, but these α-helices are not stable. Removing the heme group destabilizes not only the tertiary structure but also the secondary structure. The conformational states of apo-Mb molecules, or individual α-helices, will then interconvert rapidly between folded and unfolded states. At any time, there are some protein molecules or α-helices in folded states that are detected by CD. In H/D exchange experiments, the exchange is rapid during the period in which a protein, or part of the protein, is in the "unfolded" state. If the unfolded−refolded transition rate were fast, all protein molecules would spend a significant portion of their time in the "unfolded" or partly "unfolded" state during which H/D exchange would occur. Thus, all protein molecules, regardless of their conformation at the instant of forming gas phase ions in the ESI process, would appear to exchange at a fast rate like that of an unfolded protein. The apparent discrepancy between the CD results and the H/D exchange results reflects the differing and complementary nature of the two techniques. CD measures the average amount of secondary structure (α-helices here). In contrast, the amount of H/D exchange monitored by ESI-MS is cumulative, and the number of unexchanged labile hydrogens reflects the amount of *stable* folded structures.

Apo-Mb in Buffered Solution at pH 7 Has a Set of Rapidly Interconverting Conformational States. The conformational stability and compactness of apo-Mb may increase in the buffered solution. The mean residue ellipticity at 222 nm of apo-Mb in 10 mM ammonium acetate is −19 353, a ∼19% increase compared with that of apo-Mb in H_2O. The mean residue ellipticity at 222 nm of holo-Mb in 10 mM ammonium acetate is −22 712, which is very close to that of holo-Mb in H_2O. The charge state distribution (Figure 3b) for apo-Mb in 10 mM ammonium acetate displays a bimodal distribution, with one charge distribution similar to that of the native state of holo-Mb and the other corresponding to a denatured state. If the bimodal charge state distribution represents two different but stable conformations,

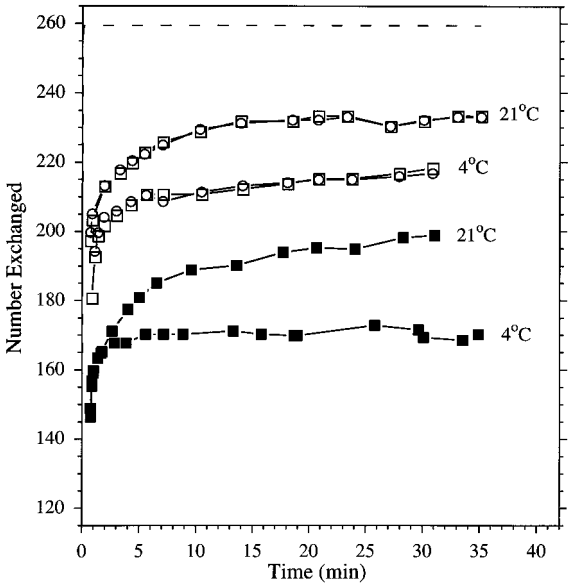

FIGURE 5: Plot of the number of H/D exchanges as a function of time, computed from the changes in *m*/*z* of +15 (□) and +9 (○) charge states of apo-Mb and of +9 and +8 charge states of holo-Mb (■) in D_2O containing 10 mM CH_3COOND_4 (pH 7), at temperatures of 4 and 21 °C, respectively. The dashed line with no symbols is the predicted exchange curve for the random coil apo-Mb at pH 7 and 21 °C.

they are expected to exchange at different rates upon exposure of the protein to the buffered D_2O solvent. H/D exchange rates of buffered apo-Mb monitored on the charge states 15+ and 9+ of the protein ions are shown in Figure 5. Surprisingly, the two exchange curves coincide, indicating that the two charge states have the same deuterium exchange rate. Interestingly, such bimodal charge state distributions have also been observed in ESI mass spectra of several small proteins including myoglobin and cytochrome *c* under mild acid or heat denaturation (Mirza et al., 1993; Feng & Konishi, 1993; Wagner & Anderegg, 1994). It has been shown that, for bovine cytochrome *c*, the deuterium exchange rates of the two distinct charge states in a bimodal distribution are the same under the acid denaturing condition, but are different under the heat denaturing condition because the rapid interconversion between the two conformations would be blocked under the heat denaturing condition (Wagner & Anderegg, 1994). As discussed in the case of apo-Mb in water, we attribute the phenomenon of a single exchange rate, for the two representative charge states in a bimodal distribution of apo-Mb in the buffered solution, to the instability of the conformations. If the two conformations are interconverting rapidly, the exchange will appear at the faster rate representing the more open conformation. The number of deuterons incorporated in apo-Mb in the buffered D_2O solution is 232 (or 88%) in 30 min, which is 20 less than that in unbuffered D_2O, indicating that apo-Mb in the buffered D_2O solution has a more compact conformation than in D_2O, consistent with the CD and the charge state distribution data. However, apo-Mb still incorporated 33 more deuterons in 30 min than holo-Mb under the same solution conditions (Figure 5), and the difference between the number of deuterons incorporated in apo-Mb and in holo-Mb remains ~33 even after 2 h of exchange. The results of measurements of charge state distribution and H/D exchange indicate that apo-Mb in the buffered solution still

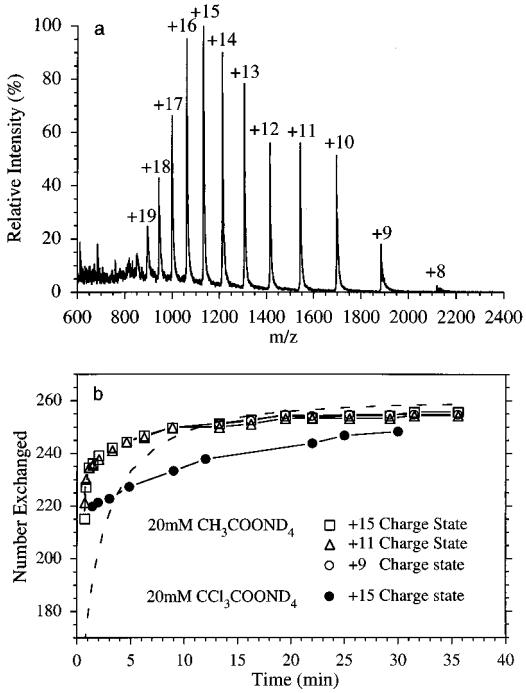

FIGURE 6: (a) Positive ion ESI mass spectrum of apo-Mb (5 μM) in 20 mM CH_3COONH_4 at pH 4.3. The labels on the peaks in (a) indicate the protonation (charge) states of the protein. (b) Plot of the number of H/D exchanges as a function of time, computed from the change in *m*/*z* of +15, +11, and +9 charge states of apo-Mb (5 μM) in D_2O containing 20 mM CH_3COOND_4 at pH 4.3, and in D_2O containing 20 mM CCl_3COOND_4 at pH 4.3. The solution pH was adjusted using CD_3COOD and CCl_3COOD, respectively, and the exchanges were carried out at room temperature (~21 °C). The dashed line with no symbols is the predicted exchange curve for the random coil apo-Mb at pH 4.3.

has a less stable and, on average, more open conformation than holo-Mb.

Intermediate State Detected in the Acid Unfolding Pathway. It has been reported that apo-Mb exists as a partly folded stable intermediate state in acetate-buffered solution at pH 4−5 and at room temperature (Griko et al., 1988; Hughson et al., 1990; Griko & Privalov, 1994). The intermediate was described to be almost as compact as the native state, retaining a considerable amount of secondary structure, but not possessing a fixed tertiary structure. It has characteristics of a "molten globular" state, based on CD measurements (Griko et al., 1988; Griko & Privalov, 1994), H/D exchange, and NMR (Hughson et al., 1990). Figure 6a shows the charge state distribution of this intermediate state observed in the ESI mass spectrum. Interestingly, the charge state distribution of apo-Mb in acetate-buffered solution at pH 4.3 retains some of the bimodal character, although the two distributions moved closer and on average shifted to higher charge states (low *m*/*z*) due to the lower pH, compared with the charge state distributions of apo-Mb in the buffered solution at pH 7 (Figure 3b). However, the average charge state of apo-Mb in buffered solution at pH 4.3 is lower than that of apo-Mb in H_2O at pH 6 (Figure 3a), which suggests the buffers and salts play important roles in determining protein conformations that affect the charge state distributions. Figure 6b shows the H/D exchange rate of this intermediate state measured by ESI-MS on +15, +11, and +9 charge states. As expected, the H/D exchange curves of the three charge states coincide, indicating that, as observed for native apo-Mb, the two charge distributions

have the same exchange rate due to the rapid interconversion between the different conformations. The H/D exchange rate of the intermediate state is similar to that of apo-Mb in D_2O, and only eight labile hydrogens remain unexchanged after 30 min of exchange. Interestingly, for apo-Mb in D_2O containing 20 mM ND_4TCA salts at pH 4.3, we observed a slower exchange rate for the intermediate state, as shown in Figure 6b. The effect of the TCA anions in inducing folding (slow exchange rate) of apo-Mb in this case (pH 4.3) is not as effective as for apo-Mb at pH 2.

Comparison of the Measured H/D Exchange Kinetics with Those Predicted for a Random Coil Apo-Mb. The protection against H/D exchange imposed by protein structure can be evaluated by comparing the measured H/D exchange kinetics with those predicted for the random coil protein using model peptide data (Molday et al., 1972; Bai et al., 1993). The exchange rates expected for unprotected amide hydrogens can be calculated from the model peptide data and are dependent on the sequence of the protein, solution pH, and temperature (Bai et al., 1993). The global exchange kinetics expected for a random coil protein can then be computed by summing the predicted exchange behavior of all the individual backbone amide hydrogens and the exchangeable hydrogens on amino acid side chains of the protein. Structural and stability information of protein states can be extracted by comparing the H/D exchange rates measured in a protein with the exchange rates expected in a random coil model. The overall exchange behavior of the unprotected backbone amide hydrogens is predicted using the apo-Mb sequence and the model peptide data reported by Bai et al. (1993), which includes the PDLA reference rates in Table III, the side chain factors in Table II, and eqs 1−3. The predicted global exchange behavior of a random coil apo-Mb under various experimental conditions is shown as dashed lines (without symbols) in Figures 4, 5, and 6b. The pD values used in the calculations of the predicted rate constants are pD 2 and 6.4 for Figure 4, pD 7.4 for Figure 5, and pD 4.7 for Figure 6b, reflecting the proper corrections for pH meter anomaly in D_2O ($pD_{corr} = pD_{read} + 0.4$) (Glasoe et al., 1960). Since the unprotected exchangeable hydrogens on amino acid side chains are exchanged very rapidly (with the exception of the NδH side chain proton of arginine) (Molday et al., 1972; Bai et al., 1993), they are assumed to exchange instantaneously for the purposes of the computation of the predicted global exchange curves. Good agreement is found between the measured and the predicted global exchange curves of apo-Mb at pH 2 (Figure 4), since apo-Mb is in a maximally unfolded state at pH 2. At pH 6 and 7, the measured exchange rates are much slower than the predicted exchange rates (Figures 4 and 5). This is expected since apo-Mb at pH 6 or 7 has partially folded or folded conformations, although they are unstable. At pH 4.3, the measured exchange rates are faster than the predicted random coil exchange rates for the first few minutes (Figure 6b). The cause of the disagreement is unclear at present.

Conformations and Stability Comparison between Apo- and Holo-Mb. The structure of apo-Mb at pH 6−7 has been reported to resemble that of holo-Mb. It has a compact and unique spatial structure with an extended hydrophobic core (Griko et al., 1988). Hughson et al. have reported the NMR measurement of amide hydrogen exchange rates at about 50 sites, and have concluded that the structure of apo-Mb is similar to that of holo-Mb since the pattern of protection of these amide hydrogens observed in holo-Mb is largely preserved in apo-Mb. It is difficult to make a fair assessment here since the NMR measurements of the hydrogen exchange rates (Hughson et al., 1990) were made under conditions different from those used for our ESI-MS measurements. In the NMR case, the exchange of about one-third of the amide hydrogens of the apo- and holo-Mb, prepared from sperm whale myoglobin, was monitored (Hughson et al., 1990), and the exchange was carried out in 10 mM sodium acetate buffered D_2O solution at 5 °C for the apo-Mb, and at 35 °C for the holo-Mb. In our case, the global exchange rates of both apo-Mb and holo-Mb, prepared from horse heart myoglobin, were measured in the same deuterated solvents at room temperature (21 °C). Since the primary sequence and the crystallographic backbone conformations of horse heart myoglobin are very similar to those of sperm whale myoglobin (Evans & Brayer, 1990), we do not expect any significant difference between the solution conformations and stability of the apo-Mbs prepared from horse heart and sperm whale myoglobin. It is known that salts and low temperature stabilize the protein conformation. Moreover, the exchange rate decreases significantly with temperature (Englander et al., 1979). We have demonstrated above that, in H_2O, the conformation of apo-Mb is different from, and is much less stable than, that of holo-Mb. The addition of ammonium acetate buffer to apo-Mb solution increased the degree of folding of the apo-Mb as judged by the 19% increase of the mean residue ellipticity at 222 nm, the slower H/D exchange rate (Figure 5), and the appearance of native-like charge state distribution (Figure 3b). However, even in the acetate-buffered solution, the structure of apo-Mb is a conformational mixture consisting of denatured and native-like states, which are not stable.

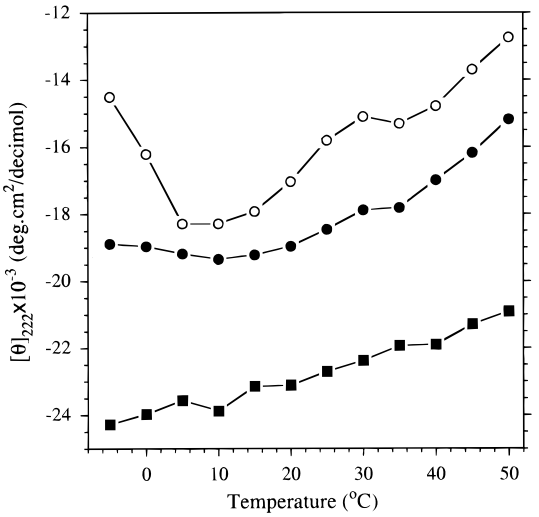

FIGURE 7: Temperature dependence of mean residue ellipticity at 222 nm of apo-Mb in deionized H_2O at pH 6 (○), in 10 mM CH_3COONH_4 at pH 7 (●), and of holo-Mb in 10 mM CH_3COONH_4 at pH 7 (■). The protein concentrations were 5 μM.

In order to assess the effect of temperature on the conformational stability of apo-Mb in buffered solution, we measured CD spectra and H/D exchange rates of apo-Mb and holo-Mb in D_2O buffered with 10 mM ammonium acetate at 21 and 4 °C, respectively. The mean residue ellipticities at 222 nm of the buffered apo-Mb, measured either at 21 °C or at 4 °C, are about the same (see Figure 7). The ESI mass spectra observed for apo-Mb in 10 mM

ammonium acetate at 4 °C (not shown) and 21 °C are essentially the same, each displaying a bimodal charge state distribution as described above. However, H/D exchange for apo-Mb is slower at 4 °C than at 21 °C as expected. As indicated in Figure 5, within 51 s of exchange apo-Mb incorporates 49 more deuterons than holo-Mb at 21 °C, but at 4 °C it incorporates only 27 more deuterons. Moreover, 46 labile hydrogens remain after 30 min of exchange at 4 °C, but only 30 labile hydrogens (corresponding to 20% of the backbone amide hydrogens) remain at 21 °C, and the exchange curve at 4 °C moves closer to that of holo-Mb at room temperature. Our results from the direct measurement of the H/D exchange rates of apo-Mb in the buffered solution at pH 7 and at room temperature are consistent with those from the ESI-MS measurement of the quenched H/D exchange of apo-Mb (Johnson & Walsh 1994), where apo-Mb was found to incorporate 52 more deuterons than the holo-Mb within 30 s and only ∼20% of the backbone hydrogens remain after ∼30 min of exchange. Thus, the slower H/D exchange rate of apo-Mb in the buffered solution at 4 °C may be due to increased conformational stability of apo-Mb in the buffered solution and a slower exchange reaction rate at low temperature. However, by comparing the exchange curve obtained from apo-Mb with that from holo-Mb under the same temperature and solution conditions, there were still 48 and 33 more deuterons incorporated in apo-Mb than in holo-Mb even in buffered D_2O at 4 and at 21 °C, respectively. It is clear that, under the same conditions, apo-Mb is less tightly folded and its structure is less stable than that of holo-Mb due to removal of the heme group. Furthermore, apo-Mb at neutral pH has a population of rapidly interconverting conformations, while the holo-Mb has a folded conformation which is stable on the time scale of these experiments.

Cold Denaturation of Apo-Mb. Griko et al. (Griko et al., 1988; Griko & Privalov, 1994) have reported that the native structure of apo-Mb is disrupted at temperatures either above or below the range of 25−30 °C. They have also shown that at pH 4−5 the temperature dependence of the apo-Mb mean residue ellipticity at 222 nm shows a maximum at about 20−30 °C, and the ellipticity decreases as the temperature increases or decreases from the maximum ellipticity temperature. This phenomenon was termed heat or cold denaturation of apo-Mb. However, the mechanism of cold denaturation of apo-Mb is unclear. We have measured the CD spectra of holo-Mb and apo-Mb over the temperature range from −5 to 50 °C. Figure 7 shows the temperature dependence of the mean residue ellipticity at 222 nm of holo-Mb and apo-Mb. As can be seen, the ellipticity of apo-Mb in H_2O decreases sharply as the temperature decreases from ∼5 °C or increases from ∼10 °C. This suggests that apo-Mb in H_2O undergoes strong "cold denaturation" and "heat denaturation", which is very similar to the cold and heat denaturation phenomenon observed for the intermediate state of apo-Mb although the maximum ellipticity temperatures are different (Griko et al., 1988; Griko & Privalov, 1994). For apo-Mb in 10 mM ammonium acetate, the temperature dependence of the ellipticity shows similar behavior, but is far less pronounced than that of apo-Mb in H_2O at temperatures below 5 °C, suggesting that there is a weak cold denaturation effect. The temperature dependence of the ellipticity of holo-Mb in 10 mM ammonium acetate is almost linear in the temperature range investigated. The ellipticity increases with decrease of temperatures; that is, there is no cold denaturation for holo-Mb. Although more work is required to deduce the detailed mechanism, it is clear that the apparent cold denaturation of apo-Mb is related to its conformational stability as discussed above.

CONCLUSIONS

Our results provide new insight into the conformations of apo-Mb and the stability of the partially folded acidic molten globular states and the native state. Interestingly, Lin et al. (1994) have suggested recently that the native state of apo-Mb has molten globular characteristics, based on the observation of significant changes in molar ellipticity at 222 nm and in tryptophan fluorescence intensity in mutant apo-Mb with only a single amino acid substitution. The results from ESI-MS measurements of H/D exchange rates, and of charge state distributions, of apo-Mb, provided direct evidence for the first time that both the native state and the intermediate state of apo-Mb at pH 4.3 have highly plastic and unstable structures consisting of a set of two or more distinct but rapidly interconverting substates rather than a fixed conformation. However, the salt-induced acidic compact state of apo-Mb at pH 2 appears to be a partly folded stable state. Bimodal charge state distribution is a general phenomenon observed in mass spectra of acid- and heat-denatured proteins (Mirza et al., 1993; Wagner & Anderegg, 1994), and also of proteins denatured due to the removal of the ligand as we demonstrated here for apo-Mb. Such a state, consisting of two or more rapid interconverting substates rather than a fixed conformation, may well be a general intermediate state between native and unfolded states.

Although CD is the most widely used technique for monitoring protein conformation changes and for measuring the amount of secondary structure of proteins, CD alone is insufficient to determine the solution conformation of a protein. Since CD data reflect only average amounts of secondary structure, the protein conformational stability and heterogeneity (coexisting conformations) are not detected in the CD data. H/D exchange and ESI/MS combined with CD can detect not only the amount of the folded structure but also the conformational stability and heterogeneity. These parameters are important in interpreting the changes in protein conformational states during biological function and/or ligand binding.

Deuterium incorporation rates at specific amino acid residues may be derived from liquid chromatography−MS and tandem MS analysis of deuterated peptide fragments generated by peptic digestion (Zhang & Smith, 1993). If the structures of the salt-induced partly folded proteins at acidic pH (Goto & Fink, 1990a) are similar to the kinetic folding intermediates, then the H/D exchange and MS techniques in combination with the method of generating the stable partly folded proteins may become a powerful tool for studying protein folding pathways. Work to characterize the detailed folding of the salt-induced acidic compact states of apo-Mb is currently in progress.

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