Methanol-Induced Conformations of Myoglobin at pH 4.0†

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ABSTRACT: The equilibrium methanol-induced conformation changes of holomyoglobin (hMb) at pH 4.0 have been studied by circular dichroism, tryptophan fluorescence, and Soret band absorption and by electrospray ionization mass spectrometry (ESI-MS). Optical spectra show the following: (1) In 35−40% (v/v) methanol/water, the native-like secondary structure remains, the tertiary structure is lost, the heme protein interactions are decreased, and a folding intermediate is formed. (2) In 50% methanol, heme is lost from the protein, and there is a small decrease in helicity together with a loss of tertiary structure. (3) At >60% methanol, the helicity increases and the apoprotein goes into a helical denatured state. The conformations are also probed by the charge states produced in ESI-MS and by hydrogen/deuterium (H/D) exchange with mass measurement by ESI-MS. At 0−30% methanol, native hMb produces relatively low charge states (9^+−13^+) in ESI-MS and exchanges relatively few hydrogens. In 35−40% methanol, at which an intermediate is formed, there is a bimodal distribution of hMb ions with both low (9^+−13^+) and high (14^+−23^+) charge states and also a high charge state distribution (12^+−26^+) of apomyoglobin (aMb) ions. Low and high charge states of hMb and a high charge state of aMb all show the same H/D exchange rate, indicating that an unfolded hMb intermediate interconverts between folded hMb and unfolded aMb. The charge state distribution for the unfolded hMb intermediate observed here is similar to that of the recently reported transient intermediate formed during the acid denaturation of hMb. At 50% alcohol the protein produces predominantly high charge states of aMb ions and shows H/D exchange rates close to those of the acid-denatured protein. H/D exchange of the helical denatured protein at alcohol concentrations >60%, at which high charge states of aMb are produced, shows that the protein structure is more protected than at ∼50% methanol.

The folding or self-assembly (*1*) of a protein into a native, biologically active conformation can occur through intermediates (*2*). Protein folding (or unfolding) is studied in kinetic and equilibrium experiments. In kinetic experiments, folding is initiated and the time course of unfolded, folded, and any intermediate states is followed. In equilibrium experiments the concentration of a denaturant is systematically varied and the concentrations and properties of folded, unfolded, and intermediate conformations at equilibrium are measured. Because intermediates are only transiently populated in kinetic experiments, determination of their structures (*3*, *4*) requires fast reaction methods or other indirect approaches (*5*). The observation of intermediates in equilibrium experiments allows the determination of the properties of conformations that may be similar to transient folding intermediates (*6*). One partially folded intermediate is the molten globule state, which is compact with significant native-like secondary structure but which has lost rigid tertiary interactions. It has been suggested that this state is a common folding intermediate of small globular proteins (*6*−*8*). Molten globule states are formed under relatively mild denaturing conditions such as low pH, high ionic strength, or moderate concentrations of denaturants (*8*).

One recent approach to generating partially folded intermediates is to use organic solvents, especially alcohols. Alcohols stabilize the helical structure (*9*−*12*) but destabilize the tertiary structure of a protein (*13*−*16*) and thus have been shown to produce partially folded states in several proteins (*12*, *17*−*23*). These alcohol-induced states are also of interest because it has been proposed that they are similar to partially denatured states formed near membrane surfaces where the pH and dielectric constant are low (*22*).

Circular dichroism (CD),[1] fluorescence, absorption spectroscopy, calorimetry, viscometry, small-angle X-ray scattering (SAXS), and NMR have been used to study the alcohol-induced conformational changes in many proteins (*19*−*23*). Additional and complementary information can also be obtained from hydrogen/deuterium (H/D) exchange combined with ESI-MS (*24*−*26*) and from charge state distributions produced in ESI-MS (*24*, *27*−*29*). An unfolded protein generally produces more highly protonated ions in positive mode ESI-MS than the same protein in the native state [this is not necessarily the case for negative ions (*30*)]. This has been attributed to the increased availability of basic amino acid residues for protonation, or, alternatively, an increased surface area of the unfolded protein (*31*). Chowdhury

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[1] Abbreviations: aMb, apomyoglobin; $aMb18^+$, apomyoglobin + 18 H^+; CD, circular dichroism; ESI, electrospray ionization; ESI-MS, electrospray ionization mass spectrometry; H/D exchange, hydrogen−deuterium exchange; hMb, holomyoglobin; $hMb10^+$, holomyoglobin + 10 H^+; *m*/*z*, ratio of mass to charge; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering.

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et al. (*32*) first showed dramatic differences in the charge state distributions from native and acid-denatured cytochrome *c*. The native conformation in solution produced a narrow distribution of charge states from 8^+ to 12^+ and the denatured protein a broader distribution with charge states from 11^+ to 18^+. A detailed study of cytochrome *c* concluded that a shift to high charge states occurs when the tertiary structure in solution is lost (*27*). Although the mechansim which produces different charge state distributions is not well understood, it has been shown that charge state distributions can be used to probe the conformational changes in proteins in a manner analogous to other spectroscopic techniques. The effects of pH, heat, solution composition, and intramolecular disulfide bonds on protein conformation have been studied by ESI-MS (*32*−*36*). ESI-MS has been used to distinguish cooperative two-state unfolding transitions from unfolding involving multiple intermediates (*28*). If two conformations are present in equilibrium in solution, a bimodal distribution is produced in ESI (*25*, *27*, *28*, *32*, *37*, *38*). Time-resolved mass spectrometry with ESI has been used to study the changes in protein conformations during folding/unfolding reactions (*37*−*40*). For example, it was shown that the acid denaturation of holomyoglobin (hMb) proceeds through a partially unfolded intermediate that still binds heme (*38*). Although this reaction had been studied by optical methods previously, use of ESI-MS allowed the mechanism to be assigned unambiguously. The unfolding was detected in ESI-MS as a shift to a distribution of high charge states of hMb, and binding or loss of heme was readily identified from the mass-to-charge ratios of the ions. ESI-MS can detect conformation changes that are difficult to see in CD spectra. This was shown for ribonuclease A. Unfolding was detected as a shift to higher charge states and confirmed by loss of enzymatic activity, although no changes in the CD spectra could be seen (*41*).

We have recently reported an ESI-MS study of cytochrome *c*, *β*-lactoglobulin, and ubiquitin in solutions of different methanol contents (*42*). These proteins were chosen because formation of methanol-induced intermediates had previously been extensively studied by optical spectroscopy and other methods. Under conditions that promote the formation of intermediates in solution, a bimodal charge state distribution is produced in ESI-MS. H/D exchange followed by ESI-MS mass measurements on high and low charge states in the bimodal distribution showed equal exchange rates, and it was concluded that the methanol-induced intermediates fluctuate between folded and unfolded conformations.

In this work we report a study of the conformation changes of myoglobin in methanol/water mixtures. In native hMb a heme group is noncovalently bound in a hydrophobic pocket of the protein by van der Waals interactions, by coordination of the central heme iron with the proximal histidine (His^{93}), and by hydrogen bonds between the propionate groups of the heme and the protein (*43*). When the heme is removed, the protein stability is decreased. However, apomyoglobin (aMb) remains folded with extensive helical structure and a hydrophobic core (*25*, *26*, *44*−*46*). The conformational properties of aMb have been studied extensively as a model for protein folding (*25*, *47*−*49*). The details of pH- and salt-induced conformational transitions have been characterized (*50*−*52*), and the structures of the denatured states found during these transitions have been studied (*53*−*55*).

The effects of alcohols on hMb were reported by Brunori et al. in 1972 (*56*). The combined effects of alcohol and heat on the denaturation of the holoprotein at pH 9.1 were studied. It was shown that alcohols up to 25% v/v destabilized the protein by increasing ΔS for the unfolding transition, without changing ΔH. Recently, methanol-induced transitions of aMb (*21*) and hexafluoroisopropanol-induced transitions of hMb (*57*) have been reported, and it was shown that an intermediate is accumulated during these transitions. Trifluoroethanol has been shown to stabilize the pH 4.0 folding intermediate of sperm whale aMb (*58*). In this study, far- and near-UV CD, tryptophan fluorescence, Soret absorption, ESI-MS charge state distributions, and H/D exchange followed by ESI-MS mass measurements are used to investigate the methanol-induced conformational transitions of hMb at pH 4.0. These measurements show that at ∼35−45% (v/v) methanol/water and pH 4.0, hMb is transformed into an intermediate state. The similarities in the charge state distributions formed in ESI-MS by this state with the distributions formed by the transient intermediate observed during folding/unfolding of hMb by time-resolved ESI-MS (*38*) suggest that the alcohol-induced intermediate might be similar to the transient folding intermediate. Under solution conditions in which the intermediate is populated, a bimodal distribution of hMb ions is formed, along with high charge states of aMb ions. H/D exchange rates measured on low and high charge states of hMb and on a high charge state of aMb are equal. This suggests that the intermediate is in equilibrium and interconverts between folded and unfolded hMb and unfolded aMb. At ∼50% methanol the protein is transformed into a denatured state with loss of heme and an increased H/D exchange rate. At high concentrations of alcohol (>60%) the protein adopts a more helical structure. H/D exchange shows this conformation is more protected than the denatured protein. However, it still produces high charge states of aMb in ESI-MS.

MATERIALS AND METHODS

Horse heart myoglobin was from Sigma (St. Louis, MO) and was used without further purification. HPLC grade methanol, acetic acid, and hydrochloric acid were from Fisher Scientific (Nepean, ON, Canada). All deuterated solvents were from Aldrich Chemical Co. (Milwaukee, WI).

pH Measurements. Solution pH was measured with an Accumet meter (model 15, Fisher Scientific). The pH values of the protein solutions containing 0.005% acetic acid in H_2O/CH_3OH were adjusted by adding HCl. The pH in D_2O/CH_3-OD solvent was adjusted by adding DCl and is given as pD. The reported pH values are not corrected for methanol but are corrected for the isotopic effect (pD = pH meter reading + 0.4) (*59*).

CD Measurements. CD measurements were carried out at 25 °C with a Jasco spectropolarimeter, model J-710, with a quartz cell of 1 mm path length for the far UV and 10 mm path length for the near UV, at protein concentrations of 10 and 100 μM, respectively. Water circulating through a jacket around the cell controlled the temperature of the cell. The results are expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the path length in centimeters. The α-helix

content under different solution conditions was calculated approximately from $[\theta]_{224}$ as described by Chen et al. (eq 14 of ref *60*).

Soret Absorption. The Soret absorption of the heme group was monitored from 350 to 450 nm with a Unicam UV−vis spectrometer (UV4) using a 10 mm path length quartz cell (protein concentration ~70 μM).

Tryptophan Fluorescence. Fluorescence spectra were recorded with a Perkin-Elmer LS 5B spectroluminescence meter in a 10 mm path length quartz cell. Myoglobin at 10 μM was equilibrated in the presence of different concentrations of methanol at room temperature for 1 h before spectra were recorded. The excitation wavelength was 295 nm, and emission was recorded from 300 to 400 nm.

Mass Spectrometry. For ESI-MS measurements the protein concentration was 10 μM. Protein ions were generated by pneumatically assisted ESI at a liquid flow rate of 2 μL/min and mass analyzed on a quadrupole mass spectrometer constructed in-house (*27*). The ESI source was operated at +4600 V. All ESI-MS experiments were carried out at room temperature (21 ± 2 °C). The voltage difference between the ion sampling orifice and the rf-only quadrupole was +40 V. For ESI-MS experiments of H/D exchange rates, the protein was first dissolved in deionized H_2O at pH 4.0. The H/D exchange was then initiated by diluting the 1 mM protein solution 200-fold into different concentrations of CH_3-OD in D_2O at the desired pD. The sample solutions were immediately introduced into the spectrometer, and the degree of deuterium incorporation was calculated from the protein's mass shift during the time course of an experiment. A small mass range (30 *m*/*z*) encompassing an ion of selected charge state, normally the ion with the maximum intensity, was scanned continuously with a 0.1 Da step size and a 10 ms dwell time, leading to a scan time of 3 s. The small mass range and fast scan times were used during these experiments to ensure high-quality data, especially for early time points when the mass changes rapidly. The calculated molecular weight and hence the degree of deuterium incorporation for each time point could be obtained. No attempt was made to prevent the rapidly exchanging deuterium atoms from re-exchanging with hydrogens in the laboratory air.

RESULTS

CD Spectroscopy. Figures 1 and 2 show the far- and near-UV CD spectra, respectively, of myoglobin in the presence of various concentrations of methanol at pH 4.0 and at pH 2.0 at 25 °C. The spectral changes as a function of methanol concentration are represented by changes at 222 nm ($[\theta]_{222}$), reflecting the secondary structure, and by changes at 274 nm ($[\theta]_{274}$), reflecting the tertiary structure. These are shown in panels A and B of Figure 3, respectively.

At pH 4.0 in water, hMb exists in the native state as a monomer (*57*). The far-UV CD spectrum in the absence of methanol shows minima at 208 and 222 nm. From $[\theta]_{224}$ the helix content of the protein is calculated to be ~81%, close to that of the native conformation (79%). The near-UV CD spectrum shows peaks at 265, 274, 285, and 295 nm, indicating that the aromatic residues are in a specific tertiary structure. The CD transition curves induced by methanol (Figure 3A,B) can be divided into three regions. In the first region (from 0 to 25%), methanol has little effect

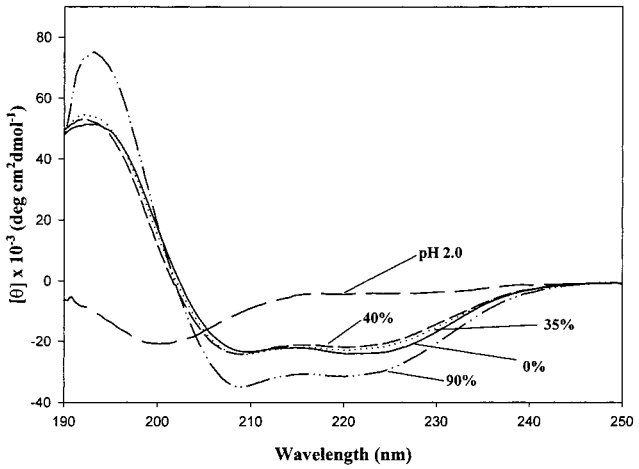

FIGURE 1: Far-UV CD spectra of myoglobin in different concentrations of methanol at pH 4.0 and spectra of the unfolded protein at pH 2.0. The protein concentration was 10 μM.

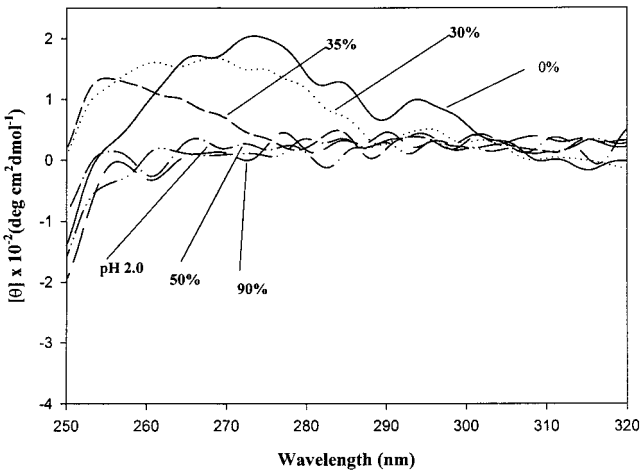

FIGURE 2: Near-UV CD spectra of myoglobin in different concentrations of methanol at pH 4.0 and spectra of the unfolded protein at pH 2.0. The protein concentration was 100 μM.

on the far- and near-UV CD spectra. This indicates that the native structure is almost fully maintained in this region. In the second region (from 30 to 50%), the $[\theta]_{222}$ and $[\theta]_{274}$ values show sharp changes suggesting that significant structural changes occur in a cooperative fashion. The $[\theta]_{274}$ data indicate that all of the tertiary interactions are lost by 40% methanol. From $[\theta]_{224}$, the average helix content is calculated to be ~71% in 40% methanol. In the third region (>50%), the magnitudes of $[\theta]_{222}$ values increase, suggesting that a transition into a more helical state takes place by an increase in the secondary structure. There is no change in the $[\theta]_{274}$ value, indicating that methanol does not induce any tertiary structure. The spectra for myoglobin at pH 2.0, where the protein is totally unfolded, are also shown in Figures 1 and 2 for comparison.

Tryptophan Fluorescence. Tryptophan fluorescence of myoglobin was obtained in water−methanol mixtures from 0 to 90% v/v at pH 4.0. Figure 3C illustrates the methanol dependence of fluorescence intensity at the maximum of the fluorescence spectrum, as well as the wavelength of maximum emission. As with the CD spectra, the data can be divided into three regions. In the first region, from 0 to 25% methanol, there is little change in the intensity or in the wavelength of maximum emission, suggesting that the native

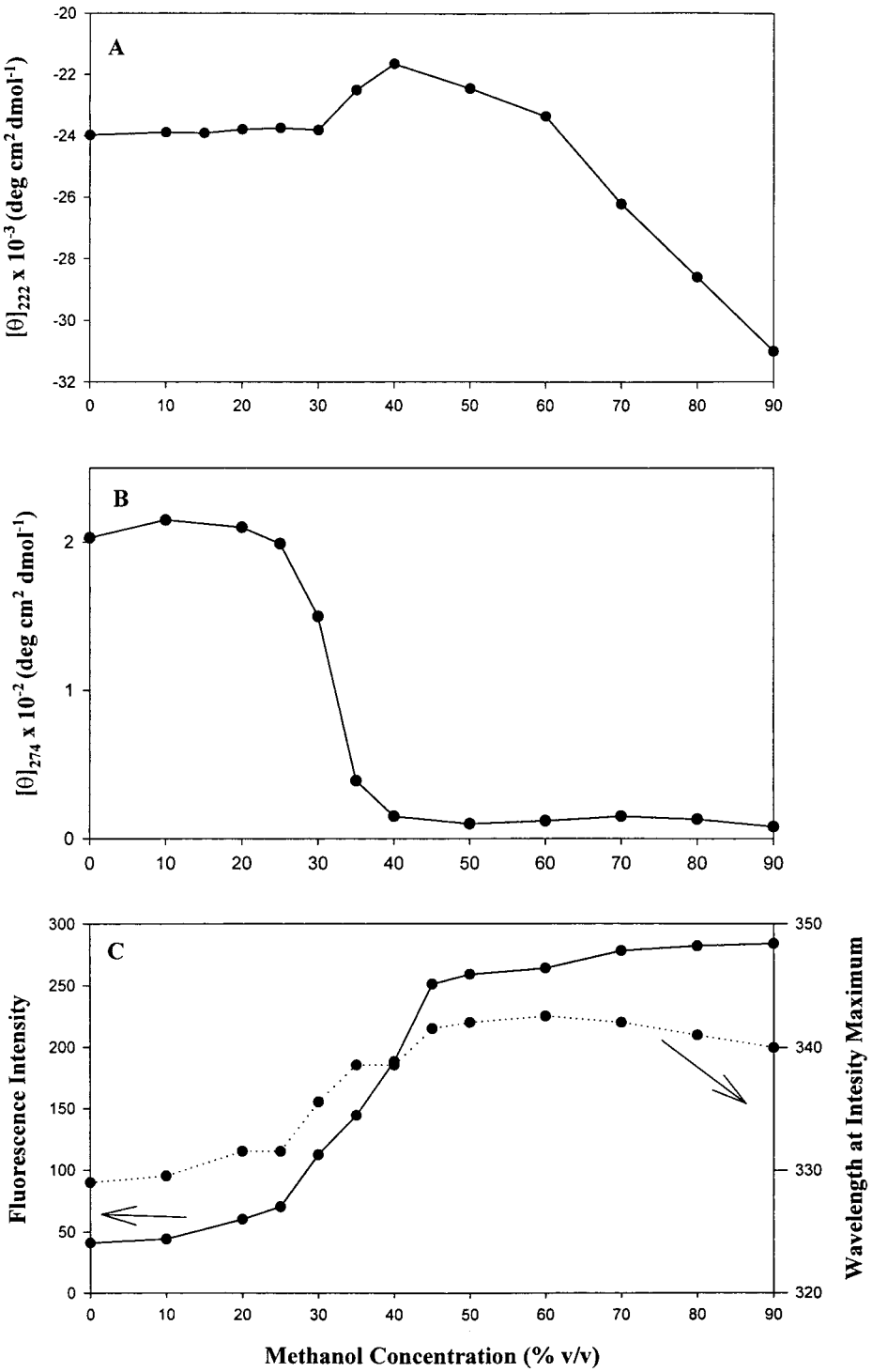

FIGURE 3: Changes in ellipticity of myoglobin with methanol concentration at pH 4.0: (A) at 222 nm; (B) at 274 nm. (C) Methanol concentration dependence of maximum fluorescence intensity and wavelength maxima at pH 4.0 and 25 °C for myoglobin. The excitation wavelength was 295 nm. The protein concentration was 10 μM.

tertiary structure is maintained, with the tryptophans buried in the hydrophobic core. From 25 to 50% methanol (the second region), both the intensity and the wavelength maximum increase significantly, indicating the unfolding of the protein and the exposure of tryptophan to the solvent. Above 50% methanol (the third region), these values became nearly constant. At pH 2.0, where the protein is in a completely unfolded conformation, the tryptophan emission maximum is ~350 nm (data not shown).

Soret Absorption. The Soret region of the myoglobin absorption spectrum provides a probe to study changes in the binding of the heme group to the protein. Figure 4 presents the data for myoglobin at different concentrations of methanol at pH 4.0. From 0 to 25% methanol, there is no change in the Soret band, which has a maximum at 409 nm. From 30 to 45% methanol, there is a gradual decrease in the intensity at 409 nm, suggesting a decrease in the heme binding to protein. Above 45% methanol there is a blue shift

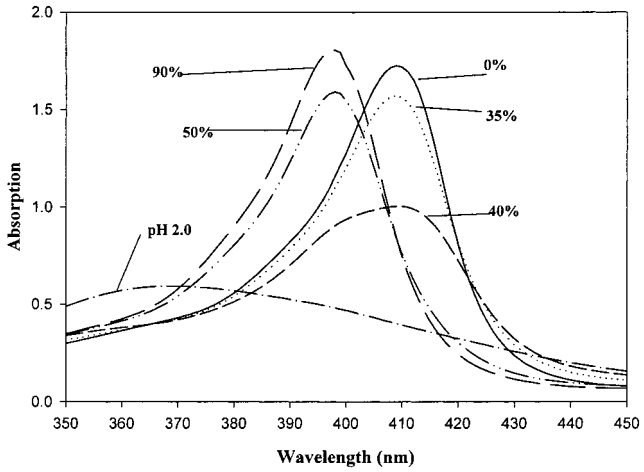

FIGURE 4: Soret band absorption spectra for myoglobin in different concentrations of methanol at pH 4.0 and for the unfolded protein at pH 2.0.

in the absorption maxima to ~397 nm. This spectrum is identical to that of the free heme in methanol at pH 4.0 (data not shown), suggesting the total loss of binding of heme to the protein at >50% methanol concentration, producing aMb. At pH 2.0 the Soret band absorption is strongly diminished.

ESI-MS Charge State Distributions. ESI-MS spectra of myoglobin at different concentrations of methanol at pH 4.0 and for the protein at pH 2.0 are shown in Figure 5. At 0% methanol, the protein shows a relatively narrow distribution of charge states of hMb ranging from $hMb9^+$ to $hMb14^+$, with $hMb11^+$ being the most intense peak. Increasing the concentration of methanol up to 30% causes only minor changes in the charge state distribution. The maximum shifts from $hMb11^+$ to $hMb10^+$. This small shift need not be due to a change in conformation. More likely, it is due to a change in ESI conditions with increasing methanol content ["secondary solvent effects" (*27*, *28*)]. Addition of 35% methanol transforms the protein into an intermediate state with considerable native secondary structure (~74% helix content) and with loss of tertiary interactions as studied by CD. Under these conditions a bimodal charge state distribution of hMb ions is produced in ESI-MS and also high charge states of aMb ions. For the hMb ions, one distribution is centered at $hMb10^+$, similar to that of the native protein sprayed from a solution with low methanol content, and the second is centered at $hMb16^+$. At methanol concentrations >50% the mass spectrum is dominated by high charge states of aMb. These also show some evidence of a bimodal distribution, with maxima at $aMb18^+$ and $aMb23^+$. Low levels of hMb ions in high charge states are also evident.

ESI-MS Measurements of H/D Exchange Rates. The H/D exchange of myoglobin at different concentrations of methanol was measured at pD 4.0 and for aMb at pH 2.0. Between 0 and 30% methanol, exchange was measured on the $hMb10^+$ ions. At 35−40% methanol, exchange was measured on the $hMb10^+$, $hMb16^+$, and $aMb18^+$ ions. At ≥50% methanol, exchange was measured on $aMb17^+$ and at pH 2.0 on $aMb19^+$. The results are shown in Figure 6. There is no increase in the exchange rate up to 25% methanol. There is a slight increase in the exchange rate at 30%, suggesting that the protein has started to become slightly more flexible or unfolded. At 35% methanol, at which the hMb charge state distribution is bimodal, $hMb10^+$, $hMb16^+$, and $aMb18^+$ were selected because these represent the different charge envelopes. The rates of exchange measured on these three ions were found to be equal. At 35% methanol, the exchange is substantially increased compared to that of the native state but lower than that of the fully unfolded protein. At 50% methanol, the exchange rate is almost equal to that of the acid-denatured state. This shows that at 50% methanol the protein goes into a denatured state. Increases in the methanol concentration above 60% result in reduced exchange rates. Figure 6B shows the number of hydrogens exchanged at 60 min versus methanol concentration.

DISCUSSION

Optical Spectroscopy. The far- and near-UV CD and fluorescence spectroscopy data (Figure 3) and Soret band absorption data (Figure 4) show that the methanol-induced transitions for myoglobin do not occur simultaneously and therefore suggest the existence of at least one intermediate state. This intermediate is most populated at ~35−45% methanol. The intermediate state has helical content reduced from ~80% to ~73%. In comparison, native aMb has ~55% helix content (*61*). Thus, the presence of the heme group appears to help stabilize the protein (*62*). The rigid tertiary interactions are lost by 35% methanol ($[\theta]_{274}$, Figure 3B). Myoglobin contains two tryptophan residues, both in the A helix. Fluorescence data show that the emission maximum has increased from 330 nm for the native protein to ~340 nm for the protein in >40% methanol. This red shift of the emission maximum shows that the tryptophans are not completely exposed to the solvent. However, the emission maximum of the acid unfolded protein is around 350 nm (data not shown). This suggests that the protein is not fully unfolded but in some partially compact conformation. In aMb, the partially unfolded intermediate shows an increase in fluorescence intensity coincident with loss of $[\theta]_{222}$ signal and it has been argued that this is consistent with loss of tertiary structure in the A helix (*63*). The situation here is more complex. In hMb tryptophan fluorescence is quenched by the heme group, and fluorescence intensity mainly probes heme loss (e.g. *62*). In this regard, the mass spectra and fluorescence data are consistent. The fluorescence intensity increases between 30 and 50% methanol, and the mass spectra show that over this range, hMb is converted to aMb. There is a mixture of folded and unfolded hMb and unfolded aMb. The contributions of these three species to the increase in fluorescence are not clear. The Soret band absorption is reduced for the protein in 35−40% methanol, suggesting reduced heme-protein interactions, but this intermediate still binds the heme group. All these characteristics, i.e., loss of tertiary interactions, native secondary structural elements and a compact structure are typical of a molten-globule-state (*7*, *8*). At concentrations of methanol greater than 60% the CD data indicate an increase in helical content, to a very high value at 90% methanol (approximately 100% calculated from $[\theta]_{224}$). However the $[\theta]_{274}$ data and fluorescence data show that protein remains somewhat unfolded, relative to the native structure. The Soret band shows that at 50% or more methanol the heme group is mostly dissociated from the protein.

Charge State Distributions. The charge state distributions show that for the native protein a distribution of relatively low charge states is obtained, as expected. For the protein

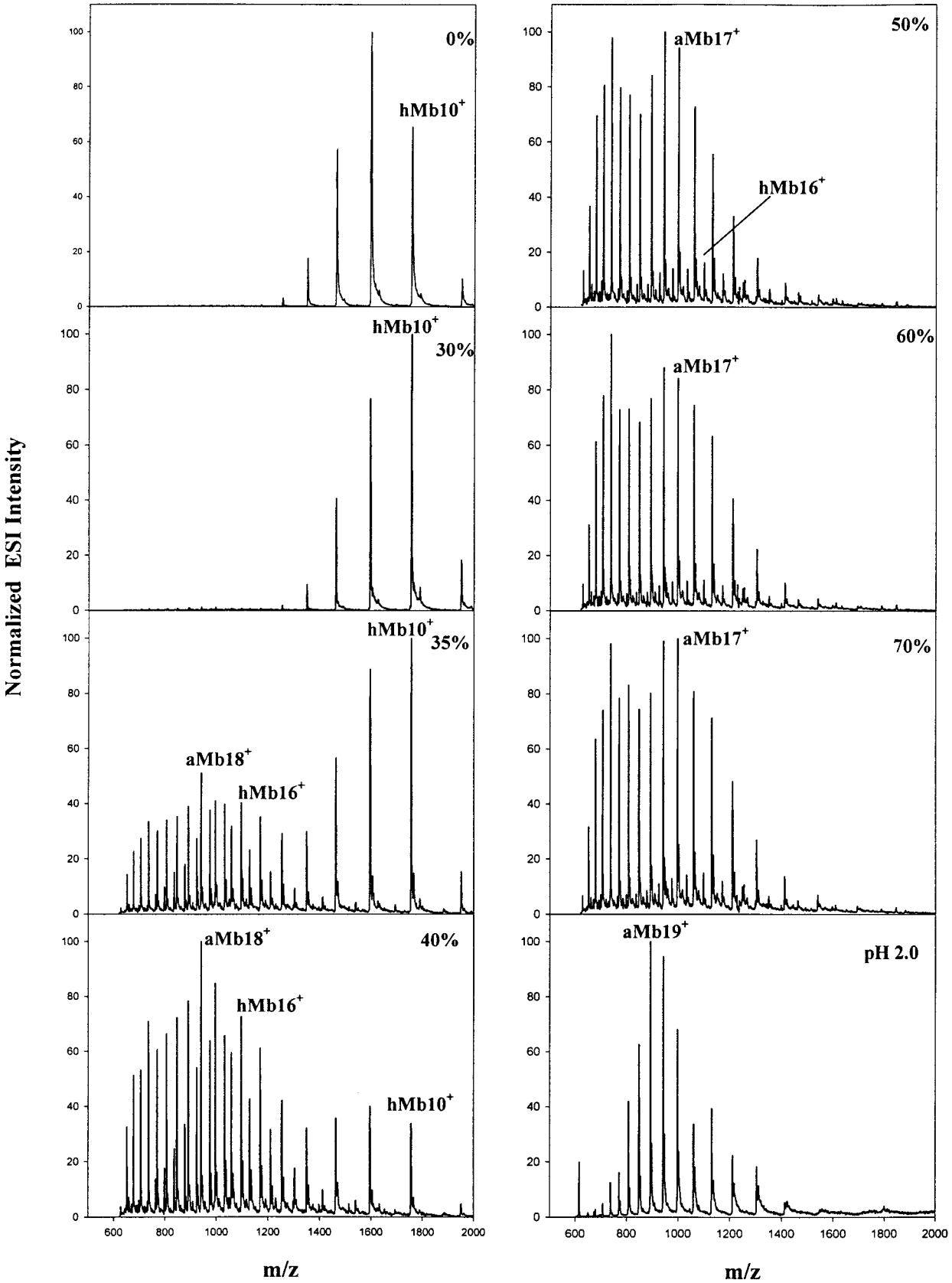

FIGURE 5: ESI mass spectra of myoglobin in different concentrations of methanol at pH 4.0 and for the unfolded protein at pH 2.0. The protein concentration was ~10 μM. Notation: $hMb10^+$ is holomyoglobin + 10 H^+; $aMb18^+$ is apomyoglobin + 18 H^+, etc.

conformation at intermediate concentrations of methanol (35−45%), where the optical data indicate that a folding intermediate is accumulated, the charge state distribution for hMb is bimodal, with one charge state distribution similar to that formed by the native protein and the other a distribution of high charge states. A high charge state distribution for aMb similar to that of the acid denatured form is also produced. The optical studies show that further

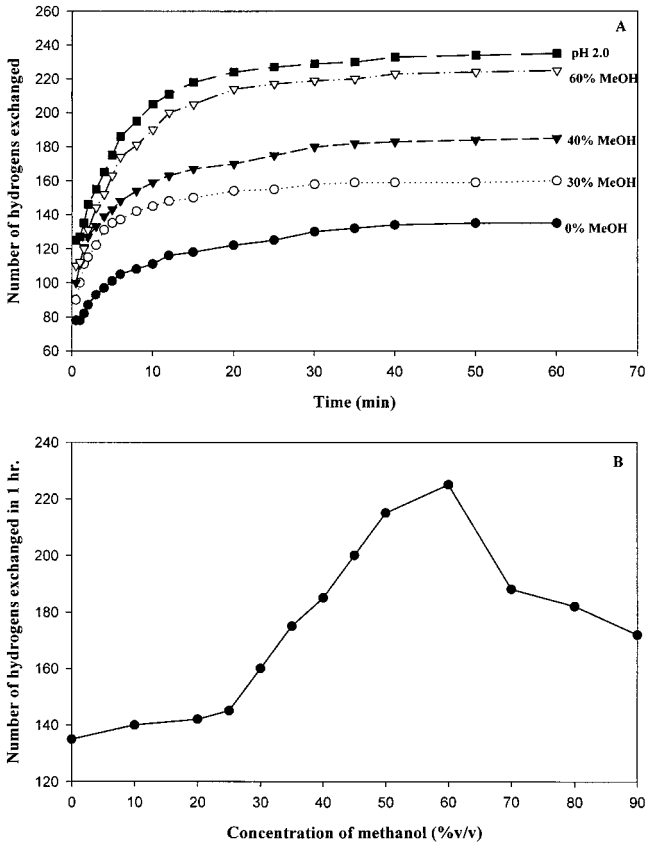

FIGURE 6: (A) Number of H/D exchanges as a function of time, at different concentrations of methanol for myoglobin at pH 4.0 and for the unfolded protein at pH 2.0. (B) Number of H/D exchanges in 1 h at different concentrations of methanol.

increases in the concentration of methanol above 60% transform the protein into a state with loss of heme, complete loss of tertiary structure but an increase in helical content (a helical denatured state). This behavior is similar to that reported recently for several different proteins (*19*−*21*). Under these conditions the mass spectrum consists mostly of a bimodal distribution of high charge states of aMb. Ions of aMb are expected, because the Soret data show the heme is dissociated from the protein. Despite the increase in helical structure, the high charge states indicate that aMb has lost its tertiary structure, consistent with the $[\theta]_{274}$ data, and remains unfolded in solution. The low levels of hMb ions in high charge states suggest that a low concentration of unfolded holomyoglobin also remains in solution.

The bimodal distribution of hMb and the high charge states of aMb produced at 35% methanol concentration were unexpected. A bimodal charge state distribution is often attributed to a mix of folded and unfolded conformers in solution (*25*, *27*, *28*, *32*, *37*, *38*, *64*). Previously, a bimodal charge state of hMb was observed with a flow-mix apparatus used to study the kinetics of the acid denaturation of holomyoglobin (*38*). The low charge states were attributed to hMb in its native state in solution, and the high charge states were attributed to a transient intermediate state of myoglobin that was unfolded but still retained the heme group. The bimodal distribution of hMb at 35% methanol observed here suggests that there may be an equilibrium between folded hMb and an unfolded hMb intermediate or, equivalently, that the intermediate is highly flexible, fluctuating between folded and unfolded states.

The appearance of high charge states of aMb in the spectrum also suggests that the intermediate can lose heme to form unfolded aMb in solution. The aMb ions could in principle be formed from dissociation of high charge states of hMb in the ion sampling region of the mass spectrometer. However, the charge state distribution of aMb rules against this. When gas phase hMb dissociates, heme is lost predominantly as a 1^+ ion (*65*, *66*). Dissociation of hMb with a charge state distribution centered on 16^+ would then give an aMb charge state distribution centered at 15^+, not 18^+ as is observed. It follows that the high charge state aMb ions are formed in the ESI process from solution myoglobin and that the intermediate that produces the high charge states of hMb may also be in equilibrium with aMb. The bimodal distribution of aMb suggests that it may also exist in at least two conformations.

H/D Exchange. H/D exchange experiments were used to search for two coexisting conformations at intermediate methanol concentrations and also to study the various conformations produced in the presence of methanol. H/D exchange is discussed in references *67*−*70*. Although both NMR and ESI-MS can measure H/D exchange, the information provided by these two techniques is complementary (*70*−*73*). If two conformations that do not interconvert are present in solution, they generally will exchange at different rates and two peaks will appear in the mass spectrum (EX1 behavior) [e.g., heat-denatured cytochrome *c* (*24*) and cellular retinoic acid-binding protein in 9:1 water/ethanol (*74*)]. If two conformations are present that interconvert rapidly relative to the time scale for H/D exchange (minutes in this case), only a single peak appears in the mass spectrum and the mass shifts with time (EX2 behavior) [e.g., acid-denatured cytochrome *c* (*24*) and aMb (*25*)].

The rates of deuterium incorporation at various concentrations of methanol observed here are generally in accord with expectations from the optical spectroscopy studies. For the native conformation the exchange rate is relatively low, consistent with the protein being in a tightly folded conformation.

The H/D exchange rate at 35% methanol is intermediate between that of the native protein and that of the unfolded protein at 60% methanol. Exchange rates measured on the charge states $hMb10^+$ and $hMb16^+$, representing two populations of hMb, are equal. Thus, if the bimodal charge state distribution represents two different conformations of the holoprotein, they must interconvert rapidly relative to the exchange rate. The $aMb18^+$ peak also showed the same exchange rate as the hMb peaks. The H/D exchange data then suggest that under conditions for which the optical spectra indicate that an intermediate is formed, there is an equilibrium between folded hMb, unfolded hMb, and unfolded aMb. The protein interconverts between these forms, and the exchange rate is averaged over these species in solution.

The distributions of charge states of hMb and aMb at 35−40% alcohol in Figure 5 are remarkably like the distribution seen 0.34 s after the initiation of unfolding of hMb with a pH jump from 6.5 to 3.2 (*38*). The unfolded intermediate at 35% methanol produces a high charge state distribution of hMb ions with a maximum at $hMb16^+$. The transient unfolding intermediate observed in the acid denaturation of hMb produces a high charge state distribution of hMb ions

with a maximum at $hMb14^+$ (*38*). The transient intermediate also shows a decrease in absorption at 441 nm, as does the intermediate in 35% methanol. Thus, the intermediate in methanol has at least some properties in common with the transient unfolding intermediate. This intermediate may be related to the partially unfolded intermediate of hMb formed at pH <4, described by Sage et al. (*75*). The Fe bond to the proximal histidine is broken and the protein unfolds, but the heme pocket is at least partially intact so that the heme remains bound to the protein.

For the denatured state populated at ~50% methanol, the exchange rate is similar to that of the fully unfolded state, suggesting that under these conditions the protein is more flexible and less protected, like unfolded states (*19*−*21*). This again is consistent with the optical data, which show a loss of tertiary structure ($[\theta]_{274}$) and unfolding of the protein (Trp fluorescence). Many proteins transform into helical denatured states at high concentrations of organic solvents (*19*−*21*). On the basis of SAXS, the Akasaka group has recently shown that a variety of proteins in helical denatured states have expanded conformations, which are close to those of urea-denatured states (*21*). These SAXS studies were done for the conformations in 60% methanol. Our H/D exchange results are consistent with these observations, as the exchange rate at ~50−60% methanol for aMb is similar to that of the denatured state at pH 2.0.

Further increases in methanol >60% do not change the charge state distribution in ESI-MS. However, the H/D exchange rates decrease, and at 90% methanol concentration they are similar to the rates observed for the protein at ~40% methanol. The slow exchange observed at high concentrations of methanol relative to the exchange rates at 50−60% methanol may be because of the protection of exchangeable hydrogens due to persistent folded conformations or, alternatively, a decrease in intrinsic exchange rates by the replacement of D_2O with an organic solvent. Englander et al. (*76*) reported that acid-catalyzed intrinsic exchange rates do not change in the presence of organic solvents at acid pH, as is the case here. Recent studies of gramicidin A in the presence of trifluoroethanol (TFE) (*77*) and studies of bradykinin, an α-melanocyte stimulating hormone, and mellitin in the presence of organic solvents (*78*) showed that the decrease in the exchange rates in the presence of organic solvents is not due to the replacement of D_2O with the organic solvents but is due to the formation of protected structures. The decrease in exchange rate at up to 90% methanol then suggests that the protein is in more compact protected conformations as compared to the protein in 50% methanol. This is consistent with the increase in helicity evident in the $[\theta]_{222}$ data of Figure 3A. The conformations at high alcohol contents remain sufficiently unfolded to produce high charge states in ESI-MS.

Unfolding of aMb has been modeled as a three-state, two-transition, process (*62*, *79*). By assuming the free energies of the species involved vary linearly with denaturant concentration, fits to changes in CD spectra can used to derive equilibrium constants and hence free energy differences. This has not been attempted here because the situation is more complex. The mass spectra show that at least four species must be modeled (folded and unfolded hMb and two conformations of unfolded aMb). In principle, the mass spectra could be used to determine the relative abundances of these species. The combined effects of heat and temperature might also be studied, as was done by Brunori et al. (*56*) to obtain ΔH values for transitions between solution conformations. The ESI source used here could not be heated, but such experiments are possible in principle (*36*). However, it has not yet been demonstrated that the relative abundances of ions in the different charge state distributions match the relative solution abundances of different conformations, so it is not evident that equilibrium constants can be derived from the mass spectra.

The conformation changes described here might also be detected by chromatography. However, chromatography probes not just conformation but also binding properties to the column material, which can also induce conformation changes. For example, it has been shown that when sperm whale hMb is adsorbed on C18 or C4 sorbents, it loses heme, and separation of hMb and aMb can be difficult (*80*). In contrast, hMb and aMb are easily distinguished in ESI-MS.

The similarity between the intermediate state populated at ~35−45% methanol with the transient intermediate state of myoglobin (*38*−*40*) suggests that the alcohol-induced intermediate might be similar to on-path way intermediates. When the intermediate state of myoglobin is present in solution, there is a bimodal distribution charge state for hMb and a high charge state distribution for aMb. Bimodal charge state distributions are observed for other proteins under conditions that permit the identification of alcohol-induced intermediates by optical spectroscopy (*42*). The bimodal charge state distribution in ESI-MS may be a characteristic of intermediate states in general in which the tertiary interactions are lost and the secondary structural elements are present, indicating that the intermediates interconvert between folded and partially unfolded states. This is not detected in optical spectra which average over all species in solution. Thus, ESI-MS complements the optical techniques is two useful ways. The charge states allow detection of coexisting conformers that are not easily separated in optical spectra, and loss of heme from the protein is easily detected as a change in mass.

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