Conformational Stability of Heme Proteins *in Vacuo*†

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ABSTRACT: The function and conformational stability of myoglobin were studied *in vacuo* by using an electrospray mass spectrometer. The electrospray technique gently transfers protein molecules from the solution phase to the gas phase, and solvent-free protein ions are produced in the mass spectrometer. Horse myoglobin was dissolved at neutral pH, Fe^{3+} in the heme was reduced to Fe^{2+} to produce the biologically active oxymyoglobin in solution, and then the protein was isolated *in vacuo*. A molecular ion (17 601 Da) corresponding to the molecular mass of oxymyoglobin (17 600.0 Da) was observed in the mass spectrum. This demonstrates that the protein retains a heme and an oxygen molecule in the gas phase. Since the biological function of myoglobin is to carry an oxygen molecule, this is the first observation that a protein is functional in the absence of solvent. Gas-phase "unfolding" of myoglobin was also studied. Collisions of accelerated protein ions with nitrogen curtain gas at a quadrupole guidance lens or argon gas introduced at a second quadrupole increase the "molecular temperature" of myoglobin, resulting in release of the heme from myoglobin. Apomyoglobin produced at the quadrupole guidance lens showed a larger collisional cross section than that of myoglobin, revealing conformational disordering of the protein. The gas-phase unfolding of horse and whale myoglobins and the α-chain of human hemoglobin induced at the second quadrupole were studied as a function of the argon gas thickness. Horse and whale myoglobins showed the same gas-phase stability, whereas the α-chain of human hemoglobin was less stable. Consequently, the amino acid sequence by itself has sufficient information to stabilize the gas-phase conformation of the heme proteins.

Three distinct conformations have been elucidated as stable states of proteins. These are the native, disordered, and molten globular structures. The native conformation has a well-defined rigid structure. It is stabilized by hydrophobic interactions, electrostatic interactions, and hydrogen bonds. Protein unfolding accompanies a large increase of the conformational entropy and the molecular volume. Molten globular structure was named by Ohgushi and Wada (1983) for the structure that is compact, has secondary structures, and is missing tertiary structure (Kuwajima et al., 1976; Ahmad & Bigelow, 1979; Contaxis & Bigelow, 1981; Dolgikh et al., 1981; Denton et al., 1982). Proteins may taken any of the three states depending on the solvent composition (Denton et al., 1982); however, the free energy difference of these states is small; e.g., the native conformation is 5–15 kcal/mol more stable than the disordered conformation under physiological conditions (Privalov, 1979). These small differences in the free energy make it difficult to determine which of these states is preferred by the amino acid sequence alone. The properties of a protein solely determined by the amino acid sequence can be extracted when the protein is isolated *in vacuo*. The lack of solvent and counterions changes the interaction energies significantly. Electrostatic interaction is enhanced *in vacuo* due to the low dielectric constant ($\epsilon = 1.0$), whereas it is relatively weak in solution due to the high dielectric constant of water ($\epsilon = 78.54$) and the shielding effect of buffers and salts. The hydrogen bond is a real gain of stability in *vacuo*, whereas it is frequently countered by the intermolecular hydrogen bonds with water molecules. The hydrophobic interactions are solely contributed by van der Waals interactions *in vacuo*, whereas desolvation is another factor in solution. Due to these significant differences of the interaction energies in solution and in the gas phase, a commonly accepted assumption that all the information necessary to determine the native structure of a protein is contained in its amino acid sequence has to be examined in the isolated system of the gas phase.

Although proteins are nonvolatile, soft ionization techniques have been developed to isolate protein molecules in the gas phase for mass spectrometry. These are fast atom bombardment (Barber et al., 1981), electrospray (Dole et al., 1968; Fenn et al., 1989), ionspray (Covey et al., 1988), ^{252}Cf plasma desorption (Torgerson et al., 1974; Sundqvist & Macfarlane, 1985), and laser desorption techniques (Karas & Killenkamp, 1988). Although the mechanism of protein evaporation in the electrospray technique has not been completely elucidated (Fenn et al., 1990; Smith & Light-Wahl, 1993), the first step is generation of tiny droplets, which contain multiply charged protein molecules. While the solvent evaporates from the droplet, the electrostatic repulsions among the charged ions exceed the solvation energy of the protein ions and emit the protein ions from the droplet. The protein molecules are then introduced into the vacuum chamber of the mass spectrometer. A precise match of the protein mass with its sequence molecule mass (MMcalc),[1] e.g., an error of less than 1 Da for a 20 kDa protein (Feng et al., 1991), proves complete isolation of the protein molecules from any solvent or salts. These proteins are multiply charged so that even a large protein, complement C4 (observed molecular mass 196 863 ± 29 Da, 80–150 net

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[1] Abbreviations: DTT, dithiothreitol; MMcalc, sequence molecular mass; MMobs, molecular mass observed; m/z, mass-to-charge ratio; Q0, quadrupole guidance lens; Q1, first quadrupole; Q2, second quadrupole; Q3, third quadrupole; *R0*, Q0 AC rod offset voltage; *R2*, second quadrupole rod offset voltage; *R3*, third quadrupole rod offset voltage.

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positive charges), was measured at 1300–2400 mass-to-charge ratio (m/z) in a quadrupole mass spectrometer (Feng & Konishi, 1992).

Interestingly, the charge state of a gas-phase protein reflects its solution conformation; e.g., native cytochrome *c* at pH 5.2 showed net positive charges of 8–12, whereas unfolded cytochrome *c* at pH 2.6 showed more positive charges of 12–18 (Chowdhury et al., 1990). An interpretation is that the native conformation may have a limited capacity to hold large net charges because of its compactness, whereas the disordered conformation may be stretched out to reduce the electrostatic repulsion and retain more net charges. Furthermore, some proteins *in vacuo* were observed to retain noncovalently associated ligands (Smith & Light-Wahl, 1993), which are characteristics of the native proteins, e.g., apomyoglobin–heme complex (Katta & Chait, 1991; Li et al., 1993; Feng & Konishi, 1993b), cytoplasmic receptor (FKBP)–immunosuppressive agent (FK506 or repamycin) complexes (Ganem et al., 1991), ribonuclease S-protein–S-peptide complex (Loo et al., 1993), metallothionein–metal (Zn^{2+} or Cd^{2+}) complexes (Yu et al., 1993), and H-ras protein–GDP complex (Ganguly et al., 1992). Also, some gas-phase proteins showed a limited number of exchangeable hydrogens as well as small collision cross sections (compact structures) compared to those of the corresponding denatured proteins (Suckau et al., 1993; Covey & Douglas, 1993). These data imply that the native or at least the native-like solution conformation is retained *in vacuo*. In this report, we studied the activity of myoglobin and the conformational stability of heme proteins *in vacuo*.[2]

EXPERIMENTAL PROCEDURES

Myoglobin from horse skeletal muscle was purchased from Calbiochem. Myoglobin from sperm whale skeletal muscle, hemoglobin from human blood, and dithiothreitol (DTT) were purchased from Sigma. The proteins were used without further purification. The iron ion in these heme proteins was Fe^{3+} unless it is specifically reduced and named as ferro-, oxy-, and deoxymyoglobin.

Mass Spectrometric Measurements. A triple quadrupole mass spectrometer (a SCIEX API III System) was used for all experiments. The instrument has a m/z range of 2470 and is fitted with an ionspray (pneumatically-assisted electrospray) interface. All of the experiments were performed at room temperature. Proteins were dissolved at neutral (water or 10 mM ammonium acetate) or acidic (10% acetic acid) pH; DTT was added to prepare ferromyoglobin. A protein solution (0.1 mg/mL) was delivered to a sprayer, which generates positively charged small droplets. The protein ions emitted from the surface of the droplet are guided to the mass spectrometer. The protein ions entered into the mass spectrometer through an orifice traverse a quadrupole guidance lens (Q0) and first, second, and third quadrupoles (Q1, Q2, and Q3, respectively) to a detector for counting. The molecular mass of protein ions was determined by either a Q1 or a Q3 mass analyzer. The m/z scales of Q1 and Q3 analyzers were calibrated with the ammonium adduct ions of poly(propylene glycols). Argon gas was introduced at Q2 for collision experiments (Covey & Douglas, 1993), and its thickness is defined as (the gas density at Q2) × (the Q2 cell length). The details of the electrospray mass spectrometer are described elsewhere (Bruins et al., 1987; Covey et al., 1988; Fenn et al., 1989, 1990; Feng & Konishi, 1993a).

"Unfolding"[2] of Myoglobin at Q0. Myoglobin from horse skeletal muscle was dissolved in water at a concentration of 0.1 mg/mL and was partially reduced by 5 mM DTT. A moderately high orifice voltage (160 V) was applied to accelerate myoglobin ions during the free jet expansion at Q0. Upon the collisions with nitrogen gas at Q0, a fraction of collision energy is converted to the internal energy of the protein ions. The increase of the "molecular temperature" of the protein ions may induce unfolding of the gas-phase myoglobin. All of the protein molecules heated at Q0 were transported to Q2 using Q1 as an ion transporter for further characterization. The ions were slightly accelerated by a low potential difference (ΔV = 53 V) between Q0 (rod offset voltage $R0$ = +30 V) and Q2 (rod offset voltage $R2$ = –23 V) and passed Q2 in the absence or the presence of collision argon gas. Collisions of the low-velocity protein ions with argon gas predominantly slow down the velocity of the protein ions. The Q3 rod offset voltage ($R3$) was set to +28 V so that only the ions with sufficient translational energy could pass Q3 and reach the detector. Since the ions lose kinetic energy exponentially with increasing collision cross section (Covey & Douglas, 1993), the relative intensity loss of the ions due to the collisions was monitored to compare the collision cross sections of myoglobin and apomyoglobin generated at Q0.

Unfolding of Heme Proteins at Q2. Heme proteins (0.1 mg/mL) were dissolved in 10 mM ammonium acetate at neutral pH, and 5 mM DTT was then added for the partial reduction of the heme iron. The native conformations of the heme proteins are stable in solution at neutral pH. The protein ions introduced into the mass spectrometer were minimally heated during the free jet expansion at Q0 by lowering the orifice voltage to +100 V. Quadrupole Q1 was operated in the mass-resolving mode to pass only the protein ions with certain net charges to Q2. The ions were moderately accelerated by the potential difference (ΔV = 105 or 150 V) between Q0 and Q2. Collisions of the moderately accelerated protein ions with argon gas at Q2 convert a portion of the collisional energy to the internal energy of the protein ions and elevate the molecular temperature of the protein ions. This increase of the molecular temperature was regulated by argon gas thickness. Quadrupole Q3 was operated in the mass-resolving mode to identify the collision products and to quantitate them on the basis of their relative peak intensities.

RESULTS AND DISCUSSION

Denatured Horse Apomyoglobin in Vacuo. It is well established that horse myoglobin is denatured at low pH (Puett, 1973; Bismuto et al., 1983). The denaturation is accompanied by the release of the heme. A mass spectrum of horse apomyoglobin and the heme in 10% acetic acid showed a series of multiply charged ions at m/z 650–1420 and a peak at m/z 616.5 (Figure 1A). The charge states of the multiply charged ions are labeled at the top of the peaks. The average molecular mass derived from these multiple peaks is 16 950.5 ± 2.4 Da, which is consistent with the sequence molecular mass (MMcalc = 16 951.5 Da) of horse apomyoglobin. The spectrum showed no peak corresponding to horse myoglobin, revealing the complete dissociation of apomyoglobin and the heme *in vacuo*. Figure 1A also shows a peak at m/z 616.5, of which the charge state was determined as 1+ based on the isotope peaks at m/z 617.5 and 618.5. This peak corresponds to Fe^{3+}-protoporphyrin IX (z = 1+, MMcalc = 616.5 Da) dissociated from myoglobin upon its denaturation.

[2] It should be noted that the conformations and conformational changes of gas-phase proteins are described using the terminologies of proteins in solution in this early study of gas-phase proteins. Some of them may not be appropriate for gas-phase proteins and may be replaced by the terminologies specific to the gas-phase proteins when sufficient amounts of information of gas-phase proteins are accumulated.

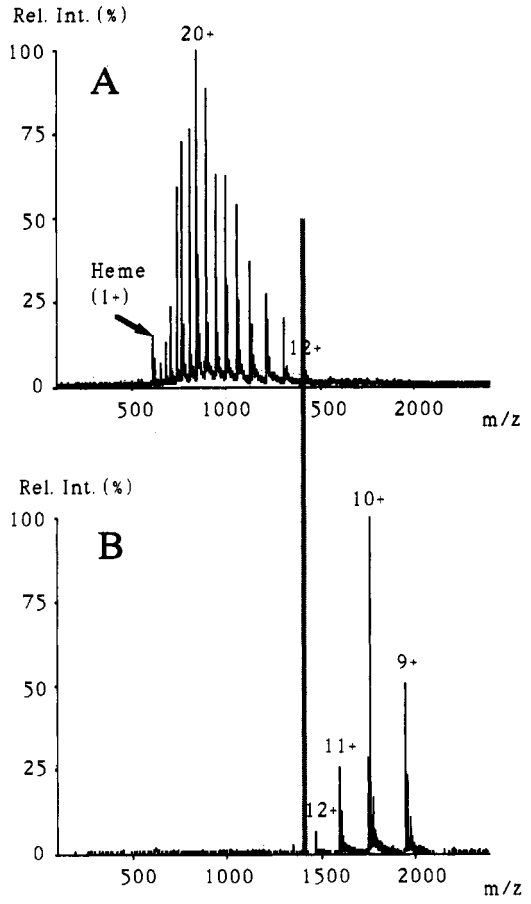

FIGURE 1: Mass spectra of (A) horse skeletal muscle apomyoglobin (0.1 mg/mL) and a heme in 10% acetic acid and (B) horse skeletal muscle myoglobin (0.1 mg/mL) in water. The charge states of the proteins are labeled for some peaks. A shaded line at the peak (m/z 1413.6) of apomyoglobin^{12+} illustrates that no corresponding peak was observed in Figure 2B.

Each charge state of apomyoglobin showed minor multiple peaks besides the predominant peaks corresponding to apomyoglobin. These are sulfate or phosphate adduct(s) of the protein with 98 × n (n = 1, 2, ...) Da higher than the molecular mass of the protein. Other common adducts in electrospray mass spectrometry are ammonium, sodium, and potassium. These minor adduct peaks were disregarded in this study unless specified.

Native Horse Myoglobin in Vacuo. Horse ferrimyoglobin (Fe^{3+}) was dissolved in water. The protein in solution has a stable native conformation at neutral pH and room temperature, and the heme is associated with apomyoglobin. The protein injected into the mass spectrometer showed a mass spectrum with a series of multiply charged ions between m/z 1350 and 2000 (Figure 1B). The molecular mass determined (MMobs = 17 567.2 ± 1.3 Da) was consistent with that (MMcalc = 17 568.0 Da) of horse ferrimyoglobin. As shown by the shaded bar at m/z 1413.5 corresponding to apomyoglobin^{12+}, no peak corresponding to apomyoglobin was observed. The peak at m/z 616.5 corresponding to the heme was also not observed. Thus, the ionization and the desolvation of myoglobin in the ionspray technique seem to be soft enough not to dissociate the heme from the protein. Furthermore, it is interesting that horse myoglobin does not require hydration to retain the heme during its millisecond traverse in the mass spectrometer, while heme association is characteristic of native myoglobin in solution.

It is a fundamentally critical question whether the amino acid sequence has sufficient information to retain the biological activity of a protein or not. The biologically functional form of myoglobin, which serves as a reserve supply of oxygen and facilitates the movement of oxygen within muscle, is oxymyoglobin, which holds the heme (Fe^{2+}) and an oxygen molecule. Thus, the mass spectrometer was used to examine the oxygen binding capability of myoglobin isolated *in vacuo*. First, oxymyoglobin was prepared in solution by reducing ferrimyoglobin (1 mg/mL) in water with 50 mM DTT at 37 °C for 1 h and by diluting it subsequently 10-fold with air-saturated water. The protein showed a characteristic absorption spectrum of oxymyoglobin with two maxima at 544 and 582 nm compared to the maxima of ferrimyoglobin and deoxymyoglobin at 500 and 630 nm and at 555 nm, respectively (Fasman, 1989). Immediately after the dilution, the protein was injected into the mass spectrometer. The mass spectrum showed a new peak at m/z 1761.1 with a 10+ net charge (Figure 2), which corresponds to oxymyoglobin (MMobs = 17 601 Da and MMcalc = 17 600.0 Da). The peaks at m/z 1757.8 and 1767.8 corresponded to deoxymyoglobin (MMobs = 17 568 Da and MMcalc = 17 568.0 Da) and deoxymyoglobin with a sulfate or phosphate adduct (98 Da), respectively. It should be mentioned that no oxygen attachment was observed in ferrimyoglobin (Fe^{3+}) as expected. Therefore, myoglobin is able to retain the function *in vacuo* in defining its function as to hold the heme (Fe^{2+}) and an oxygen molecule.

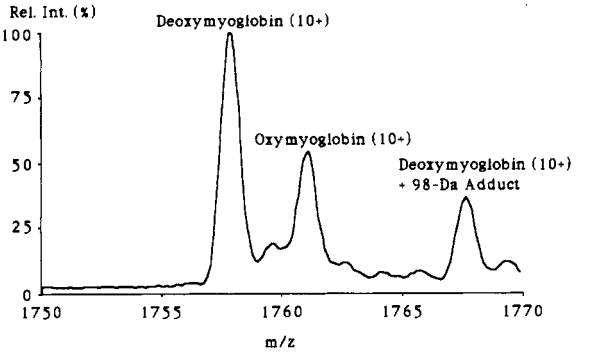

FIGURE 2: Mass spectrum of ferromyoglobin (0.1 mg/mL) in 5 mM DTT/H_2O. The iron ion (Fe^{3+}) was reduced by incubating the protein (1 mg/mL) in 50 mM DTT at 37 °C for 1 h. The protein solution was then diluted 10-fold with air-saturated water just before the measurement. The three major peaks at m/z 1757.8, 1761.1, and 1767.7 were identified as deoxymyoglobin^{10+}, oxymyoglobin^{10+}, and deoxymyoglobin^{10+} with an adduct of phosphate or sulfate, respectively, based on their molecular masses.

Gas-Phase Unfolding of Myoglobin. The primary function of nitrogen curtain gas at the orifice is to remove the residual solvent molecules from the protein. However, when myoglobin ions were further accelerated by increasing the orifice voltage to 160 V, the ions moderately collide with nitrogen gas during the free jet expansion at Q0, and some of the ions were subsequently dissociated to apomyoglobin and the heme (Katta & Chait, 1991; Feng & Konishi, 1993b) as shown in Figure 3A, where the peaks corresponding to myoglobin are labeled with (●) and the peaks corresponding to apomyoglobin are labeled with (◊). In Figure 3, the data were recorded only around the peaks in order to improve the signal/noise ratio, ignoring the spaces between the peaks (the appearance of no other peaks in the spaces unrecorded was confirmed by a separate experiment). The data were collected every 0.3 m/z interval, and the intensities of each data point were added to integrate the peak intensities. The peaks due to multiple adducts of potassium ion complicated the spectrum and were more enhanced in myoglobin than in apomyoglobin, especially for the peaks at around m/z 2100–2240. Due to the contributions of these adduct peaks, the intensity ratioes

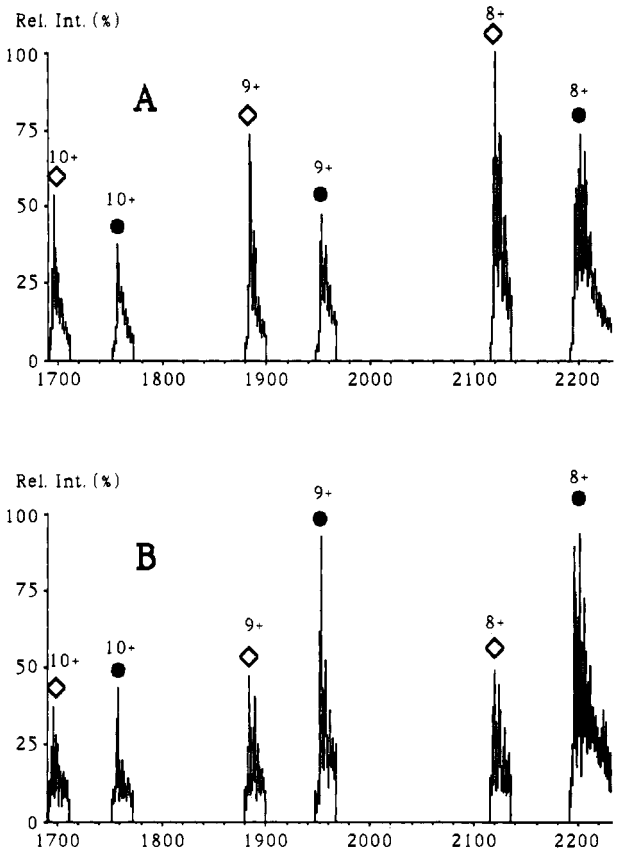

FIGURE 3: (A) Mass spectra of myoglobin (●) and apomyoglobin (◇). Apomyoglobin was generated by dissociating myoglobin at Q0 by a high orifice voltage (160 V) without Q2 collisions. The integrated peak intensity ratios of myoglobin and apomyoglobin were 55% and 45%, respectively. The protein was dissolved in water at a concentration of 0.1 mg/mL and was partially reduced by 5 mM DTT. Q3 was used to scan the spectrum. (B) Mass spectra of myoglobin (●) and apomyoglobin (◇) with Q2 collisions. The condition is the same as Figure 4A, except that argon collision gas was introduced at Q2 to slow down the protein ions. Apomyoglobin was slowed down more than myoglobin so that fewer apomyoglobin ions (28%) passed the potential barrier ($R3 = +28$ V) of the selective ion stopper Q3 than myoglobin ions (72%). The spectrum was scanned using Q3.

including the peaks of the adducts were 55% for myoglobin and 45% for apomyoglobin. An interpretation of myoglobin dissociation at Q0 is that a portion of the collision energy is converted to the internal energy of myoglobin and the increased molecular temperature subsequently unfolds the protein. Then, the disordered polypeptide, which has a low affinity to the heme, releases the heme. Or the nitrogen gas might simply knock the heme out of the binding pocket without inducing conformational change, in a similar way to dissociating the residual solvent molecules from the protein. These two possible mechanisms (the former unfolds the protein, and the latter retains the native conformation) may be examined by a method sensitive to the conformational changes of gas-phase proteins. Suckau et al. (1993) reported an elegant method to monitor hydrogen–deuterium exchange of gas-phase protein ions. The number of exchangeable hydrogens was nearly independent of charge state but sensitive to the conformational states; e.g., they observed at least three distinct gaseous conformers of cytochrome *c* ions. Covey and Douglas (1993) reported another method to determine the collision cross section of gas-phase protein ions and found two distinctive conformers of gas-phase cytochrome *c* ions. In this study, the cross section of apomyoglobin relative to that of myoglobin was examined as described under Experimental Procedures. The intensity

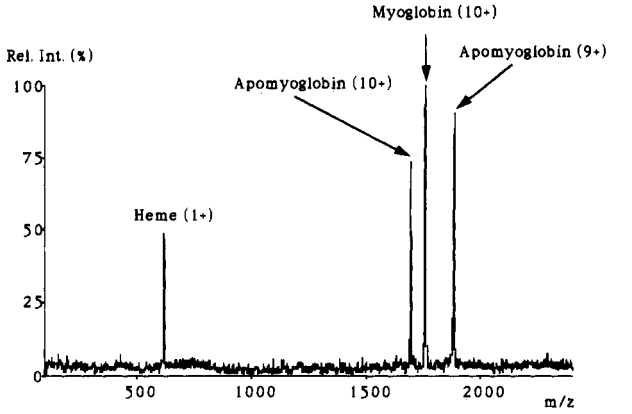

FIGURE 4: Unfolding of horse skeletal muscle myoglobin at Q2. The protein was dissolved in water at a concentration of 0.1 mg/mL and was partially reduced by 5 mM DTT. Myoglobin^{10+} (m/z 1758) was selectively passed by Q1 and collided with argon collision gas (3.16×10^{14} cm^{-2}) at Q2 after moderate acceleration by Q0 and Q2 rod offset voltages (35 and −70 V, respectively). Ferrimyoglobin^{10+} (Fe^{3+}) was dissociated to apomyoglobin^{9+} (m/z 1884) and heme^{1+} (m/z 616.5), whereas ferromyoglobin^{10+} (Fe^{2+}) was dissociated into apomyoglobin^{10+} (m/z 1696) and heme^{0+}, which was not detectable in the mass analyzer due to the lack of net charge.

ratio of myoglobin and apomyoglobin was 55 and 45%, respectively, in the absence of argon gas and 72 and 28%, respectively, in the presence of argon gas (the gas thickness was 3.0×10^{14} cm^{-2}). Since the collision gas induces the kinetic energy loss of the ions exponentially with increasing collision cross section (Covey & Douglas, 1993), apomyoglobin, which lost more kinetic energy than myoglobin by the collisions, must have a larger collision cross section than that of myoglobin at Q2. Consequently, the loss of the heme at Q0 induced conformational disordering of the polypeptide before it entered Q2, and the gas-phase unfolding of myoglobin may be monitored by the loss of the heme.

Gas-Phase Unfolding at Q2. As suggested above, the gas-phase unfolding of myoglobin may also occur at Q2 through the collisions with argon gas introduced at Q2. The gas-phase unfolding experiments at Q2 have the following advantages: (1) the acceleration of the protein molecules is more precisely controlled by the difference of the Q0 and Q2 rod offset voltages than that at the orifice, where their energies are not well controlled due to the high pressure; (2) the number of collisions (N) is given by

$$N = S\sigma \qquad (1)$$

where S is the collision gas thickness and σ is the collision cross section of the protein (Covey & Douglas, 1993). On the other hand, the collisions with nitrogen gas at the orifice during the free-jet expansion are difficult to quantify. (3) The unfolding of a protein with a certain m/z can be studied by selective transmittance of an ion using Q1 in the mass-resolving mode, whereas all of the protein ions are subject to unfold at Q0 and it is difficult to study the effect of the charge state of the ions; (4) the products of protein unfolding at Q2 can be identified and characterized by using Q3 in the mass-resolving mode. Figure 4 shows the unfolding of ferro- and ferrimyoglobin^{10+}, which were selected at Q1 and were partially unfolded through collisions with argon gas ($S = 3.16 \times 10^{14}$ cm^{-2}) at Q2. Myoglobin was moderately accelerated by the voltage difference ($\Delta V = 105$ V) between Q0 ($R0 = 35$ V) and Q2 ($R2 = -70$ V) so that some of the molecules were heated hot enough to unfold and release the heme. Upon unfolding, ferrimyoglobin^{10+} (Fe^{3+}) was dissociated to apomyoglobin^{9+} and heme^{1+}, whereas ferromyoglobin^{10+} (Fe^{2+}) was

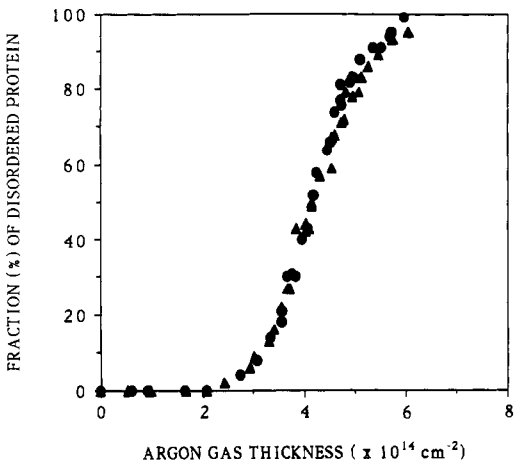

FIGURE 5: Gas-phase stabilities of horse skeletal muscle myoglobin (●) and sperm whale myoglobin (▲). The proteins were dissolved in 10 mM ammonium acetate (pH 8) at concentrations of 0.1 mg/mL and were partially reduced by 5 mM DTT. Horse myoglobin^{9+} (m/z 1953) and whale myoglobin^{9+} (m/z 1980) were selectively passed by Q1 and collided with argon gas at various thickness [(0–6) × 10^{14} cm^{-2}] after moderate acceleration by Q0 and Q2 rod offset voltages (30 and −120 V, respectively). The peak intensities of myoglobin and apomyoglobins were integrated to estimate the fraction of disordered proteins.

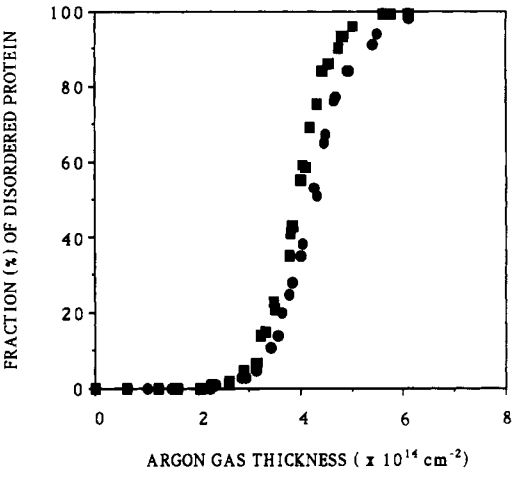

FIGURE 6: Gas-phase stabilities of horse skeletal muscle myoglobin (●) and the α-chain of human hemoglobin (■). The proteins were dissolved in 10 mM ammonium acetate (pH 8) at a concentration of 0.1 mg/mL and were partially reduced by 5 mM DTT. Horse myoglobin^{9+} (m/z 1953) and the α-chain of human hemoglobin^{8+} (m/z 1980) were selectively passed by Q1 and collided with argon gas at various thickness [(0–6) × 10^{14} cm^{-2}] after moderate acceleration by Q0 and Q2 rod offset voltages (30 and −120 V, respectively). The peak intensities of myoglobin or hemoglobin and the corresponding apoproteins were integrated to estimate the degree of unfolding.

dissociated to apomyoglobin^{10+} and heme^{0+} (not detected in the mass spectrometer). As a result, three new peaks (heme^{1+}, apomyoglobin^{10+}, and apomyoglobin^{9+}) were observed at m/z 616.2, 1696.1, and 1884.2, respectively. The relative intensities of these peaks were sensitive to the kinetic energy of myoglobin at Q2 and the density of argon gas; i.e., higher acceleration and higher argon gas density increased the peak intensities of apomyoglobin^{9+}, apomyoglobin^{10+}, and heme^{1+}. However, the relative peak intensity of apomyoglobin^{9+} to that of apomyoglobin^{10+} was constant at various degrees of unfolding. This means that ferrimyoglobin^{10+} (Fe^{3+}) and ferromyoglobin^{10+} (Fe^{2+}) have the same gas-phase stability. Although the electrostatic interactions are enhanced in the gas phase, the net electrostatic interactions of these two species seem to be very close, in spite of their different charge distribution due to the localized one positive charge on the heme of ferrimyoglobin^{10+}.

Conformational Stability of Heme Proteins in Vacuo. The gas-phase unfolding of horse myoglobin at Q2 was monitored by the peak intensities of myoglobin^{9+} at m/z 1953 and two products of apomyoglobins^{8+} at m/z 2120 and apomyoglobin^{9+} at m/z 1885. The fraction of disordered apomyoglobin was estimated from the intensities of two apomyoglobin peaks normalized by the intensities of all three peaks (one myoglobin and two apomyoglobin peaks) and was plotted as a function of the argon gas thickness (Figure 5). The density of the argon gas varied up to 6.1 × 10^{14} cm^{-2}, while the Q0 and Q2 rod offset voltages were fixed to 30 and −120 V, respectively. At low argon gas thickness (below 2 × 10^{14} cm^{-2}), no unfolding of the protein was observed. The unfolding started above 2 × 10^{14} cm^{-2} of gas thickness and was completed at around 6 × 10^{14} cm^{-2}. The 50% unfolding of gas-phase myoglobin^{9+} occurred at 4.2 × 10^{14} cm^{-2} of collision gas thickness. A myoglobin molecule that passes the collision cell filled with argon gas at a thickness of 4.2 × 10^{14} cm^{-2} has an average of 67 collisions according to eq 1 (approximate cross section of myoglobin is 1.6 × 10^3 Å^2 based on the X-ray-determined dimension of myoglobin of 44 Å × 44 Å × 25 Å; Covey & Douglas, 1993). The center-of-mass collisional energy (E_{CM}) is given by

$$E_{CM} = \{MM_{Ar}/(MM_{protein} + MM_{Ar})\}ZV \quad (2)$$

where MM_{Ar} is the molecular mass of argon, $MM_{protein}$ is the molecular mass of the protein, Z is the net charge of the protein, and ΔV is the difference of the Q0 and Q2 rod offset voltages. E_{CM}, which is the maximum energy that can be converted to the internal energy of the protein at each collision, is 71 kcal/mol for myoglobin^{9+} at a ΔV of 150 V. Thus, the maximum energy that can be converted to the internal energy is 4.7 × 10^2 kcal/mol after the 67 collisions at Q2.

Similarly, the unfolding of sperm whale myoglobin, which has 99% sequence homology (87% identity) to horse myoglobin, was studied. The unfolding of myoglobin^{9+} (m/z 1980) was monitored under the same set of Q0 and Q2 rod offset voltages ($R0$ = 30 V and $R2$ = −120 V, respectively), where E_{CM} is 70 kcal/mol. The gas thickness required for 50% unfolding of whale myoglobin^{9+} was 4.2 × 10^{14} cm^{-2} (Figure 5). Assuming the same molecular shape and partial specific volume of whale myoglobin^{9+} as those of horse myoglobin^{9+}, whale myoglobin^{9+} has 0.92% larger cross section than that of horse myoglobin^{9+} and makes on average 69 collisions. The maximum energy to be transferred to the internal energy was 4.8 × 10^2 kcal/mol, which is only 1.2% higher than that of horse myoglobin. Assuming the same efficiency of energy transfer for these homologous proteins, horse and whale myoglobins have very similar gas-phase stability.

The gas-phase stability of the α-chain of human hemoglobin^{8+} (m/z 1969), which has 70% sequence homology (22% identity) to horse myoglobin, was also studied in comparison to that of horse myoglobin^{9+} (Figure 6). The gas thickness required for 50% unfolding of horse myoglobin and the α-chain of human hemoglobin was 4.3 × 10^{14} and 3.9 × 10^{14} cm^{-2}, respectively. The Q0 and Q2 rod offset voltages were fixed to 30 and −120 V, respectively, while the collision gas thickness varied up to 6.1 × 10^{14} cm^{-2}. The center-of-mass collisional energy was 70 kcal/mol. Assuming the same molecular shape

and partial specific volume of human hemoglobin^{8+} as those of horse myoglobin^{9+}, human hemoglobin^{8+} has a 7.0% smaller cross section than that of horse myoglobin^{9+} and makes on average 58 collisions. The maximum energy that can be converted to the internal energy was 4.1×10^2 kcal/mol, which is 15% less than that (4.8×10^2 kcal/mol based on the gas thickness of 4.3×10^{14} cm^{-2} for 50% unfolding in Figure 6) of horse myoglobin. Assuming the same efficiency of energy conversion for the homologous proteins, the α-chain of human hemoglobin^{8+} is less stable than horse or whale myoglobins *in vacuo*.

CONCLUSION

Accurate molecular determination of myoglobin using an ionspray mass spectrometer revealed that horse myoglobin is functional to carry an oxygen molecule in the absence of solvent. Collisions of myoglobin with nitrogen gas at the orifice or with argon gas at the second quadrupole could induce the gas-phase disordering of the protein, which was verified by the increase of the cross section and the release of the heme. A study of the conformational stability of gas-phase heme proteins, that is exclusively determined by their amino acid sequences, revealed that horse and whale myoglobins have the same stability, whereas the α-chain of human hemoglobin is less stable.

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