Dissociation of Heme from Myoglobin and Cytochrome b_5: Comparison of Behavior in Solution and the Gas Phase[†,‡]

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ABSTRACT: The relationship of the structure of a protein in solution to the structure of a gas-phase protein ion and the manner in which gas-phase protein ions bind small molecules noncovalently are topics of current debate. To address these issues, the stability of heme binding to wild-type and variant forms of apomyoglobin and apocytochrome b_5 has been studied in the gas phase by electrospray mass spectrometry (ES-MS) and compared with the stability of heme binding to the same proteins in solution. The voltage required to dissociate ions of the heme−protein complexes in the orifice−skimmer region of an electrospray mass spectrometer, a measure of the complex stability, is found to be correlated with the activation energy for dissociation of the complexes in solution across a series of proteins in which the number of hydrogen bonds between the heme propionate groups and surface residues is systematically reduced. However, variants in which the hydrogen bonds to the proximal histidine have been removed are destabilized in solution but stabilized in the gas-phase ions. These results suggest that on the millisecond time scale of the ES-MS experiment, the gas-phase protein ion may retain much of the structure of the protein in solution, at least for those residues surrounding the heme group. Furthermore, the ability of ES-MS to detect relatively subtle differences in protein−small molecule complex stability demonstrated in this work suggests that this technique may be a convenient, sensitive, and generally useful strategy for physical characterization of such complexes.

Electrospray mass spectrometry (ES-MS) allows the production of intact protein ions in the gas phase (Fenn et al., 1989, Smith et al., 1990; Chait & Kent, 1992). The relationship of the structure and properties of these ions to those of proteins in solution is an issue of extensive debate (e.g., Wolynes, 1995). Current methods for structural characterization of gas-phase protein ions include assessment of H/D exchange of trapped ions (Suckau et al., 1993; Wood et al., 1995), relative reactivity (McLuckey et al., 1990; Winger et al., 1992; Orgorzalek Loo & Smith, 1994) and basicity (Schnier et al., 1995; Gross et al., 1996) of various charge states, sizes and shapes of ion impact damage on surfaces (Quist et al., 1994; Reimann et al., 1994; Sullivan et al., 1996) and measurements of ion collision cross sections (Covey & Douglas, 1993; Douglas, 1994; Cox et al., 1994; von Helden et al., 1995; Clemmer et al., 1995). These methods provide an indication of ion size or shape; however, detailed structural information derived from such work is largely inferential.

Electrospray is a gentle ionization process that permits observation in the gas phase of specific complexes formed through noncovalent association of proteins with smaller molecules and other proteins [for reviews see Smith and Light-Wahl, (1993) and Smith and Zhang (1994)]. Clusters (Shahin, 1966) and covalent bonds (Caldecourt et al., 1983; Aleksandrov et al., 1984) of ions formed in atmospheric pressure ion sources can be broken by collisionally activated dissociation (CAD) during the ion sampling process. CAD is achieved by the use of electric fields to accelerate ions through the locally high density of gas near the ion sampling orifice. The resulting collisions of ions with the gas transfer translational energy of the ions to internal energy and lead to ion dissociation (Bruins, 1991).

Recently, the method of collisionally activated dissociation has been used to dissociate noncovalently associated protein−substrate complexes (e.g., Baca & Kent, 1992; Smith & Light-Wahl, 1993; Tang et al., 1994; Lim et al., 1995; Loo, 1995). Complexes of greater stability generally require a greater voltage for dissociation. This method has been used to compare the relative stabilities of ras-GDP and interferon γ dimers (Huang et al., 1993), relative binding energies in heme−globin complexes (Li et al., 1993), and tetramers of concanavalin A and hemoglobin (Light-Wahl et al., 1994). Several groups have reported detection of ions of the heme−protein complex of myoglobin (Mb) by electrospray (Katta & Chait, 1991). These heme−protein complexes can also be dissociated under more energetic conditions in the ion sampling interface (Jaquinod et al., 1993; Feng & Konishi, 1993; Konishi & Feng, 1994). An important issue that has not been evaluated adequately and that can in principle give detailed structural information on gas-phase protein ions is whether the relative stability of noncovalently associated complexes in the gas phase correlates with the stability of such complexes in solution. A particularly attractive strategy for assessing this correlation would be to compare the contributions of individual hydrogen bonds toward the stability of a series of protein−small molecule complexes

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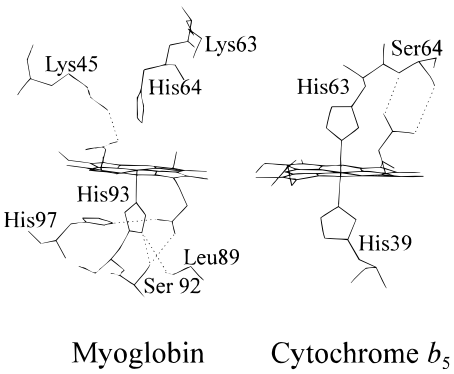

FIGURE 1: Hydrogen bonds (dotted lines) formed by the heme propionate groups of Mb and cytochrome b_5 with surface amino acid residues.

in the gas and aqueous phases.

Such a relationship has been evaluated here through the investigation of the interaction of the ferriprotoporphyrin IX prosthetic group (heme) with a series of horse heart Mb variants and bovine liver cytochrome b_5 derivatives. Both Mb and cytochrome b_5 bind the heme group noncovalently through (i) axial coordination of heme iron to protein ligands, (ii) van der Waals contact between the heme π electron system and hydrophobic protein residues, and (iii) hydrogen-bonding interactions between heme propionates and adjacent charged surface residues of the protein. These interactions allow the protein to "recognize" the heme group, and they can be modified in a controlled fashion by genetic or chemical means. The hydrogen-bonding interactions of the heme propionate groups have been defined by X-ray diffraction analysis of Mb and cytochrome b_5 (Evans & Brayer, 1990; Durley & Mathews, 1996) and are illustrated in Figure 1. In the work reported here, the hydrogen bonds formed by the heme propionates in Mb with residues 45, 92, and 97 have been removed by site-directed mutagenesis (Hunter et al., 1997). Reconstitution of Mb and cytochrome b_5 with dimethylester heme (DME-heme) (Lim, 1990; Reid et al., 1984), heme in which the propionate groups have been converted to the corresponding methyl esters, also changes the hydrogen-bonding properties of the propionates (Hunter et al., 1997). With these specifically modified proteins, a series of protein−heme complexes has been constructed in which the hydrogen-bonding interactions between the heme and the apoprotein have been removed systematically.

This ensemble of proteins provides an informative system for evaluation of the retention of molecular recognition specificity by a protein in the gas phase. The relative stability of heme binding to Mb and cytochrome b_5 in the gas phase was determined by measuring the voltage required to dissociate the protein−heme complexes by collisionally activated dissociation in the differentially pumped ion sampling interface of an ES-MS. The stability of heme binding in solution was quantified by measuring the Arrhenius activation energy, E_A, for heme dissociation from Mb and cytochrome b_5. These measurements show that (i) small differences in the affinity of proteins for ligands can be detected in the gas phase and (ii) the voltages required to dissociate the gas phase noncovalently associated complexes in the ion sampling region of an electrospray mass spectrometer are correlated with the activation energies for heme dissociation in solution for those proteins in which the number of hydrogen bonds between the heme propionate

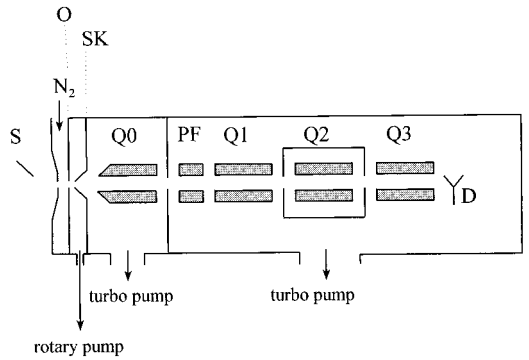

FIGURE 2: Diagram of the triple-quadrupole mass spectrometer: S, electrospray source; O, sampling orifice; SK, skimmer; Q0, RF only quadrupole; PF, prefilter; Q1, mass-analyzing quadrupole; Q2, RF-only quadrupole collision cell; Q3, quadrupole operated in RF-only mode for the orifice−skimmer dissociation measurements and in mass-analyzing mode for MS/MS experiments; D, ion detector. The background pressures were 2 Torr in the orifice−skimmer region, 6×10^{-3} Torr in Q0, and 2×10^{-5} Torr in the Q1−Q3 region.

groups and surface protein residues are systematically reduced. This observation suggests that the heme−protein complex in the gas phase may retain known solution structure, at least for residues surrounding the heme group.

EXPERIMENTAL PROCEDURES

The proteins used in this study were prepared and purified as described previously by Hunter et al. (1997).

Heme Dissociation Kinetics. The kinetics of heme dissociation from the wild-type and modified forms of Mb and cytochrome b_5 were determined as described elsewhere (Hargrove et al., 1994; Hunter et al., 1997). Dissociation of heme was monitored under pseudo-first-order conditions with the H64Y/V68F apoMb variant in excess (90 μM) relative to Mb or cytochrome b_5 (3 μM). The rate constants for dissociation were measured over the temperature range of 20−37 °C (0.15 M sodium acetate buffer, pH 5.0, and 0.45 M sucrose). Kinetic data were collected at pH 5.0 because heme dissociates from these proteins much more slowly at higher pH, thereby complicating precise determination of rate constants due to the lengthy exposure of apoMb to elevated temperatures. Rate constants were determined from single-exponential fits of the rate data using the program Scientist (version 2.0, Micromath, Inc.). Activation energies were calculated (MINSQ, version 4.02, Micromath, Inc.) from the Arrhenius equation:

$$\ln k = \ln A - E_A/RT \quad (1)$$

where E_A is the activation energy, A is the preexponential factor, R is the gas constant, and T is the temperature.

Dissociation of Heme−Protein Complexes in the Gas Phase. Ions of Mb and cytochrome b_5 in the gas phase were dissociated by collisionally activated dissociation (CAD) in the ion sampling region of an electrospray triple-quadrupole MS/MS system (ES-MS) (Figure 2). Gas-phase protein ions, formed by pneumatically assisted electrospray, are passed through a dry nitrogen curtain gas, a 0.25-mm sampling orifice into a region with a background pressure of 2 Torr, and then through a skimmer to a radiofrequency- (RF-) only quadrupole. The orifice−skimmer separation is 0.20 cm. The gas expanding through the orifice forms a free jet (Douglas & French, 1988) where the gas number density

decreases from 2.5×10^{19} cm^{-3} at the sampling orifice to 3.5×10^{16} cm^{-3} at the skimmer tip. The terminal flow speed, reached within a few orifice diameters downstream of the sampling orifice, is 7.8×10^4 cm s^{-1}. In the absence of any electric fields the ions are carried by the gas flow from the orifice to the skimmer in 2.6 μs. The voltage difference across the orifice−skimmer region (ΔV_{OS}) was varied from 30 to 150 V by changing the DC voltage on the orifice plate to dissociate the heme−protein complexes by CAD. This potential difference accelerates the ions through the gas to reduce the transit time below 2.6 μs. Ions are activated in a series of comparatively low-energy collisions, somewhat analogous to the activation process in drift tube experiments (Glosik et al., 1994). The mean free path, for a collision cross section of 2500 Å^2 (Douglas & Collings, 1996), varies from 1.6×10^{-7} cm at the sampling orifice to 1.2×10^{-4} cm at the skimmer. For a typical orifice−skimmer voltage difference of 80 V the ratio of electric field ϵ to gas number density n (ϵ/n) varies from 1.6×10^{-17} V cm^2 at the sampling orifice to 1.14×10^{-14} V cm^2 at the skimmer tip. Ions dissociate either in the orifice skimmer region or in transit through the RF-only quadrupole (Q0) before mass analysis in quadrupole one (Q1). The abundances of holoprotein, charge $(n)^+$ and the corresponding apoprotein, charge $(n - 1)^+$, were measured for each charge state pair at each orifice voltage. Because the ion transmission through the interface changes at different ΔV_{OS}, the relative abundances [holoprotein $(n)^+$]/[holoprotein $(n)^+$ + apoprotein $(n - 1)^+$] and [apoprotein $(n - 1)^+$]/[holoprotein $(n)^+$ + apoprotein $(n - 1)^+$] were calculated for each voltage, and the voltage that gave 50% heme dissociation for the holoprotein was determined, i.e., [holoprotein $(n)^+$]/[holoprotein $(n)^+$ + apoprotein $(n - 1)^+$] and [apoprotein $(n - 1)^+$]/[holoprotein $(n)^+$ + apoprotein $(n - 1)^+$] = 0.5. This was done for the most abundant charge states of the ions, 11^+−14^+ for Mb and 7^+−10^+ for cytochrome b_5. Interestingly, the mutations did not lead to shifts in the charge state distributions despite removal of up to three basic residues. Also, no significant changes in the charge state distribution were observed at higher ΔV_{OS}, other than the shift due to the loss of heme $(1)^+$. The average of these four voltages for each variant protein was taken as a measure of the voltage required to dissociate the heme−protein complex (V_D). The sprayer position was kept constant for all the measurements. Solutions were 5 μM Mb in 40 μM Tris-HCl buffer, pH 8.0/10% methanol and 5 μM cytochrome b_5 in 40 μM sodium phosphate buffer, pH 7.0/10% methanol.

Tandem Mass Spectrometry. In the tandem mass spectrometry (MS/MS) experiments, holo precursor ions were mass-selected in the first quadrupole and dissociated in the Q2 collision cell [2.2 mTorr and 10 neV collision energy for ions of charge $(n)^+$]. The resulting fragment ions were then mass-analyzed in the third quadrupole (Q3).

RESULTS AND DISCUSSION

Solution Activation Energies. The kinetics of heme dissociation from all proteins studied (20−37 °C) were described well with a single-exponential function. The results obtained for the H97L/K45E variant of Mb are representative (Figure 3) as is the Arrhenius plot (inset). Corresponding results obtained for the other proteins used in this study also produced linear Arrhenius plots over this temperature range.

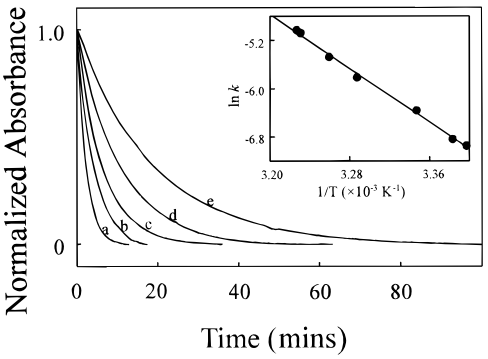

FIGURE 3: Absorbance vs time for heme dissociation from H97L/K45E Mb (pH 5.0) at temperatures of (a) 37, (b) 34, (c) 31, (d) 26, and (e) 21 °C. The activation energy, E_A, was derived from these data (inset) by fitting the rate constants to the Arrhenius equation (eq 1).

Table 1: Activation Energies and Dissociation Voltages for Wild-Type and Modified Forms of Horse Heart Mb and Bovine Liver Cytochrome b_5

protein	no. of H-bonds	ln A (s^{-1})	E_A (kJ/mol)	V_D (V)
Myoglobin				
wild type	3	34 ± 3	106 ± 7	85 ± 2
K45E	2	31 ± 2	97 ± 4	80 ± 1
H97L	2	30 ± 1	93 ± 4	81 ± 1
K45E/H97L	1	29 ± 1	87 ± 3	72 ± 2
S92D/K45E	1	27 ± 2	81 ± 4	89 ± 3
DME-Mb	0	26 ± 2	77 ± 5	66 ± 1
K45E/K63E/H97L/S92A	0	25 ± 1	73 ± 3	78 ± 4
Cytochrome b_5				
lipase-solubilized	2	38 ± 3	116 ± 7	65 ± 1
trypsin-solubilized	2	38 ± 2	115 ± 4	65 ± 1
DME−trypsin-solubilized	1[a]	34 ± 2	101 ± 4	58 ± 2

[a]Esterification of the heme propionates could result in elimination of one or both hydrogen bonds to the carboxyl group (Hunter et al., 1997).

The hydrogen-bonding interactions between the propionates of the heme prosthetic group and the protein matrix are important in stabilizing the protein−heme complex in solution. The energies of activation for heme dissociation for the variant proteins (Table 1) decrease when the functional groups involved in hydrogen bonding are removed by site-directed mutagenesis or heme substitution. The Mb variants (Figure 4, solid characters) exhibited a decrease in activation energy of ∼8−10 kJ/mol from that of the wild-type protein for each hydrogen bond removed. Surface hydrogen-bonding interactions tend to be relatively weak owing to exposure to solvent, so these results agree reasonably with the lower range of hydrogen-bond energies, ∼10−40 kJ/mol (Fersht, 1977). The systematic decrease in E_A with the number of hydrogen bonds suggests all these hydrogen bonds must be broken for dissociation to occur in solution. This hypothesis is supported by heme reorientation experiments which show all the heme contacts with the protein are broken in the transition state when reorientation occurs (La Mar et al., 1984; Yee & Peyton, 1995). All three of these electrostatic interactions contribute to ∼30% of the total activation energy for heme dissociation as seen by the 30 kJ/mol decrease in E_A for DME-Mb. These interactions are also important in cytochrome b_5 as reconstitution with the DME-heme also decreased the activation energy of dissociation (15 kJ/mol) (Figure 4, open characters). The lipase-solubilized and trypsin-solubilized forms of cyto-

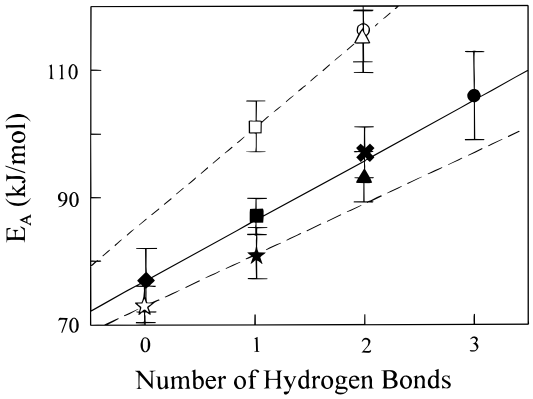

FIGURE 4: Dependence of Arrhenius activation energy in solution on the number of hydrogen bonds formed by the heme propionates and the protein. The Mb variants are labeled as follows: wild-type (●), K45E (×), H97L (▲), K45E/H97L (■), S92D/K45E (★), DME-Mb (◆), and K45E/K63E/H97L/S92A (☆) Mb. The cytochrome b_5 variants are labeled as follows: lipase-solubilized (○), trypsin-solubilized (△), and DME−trypsin-solubilized (□) cytochrome b_5.

chrome b_5 show similar activation energies as these proteins have similar heme binding pockets, the trypsin-solubilized form being shorter by 2 and 9 amino acid residues at the N- and C-termini, respectively (Strittmatter & Ozols, 1966; Ozols & Strittmatter, 1969). Work reported elsewhere has demonstrated that the rate constants for heme dissociation from Mb and cytochrome b_5 and the thermal stability in solution vary systematically with the number of hydrogen bonds formed between these propionate groups and the protein (Hunter et al., 1997).

Two of the Mb variants, S92D/K45E and K45E/K63E/H97L/S92A, involve replacement of S92, the side chain of which forms a hydrogen bond with a heme propionate and with the proximal ligand to the heme iron, H93 (Figure 1) (Evans & Brayer, 1990). As observed for both the heme reorientation and heme dissociation kinetics experiments described previously (Hunter et al., 1997), variants with substitutions at position 92 have lower activation energies than expected from the loss of a single hydrogen bond (Figure 4, stars). Replacement of the S92 residue destabilizes the heme−protein complex further either by weakening the H93−Fe(III) interaction through an electronic effect (Goodin & McRee, 1993) or increasing solvent accessibility on the proximal side of the heme pocket (Smerdon et al., 1993). This effect is reflected in the finding that the activation energy for heme dissociation from the S92D/K45E and K45E/K63E/H97L/S92A variants is 4−6 kJ/mol less than that observed for the H97L/K45E and DME-Mb variants possessing the same number of hydrogen bonds to the heme propionates. This difference in activation energy is somewhat less than the activation energy (∼10 kJ/mol) required for disruption of the His−Fe(III) bond of leghemoglobin at pH 7 (Smith et al., 1991).

The Arrhenius activation energies for heme dissociation from wild-type Mb and cytochrome b_5 were found to be 106 ± 7 and 116 ± 7 kJ/mol, respectively, at pH 5.0. Because the heme−protein complex is stabilized at higher pH, it is expected that the activation energy of dissociation will also increase as pH is raised. The activation energies for heme dissociation from horseradish peroxidase and leghemoglobin were previously reported to be 139 ± 10 and 132 ± 6 kJ/mol, respectively, at pH 7.0 (Smith et al., 1991), so the E_A values determined here compare reasonably.

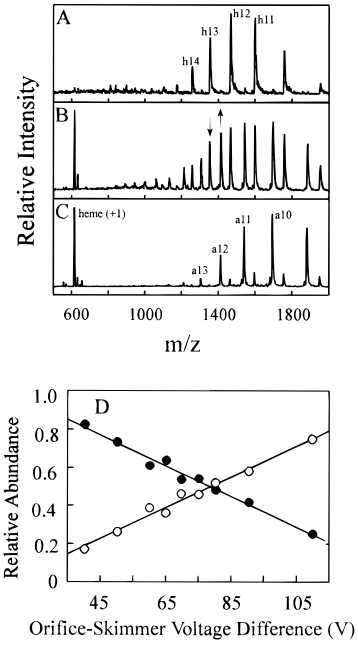

FIGURE 5: Mass spectrum of wild-type Mb at ΔV_{OS} values of (A) 40, (B) 100, and (C) 150 V. Notation: h14 is $(\text{holoMb} + 14\text{H}^+)^{14+}$, a11 is $(\text{apoMb} + 11\text{H}^+)^{11+}$ etc. The arrows indicate that the relative intensities of the holoprotein peaks (h14−h11) decrease and the apoprotein peaks (a13−a10) increase as the voltage difference between the orifice and skimmer is increased. (D) Linear dependence of complex dissociation on the orifice−skimmer voltage difference (ΔV_{OS}) for the +13 holo (●)/ +12 apo (○) charge states for wild-type Mb.

Mass Spectrometry. The relative stability of heme binding to Mb and cytochrome b_5 in the gas phase was measured by increasing the voltage difference (ΔV_{OS}) across the orifice−skimmer region (Figure 2) of the ion sampling interface to increase the dissociation of the noncovalently associated heme−protein complexes. Mass spectra of wild-type Mb recorded at several ΔV_{OS} values are shown in Figure 5. As the voltage difference is increased, the apoprotein peaks increase in intensity at the expense of the holoprotein peaks. Also, the intensty of the free heme $(1)^+$ peak increases as heme dissociation increases. The holoprotein of charge state $(n)^+$ loses heme presumably as a singly charged positive ion and produces an apoprotein with charge $(n - 1)^+$. The relative abundances of the holoprotein $(n)^+$ and apoprotein $(n - 1)^+$ peaks were found to be approximately linearly dependent on the ΔV_{OS} (Figure 5D).

The voltage difference (ΔV_{OS}) required for 50% dissociation of the heme−protein complex for each of the four individual charge states of Mb and cytochrome b_5 was determined from plots similar to that depicted in Figure 5D. The ΔV_{OS} values for wild-type and K45E/H97L Mb for each charge state are shown in Figure 6A. The ΔV_{OS} for the K45E/H97L variant shows a decrease of ∼13 V relative to wild-type Mb for each charge state. The ΔV_{OS} values decrease with increasing charge; similar results were obtained for all the proteins studied. As a convenient summary of the data for all the charge states of each protein, the average voltage for all the charge states is taken as a measure of the relative energy required to dissociate the heme−protein complex. For a constant difference for individual charge states between different variants as is seen in Figure 6A, the differences in average voltage equal the differences for individual charge states.

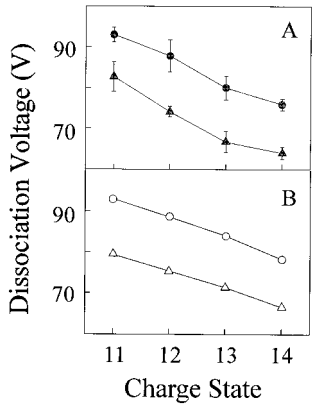

FIGURE 6: (A) Measured dissociation voltage (ΔV_{OS}) vs charge state for wild-type (●) and H97L/K45E (▲) Mb. (B) Dissociation voltage for the charge states of simulated data based on the initial experimental charge state distribution of Mb. The two sets of distributions were generated by assuming average dissociation voltages of 88 V (○) and 75 V (△) and a loss of 30% uncharged heme.

The decrease in ΔV_{OS} for 50% dissociation with increasing charge may derive from several sources. First, ions of higher charge state experience a greater acceleration in the electric field between the orifice and skimmer and thus can acquire a given internal energy at proportionately lower voltages. Regardless of the details of the activation process, this should give a constant product (charge × voltage) for a given protein. The data are consistent with this: all proteins show a constant product with standard deviations less than 6%. For example, for H97L/K45E, the products (charge × voltage) are 910, 890, 870, and 900 V for charge states 11^+-14^+, respectively. Second, higher charge states may have somewhat different structures, possibly destabilized by Coulomb repulsion. The differences in ΔV_{OS} for dissociation between variants are nearly constant over the charge states 11^+-14^+. Either the destabilization caused by increasing the charge from 11^+ to 14^+ is equal between all the proteins studied or the effects of different structures or Coulomb repulsion are small relative to the changes caused by the mutations. Third, loss of neutral heme from the holoprotein may introduce a systematic variation of ΔV_{OS} which is not related to the energy required to dissociate the complex.

The procedure for determination of ΔV_{OS} required for 50% heme dissociation is exact only when heme is lost from the complex entirely as a singly charged species. Tandem MS experiments indicated that ~20−30% of dissociated heme is lost as a neutral species for each of the charge states (data not shown; Li et al., 1993). In the tandem MS experiments, ions are activated by few relatively high-energy collisions; in the interface CAD experiment, ions are activated by many relatively low-energy collisions. The relative loss of neutral and charged heme can differ in these experiments. It is possible that neutral heme loss does not occur in the interface CAD experiments. However, we have considered in detail the effects that neutral heme loss may have on the interpretation of the experiments. Loss of neutral heme from the $(n)^+$ charge state produces the same molecular ion for apoMb as loss of heme $(1)^+$ from the $(n + 1)^+$ charge state. Because of the different abundances of the $(n)^+$ and $(n + 1)^+$ holo species, this neutral loss can cause differences in the apparent ΔV_{OS} for individual charge states.

To assess the possible contribution of neutral heme loss to the dissociation voltage for the individual charge states (ΔV_{OS}) and to the experimentally determined average dissociation voltage (V_D) for the 11^+-14^+ charge states of Mb, the experiment was simulated numerically. Starting with the initial experimental charge state distribution of holoMb, the extent of reaction (r_x) (i.e., the fraction of holoMb converted to apoMb) was calculated from

$$r_x = (0.6/V_{1/2})\Delta V_{OS} - 0.1 \qquad (2)$$

where $V_{1/2}$ is the voltage difference at which half of the holoMb ions have dissociated and ΔV_{OS} is the voltage difference between the orifice and the skimmer. This gives a linear decrease of holoMb with increasing orifice voltage as observed in the experiments (Figure 5). The same value for $V_{1/2}$ was used for all charge states. The intensity of each holoMb peak and each resulting apoMb peak was calculated assuming 0−50% of the heme was lost as neutral heme. Model peak distributions were generated for voltages covering the experimental range, and the "experimental" V_D was calculated from the simulated data using the same method used in the experiment. The calculated V_D was then compared with the value for the orifice−skimmer voltage difference for 50% heme−protein complex dissociation ($V_{1/2}$) that was used in the simulation.

The results of simulations, assuming a neutral heme loss of 30% and dissociation voltages, $V_{1/2}$, of 88 and 75 V, are shown in Figure 6B. The apparent decrease in ΔV_{OS} for the higher charge states in the data simulation (Figure 6B) is similar to the decrease seen in the experimental results (Figure 6A). This decrease reflects the shape of the initial intensity distribution of the holoMb peaks and the loss of neutral heme. Consider dissociation of the holoMb 14^+ ion to produce apoMb 13^+. If neutral heme is lost from holoMb 14^+, the contribution of holoMb 14^+ to the apoMb 13^+ is reduced. This, however, is compensated by neutral heme loss from holoMb 13^+ to produce apoMb 13^+. If the abundances of holoMb 14^+ and holoMb 13^+ are equal, the "missing" apoMb 13^+ from holoMb 14^+ is supplied by neutral heme loss from holoMb 13^+ and the correct voltage corresponding to 50% reaction is recovered. If, however, holoMb 13^+ is more abundant than holoMb 14^+, as in the experiment, the contribution from holoMb 13^+ to apoMb 13^+ exceeds the "missing" contribution from holoMb 14^+ and the ratio of (holoMb 14^+)/(holoMb 14^+ + apoMb 13^+) will reach 0.5 at a voltage lower than that which corresponds to 50% reaction. By a similar argument, ions in lower charge states will require a higher voltage than that corresponding to 50% reaction. Although there are significant differences in the dissociation voltages of the individual charge states when the simulated data are analyzed as in the experiment, the *average* dissociation voltages (V_D) determined from the simulated data for the 11^+-14^+ charge states are 86 and 73 V, within 2 V of the exact dissociation voltage, $V_{1/2}$, used in the simulation (88 and 75 V, respectively). Additional simulations for loss of uncharged heme from 0% to 50% (i.e., values that bracket the 20−30% neutral heme loss of the MS/MS experiments) result in average voltages within 4 V of that of the exact model value, $V_{1/2}$. While the loss of uncharged heme causes the various charge states to exhibit different voltages for 50% heme dissociation, the voltage (V_D) averaged over individual charge states is remarkably insensitive to the extent of uncharged heme loss and so can be regarded as a measure of the voltage required to dissociate

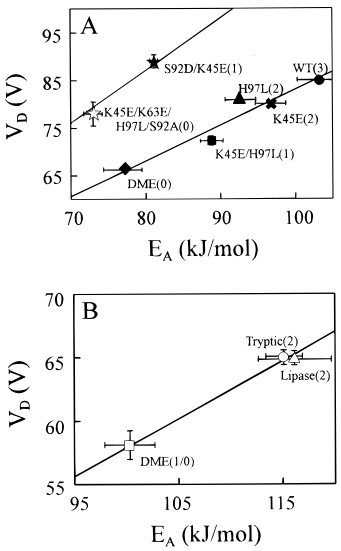

FIGURE 7: Dissociation voltage (V_D) measured in the gas phase plotted against the measured activation energy for heme dissociation, E_A, in solution for (A) Mb and (B) cytochrome b_5.

the complex. In summary, while the origin of the variation of dissociation voltage with charge state is uncertain in these experiments, the average voltage remains a useful measure of the voltage required to dissociate a given protein.

Comparison of Heme−Protein Complex Stability in Solution and in the Gas Phase. The average dissociation voltages (V_D) determined experimentally for the variants of Mb and cytochrome b_5 are shown in Figure 7 and Table 1. As seen from these results, elimination of a single hydrogen bond to a heme propionate group in Mb lowered V_D by ∼4−10 V. Similarly, the voltage required to dissociate 50% of the heme from trypsin-solubilized DME cytochrome b_5 is 7 V lower than that required for trypsin-solubilized cytochrome b_5. The dissociation voltage (V_D) for each Mb and cytochrome b_5 variant is an approximate measure of the relative internal energy required to cause the complex to dissociate within the transit time to the mass-analyzing quadrupole, Q1 (∼2 ms). It is apparent from Figure 7 that differences in stability of a noncovalently associated complex in the gas phase that result from elimination of a single hydrogen-bonding interaction can be observed with this method.

For those variants involving removal of hydrogen bonds between the heme propionates and protein residues, the dissociation voltages obtained for gas-phase ions show a positive correlation with the activation energies observed in solution and decrease in the order wild-type > K45E > H97L > K45E/H97L > DME Mb (Figure 7A, solid characters). These data appear to show a linear correlation between V_D and E_A over the experimental range. In drift tube experiments, where ions are activated in a series of low-energy collisions, the internal energy acquired by ions is proportional to the square of the drift voltage. Over the small range of voltages of these experiments, such a quadratic dependence (i.e., $V_D^2 \sim E_A$) would not be apparent within the scatter of the data but cannot be ruled out. Drift tube experiments activate ions in a region of constant gas density; it is not clear that the same dependence of ion internal energy on drift voltage applies here.

This correlation between gas-phase dissociation voltages (V_D) and solution activation energies (E_A) for Mb suggests that the protein−propionate interactions known to exist in solution are retained in the gas-phase ions in these experiments. If the protein were grossly misfolded in the gas phase, such a correlation would not be observed. Reconstitution of trypsin-solubilized cytochrome b_5 with DME-heme decreases the activation energy for heme dissociation in solution and also decreases the voltage required to dissociate heme from the holoprotein in the gas phase (Figure 7B, open characters). Again, this behavior is consistent with persistence in the gas phase of hydrogen bonds to the heme propionates in trypsin-solubilized cytochrome b_5. Lipase-solubilized cytochrome b_5 shows the same E_A in solution as trypsin-solubilized cytochrome b_5 and also the same heme dissociation voltage for the gas-phase ion. This suggests the binding energy in the gas-phase ions may be similar, but this interpretation is complicated by possible kinetic shift effects caused by the additional 11 residues in the lipase form.

In solution, the activation energy for heme dissociation from Mb is less than that observed for lipase- and trypsin-solubilized cytochrome b_5, yet a greater average voltage is required to dissociate heme from Mb (MW ∼ 17 500) than from cytochrome b_5 (MW ∼ 10 000) in the gas phase. This behavior is not surprising because larger ions are generally expected to require greater internal energies for dissociation of the heme−protein complex on the time scale of the mass spectrometry experiment (Marzluff et al., 1994; Griffin & McAdoo, 1993; McKeown & Johnston, 1991). In principle, this effect can lead to different required internal energies (kinetic shifts) for the different variants since the sizes of the proteins (number of atoms) differ slightly. Ions must acquire sufficient internal energy to dissociate in the millisecond transit time to Q1. The magnitude of this energy, E, can be estimated roughly from the RRK expression (eq 3) for the unimolecular rate constant for dissociation, k:

$$k = \upsilon \{(E - E_0)/E\}^N \quad (3)$$

where υ is a frequency factor, E_0 is the dissociation energy, and N is the effective number of oscillators (typically about $^1/_3$−$^1/_2$ of the number of degrees of freedom) (Harrison & Tsang, 1972; McKeown & Johnston, 1991; Griffin & McAdoo, 1993). Because of the large number of internal degrees of freedom of protein ions, small differences in dissociation energy can require comparatively large differences in the internal energy required to produce dissociation on a given time scale. For the Mb variants, the calculated differences in required internal energy deriving from the different number of degrees of freedom (N in eq 3) are 1% or less (where $k = 10^3$ s^{-1} and $E_0 = 106$ kJ mol^{-1}). These differences are much less than the changes in V_D seen in the experiment, i.e., the differences in kinetic shifts between variants are negligible. This is expected since the differences in the numbers of atoms are small. Similarily, the difference in required energy calculated for trypsin-solubilized cytochrome b_5 and DME-cytochrome b_5 are ∼2%, with DME-cytochrome b_5 requiring a slightly higher energy. A 10% lower voltage is observed for DME-cytochrome b_5 (Figure 7), which suggests that kinetic shifts do not contribute significantly to the differences in V_D between these ions. Calculations show the lipase-solubilized cytochrome b_5 should require a 12% higher energy for dissociation than the trypsin form due to the additional 11 residues; however, the same voltage is required to dissociate these two proteins.

This suggests either that there is a fortuitous cancelation of effects leading to the same voltage for this one pair of proteins or that the simple RRK formula is inadequate to describe the unimolecular decay of a large noncovalent complex such as cytochrome b_5 as discussed by Schlag and Levine (1995). Additional studies of related proteins of different sizes binding the same substrate may allow determination of the validity of simple expressions such as eq 3 for large molecules. In addition, CAD studies of various small protonated model ions with well-characterized bond strengths and breakdown curves may provide additional insights into the CAD process in the orifice−skimmer region. However, extrapolating these results to larger biomolecules may be difficult.

Mb variants in which S92 has been substituted exhibit a dissociation voltage greater than expected (Figure 7A), suggesting that this substitution actually stabilizes the heme−apoprotein complex in the gas phase while it destabilizes the complex in solution. This difference could conceivably reflect a different dissociation mechanism in the gas phase. While the mechanism is largely uncertain, available evidence suggests similar mechansims may apply in the gas phase and in solution. Measurements of collision cross sections for the gas-phase ions suggest the dissociation proceeds first through formation of a partially unfolded holoprotein followed by loss of heme (Douglas & Collings, 1996). A similar mechanism appears to apply to Mb in solution, i.e., formation of a complex in which the heme is unbound but contained within a partially unfolded protein, followed by heme dissociation from the complex (La Mar et al., 1984; Yee & Peyton, 1995; Konermann and Douglas, unpublished results). Additional work is required to assess the effects of other types of binding interactions such as axial ligation to the heme iron or hydrophobic interactions between the heme and the apoprotein on the stability of the heme−protein complex in the gas phase.

This report describes the first study in which the contributions of individual hydrogen-bonding interactions to the stability of a protein−small molecule complex have been characterized in both the gas and solution phases through analysis of a series of modified proteins that differ systematically in their hydrogen-bonding ability. The implications of these results are 2-fold. First, the evidence presented here demonstrates that the hydrogen bonds known to stabilize the heme−protein complex in solution persist in the gas-phase ion and contribute to the stability of the complex in the gas phase. This conclusion is consistent with the observation that the gas-phase heme−protein complex is more compact than the apoprotein, implying that the protein may remain folded around the heme (Douglas & Collings, 1996). It suggests that the gas-phase ion retains much of the solution structure around the heme over at least the millisecond time scale of this experiment.

Second, the ability to observe small differences in protein−small molecule stability in two separate proteins suggests that ES-MS may be a generally applicable method for characterizing noncovalently associated protein−ligand complexes in the gas phase. The observation that the destabilizing effects of a single mutation or bond change in noncovalently associated complexes can be detected in the gas-phase ions using this method is particularly encouraging. One notable benefit of ES-MS is the relatively small amount of sample required. The conventional electrospray source used here operated at 1 μL/min so that ∼160 pmol of each protein was consumed in the 40 min of data acquisition per protein. Developments of low-flow electrospray sources promise to lower this sample requirement at least 100-fold (Wilm & Mann, 1994, 1996; Valaskovic et al., 1996). By comparison, the method used here for determination of the activation energy for heme dissociation in solution required ∼300 times more protein and a minimum of 5 h/protein. Solution methods often require additional indirect factors to indicate the presence of complex (e.g., spectroscopic probes, indicators of loss/gain of enzymatic activity). Finally, automation could further reduce the mass spectrometric data acquisition time and sample requirements and permit development of a rapid screening technique for evaluating stability of noncovalently associated complexes.

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