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Yi-Sheng Wang, Sahadevan Sabu, Shih-Chia Wei, C.-M. Josh Kao, Xianglei Kong, Shing-Chih Liau, Chau-Chung Han, and Huan-Cheng Chang[a)]
Institute of Atomic and Molecular Sciences, Academia Sinica, P.O. Box 23-166, Taipei 106, Taiwan

Shih-Yu Tu and A. H. Kung
Institute of Atomic and Molecular Sciences, Academia Sinica, P.O. Box 23-166, Taipei 106, Taiwan and Department of Photonics, National Chiao Tung University, Hsinchu 300, Taiwan

John Z. H. Zhang
Department of Chemistry, New York University, New York, New York 10003 and Key Laboratory of Mesoscopic Chemistry of MOE, Institute of Theoretical and Computational Chemistry, College of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, People's Republic of China

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Detachment of heme prosthetic groups from gaseous myoglobin ions has been studied by collision-induced dissociation and infrared multiphoton dissociation in combination with Fourier-transform ion cyclotron resonance mass spectrometry. Multiply charged holomyoglobin ions (hMb^{n+}) were generated by electrospray ionization and transferred to an ion cyclotron resonance cell, where the ions of interest were isolated and fragmented by either collision with Ar atoms or irradiation with 3 μm photons, producing apomyoglobin ions (aMb^{n+}). Both charged heme loss (with $[Fe(III)\text{-heme}]^+$ and $aMb^{(n-1)+}$ as the products) and neutral heme loss (with [Fe(II)-heme] and aMb^{n+} as the products) were detected concurrently for hMb^{n+} produced from a myoglobin solution pretreated with reducing reagents. By reference to E_a=0.9 eV determined by blackbody infrared radiative dissociation for charged heme loss of ferric hMb^{n+}, an activation energy of 1.1 eV was deduced for neutral heme loss of ferrous hMb^{n+} with n=9 and 10. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2221696]

I. INTRODUCTION

Mass spectrometry of noncovalent protein complexes is a promising approach toward understanding the nature of molecular recognition such as protein-ligand interactions and protein-protein interactions.[1,2] One of the benchmark systems for this study is the intact holomyoglobin ion.[3–10] Holomyoglobin (hMb) is a protein responsible for oxygen transport in muscular tissues. It possesses a prosthetic heme group bound noncovalently to a single polypeptide chain. Both theoretical and experimental investigations have been carried out extensively concerning the binding characteristics of oxygen, carbon monoxide, and some other ligands to this protein.[11–13] It is known that the binding affinity of these ligands depends sensitively on the conformation of the polypeptide chain that defines the binding condition for the heme moiety.[14]

Bound noncovalently to the polypeptide chain, the heme group is prone to detachment by either acid-or alcohol-induced denaturation of myoglobin in solution.[8] Prior studies have examined the binding characteristics of heme in gaseous hMb^{n+} ions in the absence of a solvent. There are two dissociation channels leading to the formation of heme-released apomyoglobin (aMb) ions,

$$\text{charged heme loss: } hMb^{n+} \rightarrow aMb^{(n-1)+} + [Fe(III)\text{-heme}]^+, \quad (1)$$

$$\text{neutral heme loss: } hMb^{n+} \rightarrow aMb^{n+} + Fe(II)\text{-heme}. \quad (2)$$

Using an electrospray ionization (ESI)-Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer, Gross *et al.* carried out thermochemical measurements for the dissociation energy of ferric hMb^{n+} with blackbody infrared radiative dissociation (BIRD). From an analysis of the dissociation rates at different temperatures made possible by heating the ion cyclotron resonance (ICR) cell and assuming that the secondary structures of the proteins are unchanged, they determined an Arrhenius activation energy of 0.8–0.9 eV for charged heme loss in hMb^{n+} with n=9–12. This measured dissociation energy is similar to that (1.1 eV) of heme loss in solution.[15–17] The latter studies additionally showed that the interaction between heme and the proximal histidine accounts for ~25% of the total dissociation energy and the rest was contributed by van der Waals attractions and hydrogen bonding of propionic acid residues of the heme group to the specific residues in the protein.[16,17]

With respect to Eqs. (1) and (2), Chrisman *et al.* identified these two types of heme loss with collision-induced dissociation (CID) for both oxidized and reduced forms of hMb^{n+} (n=2–10) in a quadrupole ion trap. Their results, in

a)Author to whom correspondence should be addressed. Electronic mail: hcchang@po.iams.sinica.edu.tw

combination with UV-vis spectroscopic measurements, led to the conclusion that loss of charged heme primarily occurs in ferric hMb^{n+}, whereas loss of neutral heme occurs predominantly in ferrous hMb^{n+}. There is no evidence for electron transfer between heme and the polypeptide chain upon collisional activation of the charge-selected protein ions. Mark and Douglas[10] very recently studied Coulomb effects on the binding characteristic of $[Fe(III)\text{-heme}]^+$ and [Fe(II)-heme] in both positive and negative hMb ions in a high-pressure collision cell of a triple quadrupole tandem mass spectrometer. They found that the energies required to induce neutral heme loss are similar for both types of ions, while the energies required to induce charged heme loss are significantly less for the positive ions, indicating that the Coulomb repulsion between $aMb^{(n-1)+}$ and $[Fe(III)\text{-heme}]^+$ lowers the barrier for the heme release.[10] Unfortunately, information about the absolute dissociation activation energy for the neutral heme loss could not be deduced from their measurements.

In dissociation of molecular ions, sustained off-resonance irradiation (SORI) CID has been proven to be one of the most useful techniques because of its efficiency and low cost of implementation.[18,19] A large body of theories, technological developments, and application notes of SORI-CID can be found in the literature.[20–28] The technique is unique in that it repeatedly activates the ions of interest to moderate kinetic energy for multiple collisions with background atoms or molecules. Mixed ions with the same mass-to-charge ratios can be excited *simultaneously* to the same energy, thereby allowing qualitative estimation of the relative activation energies between different dissociation channels. The method is well suited for the presently proposed study of charged versus neutral heme loss from hMb^{n+} because the difference in dissociation energy between these two channels is small and needs to be measured carefully.

To determine quantitatively the relative activation energies of the processes as described in Eqs. (1) and (2), IRMPD offers an alternative approach.[28] A number of experiments[29–31] have demonstrated that continuous-wave (cw) CO_2 laser irradiation at $\sim 10\ \mu m$ is a convenient means of increasing the internal energy of trapped gaseous ions without the need of heating the ICR cell. Although understanding of the temperature/laser intensity relationship is far from complete due to the complexity of the heating mechanism involved, the infrared multiphoton dissociation (IRMPD) technique nevertheless can provide quite accurately the relative ordering of the dissociation activation energies for peptide ions with similar size and structure.[31] In view of this utility, we attempted in this work to deduce such information with both SORI-CID and IRMPD for hMb^{n+} produced from a solution containing both ferrous and ferric myoglobins. Unlike previously conducted studies,[7] the light source we use is a high-repetition-rate optical parametric oscillator (OPO) laser for excitation of NH stretches, which are highly localized vibrational modes and should have similar absorption strengths for both oxidized and reduced forms of the proteins. Results of the quasi-cw IRMPD experiments can therefore be compared closely with BIRD measurements.

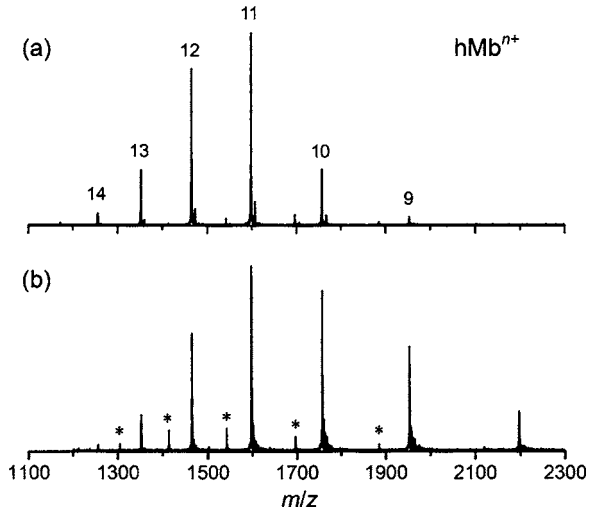

FIG. 1. FTICR mass spectra of protonated holomyoglobin ions (hMb^{n+}) produced from ESI of protein solutions (a) without any pretreatment and (b) pretreated with L-ascorbic acid for 8 h. The peaks denoted by "*" in (b) correspond to protonated apomyoglobin ions (aMb^{n+}) with $n=9-13$.

II. EXPERIMENT

The key instrument used in this experiment is FTICR mass spectrometer (APEX IV, Bruker-Daltonics) equipped with a 7 T actively shielded superconducting magnet and an external ESI source.[32,33] Gaseous myoglobin ions were generated by spraying a sample solution through an electrically grounded, gas-assisted nebulization system at an infusion rate of 100 $\mu L\ h^{-1}$. The ESI assembly was pointed 60° off-axis toward the counter electrode, which was a negatively biased capillary defining the ion entry into vacuum. A counterflowing stream of heated nitrogen gas applied to the electrode facilitated ion desolvation. After passing through a differentially pumped region, the protonated protein ions were accumulated in a hexapole ion trap for 1 s and then pulsed into the ICR cell. Mass spectra were acquired typically by coadding 30 repeated scans.

The sample solution consisted of horse-heart myoglobin obtained from the vendor (M1882, Sigma) without further purification. A stock protein solution (3.5 mM ferric hMb) was first prepared with a 1:4 methanol/water mixture containing ~0.0001% formic acid. ESI-FTICR mass spectra of the solution diluted to 3 μM (*p*H 5.38) showed a progression of multiply charged hMb^{n+} features peaking at $n=11$ [Fig. 1(a)]. While some weak features corresponding to aMb^{n+} can be found in the spectrum, most of the myoglobin ions remained intact, consistent with the observation that myoglobin retains its native conformation at *p*H greater than 4 and CH_3OH concentration lower than 30% in solution.[8] To produce mass spectra exhibiting ferrous hMb^{n+} peaks, the stock protein solution was first treated with 10 mM L-ascorbic acid for 8 h, followed by dilution to 3 μM (*p*H 4.92) for ESI. Further increase of the concentration of the reducing reagents inevitably produced myoglobin-ascorbate adduct ions and also resulted in a rapid decrease of the total ion intensities [Fig. 1(b)]. All the protein solutions after preparation were kept in the dark below 5 °C to prevent degradation before use.

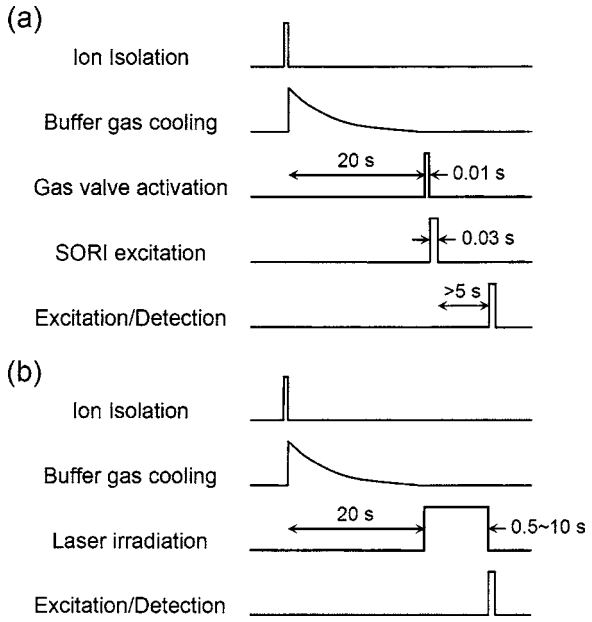

FIG. 2. Time sequences of pulse events in (a) SORI-CID and (b) IRMPD experiments.

IRMPD spectra of hMb^{n+} were obtained using a home-built pulsed OPO laser as the light source. The design and instrumentation of the infrared laser system have been described previously.[34,35] In brief, the laser is composed of a periodically poled lithium niobate (PPLN) optical parametric oscillator in a grazing-incidence grating cavity configuration for broad wavelength tuning in the mid-infrared region. The PPLN crystal was pumped by an acousto-optic *Q*-switched Nd:YAG (yttrium aluminum garnet) laser (210S, Lightwave Electronics) at a repetition rate of 4 kHz and with a pulse duration of 15 ns. The idler output wavelength of the OPO laser was tunable from 2.5 to 3.5 μm with a bandwidth of $\sim 1\ cm^{-1}$. The laser had an average output power in the range of 80 mW (i.e., 20 μJ/pulse). Prior to the IRMPD experiment, the ions of interest were first isolated by radio-frequency (rf) cleanup sweeps and thermalized with pulsed argon gas at a peak pressure of $\sim 2\times 10^{-7}$ Torr for 20 s. The charge-selected protein ions were then irradiated by the tunable OPO laser for 1 s. Upon resonant excitation, the gaseous myoglobin ions released its heme fragment. The resulting dissociation fractions were plotted as a function of laser wavelength to obtain the infrared action spectra.

Simultaneous measurements for charged heme loss and neutral heme loss were conducted using SORI-CID under mild excitation conditions in the presence of Ar buffer gas at a pressure of $\sim 1\times 10^{-6}$ Torr. The time sequence of the pulse events in the CID process is shown in Fig. 2(a), where the gas valve was activated for 10 ms prior to activation of the ions by SORI for 30 ms. In this experiment, the SORI activation was operated at a rf of $\sim$1 kHz lower than the ion cyclotron frequency of the protein complex. Control of the ion energy was made by varying the attenuation of the excitation rf amplitude (in decibel) while keeping the durations of the rf excitation and the Ar gas pulsing constant.

The relative dissociation energies of charged versus neutral heme loss were quantified by measuring the dissociation rates of ferric and ferrous hMb^{n+} ions concurrently but separately as a function of laser intensity. Figure 2(b) shows the time sequence of the pulse events used in the IRMPD measurement. Since the OPO laser was operated at a high repetition rate (4 kHz), a mechanical chopper served to control the laser irradiation period. The frequency of the laser output was fixed at 3333 cm^{-1} for excitation of the NH stretches.[33,36–40] The laser power was changed by varying the polarization of the pump laser pulses and monitored with a power meter. Constrained by the performance of the instruments and the ion source conditions, only the dissociation energies of hMb^{9+} and hMb^{10+} can be measured with sufficient accuracy in this experiment.[41]

III. RESULTS AND DISCUSSIONS

A. SORI-CID

Given the spectra shown in Fig. 1(b), we isolated hMb^{10+} produced from ESI of a myoglobin solution pretreated with reducing reagents using the rf cleanup sweep. Although the ions being isolated are of the same mass-to-charge ratio, they are actually composed of a mixture of ferric and ferrous hMb^{n+}. Both neutral heme loss and charged heme loss can, therefore, be monitored simultaneously in the SORI-CID process. In this experiment, the excitation frequency used was offset from the resonance frequency of the precursor ion by −1 kHz, which has a negligible effect on the fragment ions, as confirmed by repeating the experiment with an offset frequency of +1 kHz. To provide a more quantitative measure for the extent of the dissociation for this particular ion, we obtained the band intensity (I) of each component and calculated the corresponding dissociation fraction as $I_f/(I_p+I_f)$, where p and f denote precursor and fragment ions, respectively. Figure 3 shows the SORI-CID mass spectra obtained at different degrees of rf attenuation (in decibel). We indicate in the figure the rf attenuation instead of the absolute value of the collision energy because the exact energy involved in the SORI-induced CID process cannot be extracted from this experimental setting. However, as discussed in the Introduction, the advantage of using SORI is that both forms of the hMb^{n+} ions are equally excited with the same rf irradiation. Assuming that these two types of protein ions have the same collisional cross sections, which appears to be a reasonable assumption, an identical amount of collisional energy will be deposited into the protein complexes that undergo either charged or neutral heme loss. High precision measurement for the relative ordering of the activation energies between these two dissociation channels is then possible based on the intensities of the corresponding precursor and fragment ions.

In Fig. 4, we summarize the relative contributions of charged versus neutral heme loss as a function of the rf attenuation. The intensity ratio of the aMb^{10+} peak versus the aMb^{9+} peak is 1.2 at the highest activation energy, at which no residual parent ions are detectable in the mass spectrum (trace at 22 dB in Fig. 3). It suggests that the relative abundance of gaseous ferrous hMb^{n+} versus ferric hMb^{n+} ions in the ICR cell before the SORI-CID process is 1.2:1. The dissociation ratio of these two channels at lower excitation en-

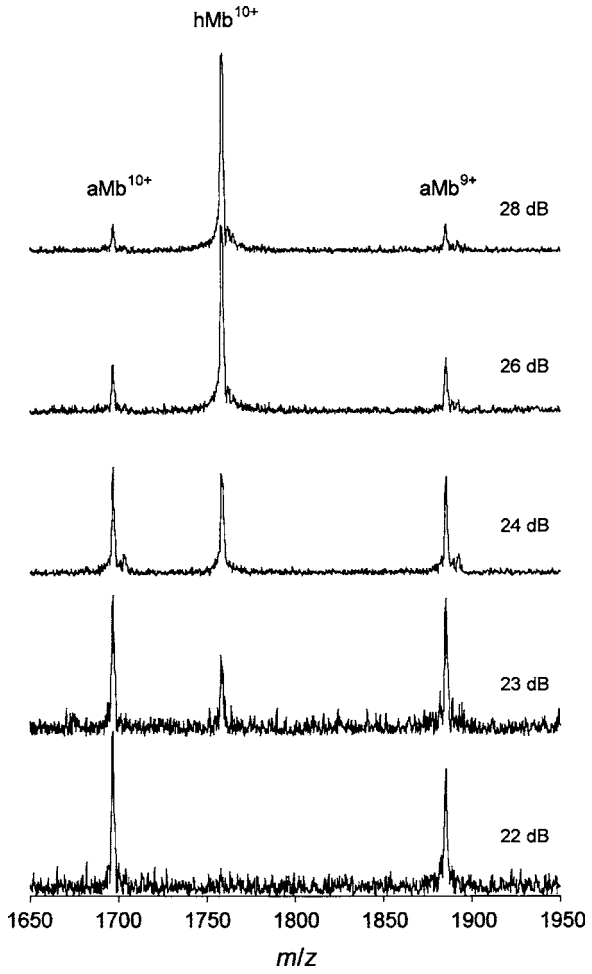

FIG. 3. SORI-CID mass spectra of hMb^{10+} at different levels of rf attenuation (in dB). The fragment ions aMb^{9+} and aMb^{10+} result from charged heme loss and neutral heme loss, respectively.

ergies should therefore reveal how their dissociation activation energies differ. As shown in the lower traces in Fig. 4, the production of aMb^{9+} is higher than that of aMb^{10+} by roughly 30% at the rf attenuation of 28 dB. Although the difference between these two fractions diminishes progressively with the decreasing rf attenuation, this trend of the change suggests that the binding energy between aMb^{9+} and $[Fe(III)\text{-heme}]^+$ is somewhat lower than that between aMb^{10+} and Fe(II)-heme. The result is in qualitative agreement with the CID measurement for hMb^{n+} ($n=5-9$) using a triple quadrupole tandem mass spectrometer by Mark and Douglas.[10]

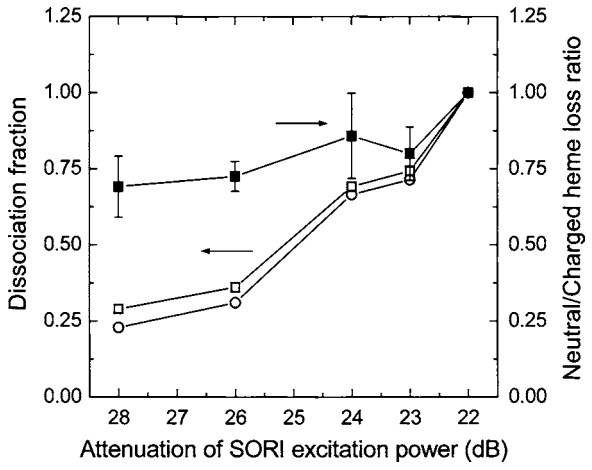

FIG. 4. Fractions of charged (□) vs neutral (○) heme loss of hMb^{10+}, and their ratios (■), as a function of SORI excitation energy. The dissociation fraction is defined as $I_f/(I_p+I_f)$, where I_p and I_f are the intensities of precursor ions and fragment ions, respectively.

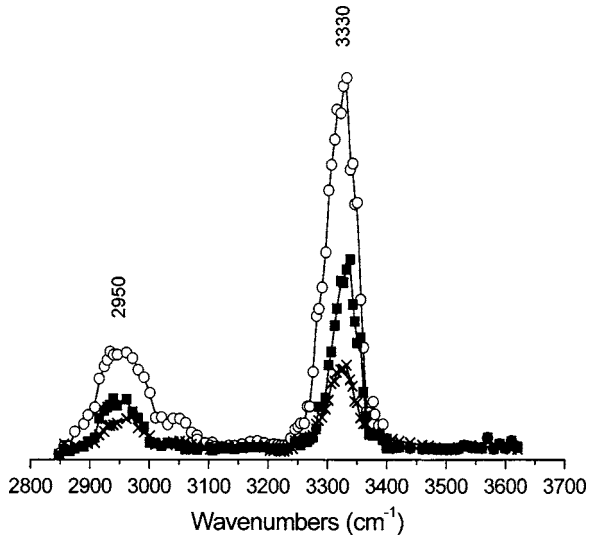

FIG. 5. IRMPD spectra of hMb^{n+} with $n=9$ (×), 10 (■), and 11 (○). The peak intensities represent dissociation fractions as defined in Fig. 4.

B. IRMPD

We started the IRMPD measurement with pure ferric hMb^{n+}. Specifically, we acquired first the infrared photodissociation spectra of these ions carrying different numbers of charges. Though there have been many experimental methods[42] employed to characterize the structures of proteins and polypeptides in the gas phase, no infrared spectra have been reported except that of McLafferty and co-workers.[36,37] These authors used a 6 T FTICR mass spectrometer and a pulsed infrared OPO laser to obtain the photodissociation spectra of multiply charged bovine ubiquitin ions in the frequency range of 3050–3775 cm^{-1}, and identified a single broad feature at 3350 cm^{-1} with a full width at half maximum (FWHM) of more than 100 cm^{-1} for free- and/or hydrogen-bonded-NH stretching vibrations of this gaseous protein ion.[33,36–40] Figure 5 shows the IRMPD spectra obtained in this work for ferric hMb^{n+} with $n=9-11$. Two prominent absorption bands were observed in the spectral scan range of 2850–3650 cm^{-1} for each ion. The band peaking at 3330 cm^{-1} again arises from N–H stretching vibrations and the weaker feature at ~2950 cm^{-1} can be ascribed to CH stretching vibrations. Compared to the spectrum observed for myoglobin in aqueous solution,[43] these two absorption bands are narrower (FWHM ~60 cm^{-1}) and much better resolved because of the absence of solvent interference.

It is noteworthy in Fig. 5 that the observed band intensity increases nearly quadratically with the charge number from $n=9$ to $n=11$. In infrared action spectra as presently acquired, the observed band intensity is a convolution of the absorption strength of the vibrational mode excited and the

dissociation yield of the individual ion within our detection time window. Since these three ions differ only in their charge numbers, they should have similar absorption strengths for both NH and CH stretches. The observed band intensity variation in Fig. 5 should therefore be associated with the difference in dissociation rate of these protein ions. Assuming that the absorbed photon energy dissipates very rapidly to other vibrational degrees of freedom and thermal equilibrium is reached before dissociation takes place, these observations suggest that the preexponential factor (A) in the first-order Arrhenius equation increases with n, given the same dissociation activation energy of $E_a \sim 0.9$ eV at $n=9-11$. A plausible interpretation for such a charge number dependence is that the gaseous myoglobin ion unfolds to a greater extent when carrying more charges, a result in close agreement with BIRD measurements.[5]

In determining the dissociation activation energy with IRMPD using a cw CO_2 laser, Jockusch *et al.*[29] demonstrated that the measured dissociation energy depends on the total laser power and yet is independent of the dimension of the laser beam used. Building on this foundation, our IRMPD-based dissociation energy measurements began with pure ferric hMb^{n+} ions of $n=9-11$. Similar to previous findings,[29,31] a short induction period was required for the laser excitation to raise the temperature of the ion population to the point of dissociation, at which the weakest bonds (i.e., the noncovalent bonds) broke. No secondary fragments other than the heme group from this protein complex were observed. Given in Fig. 6(a) is a plot for the dissociation fraction of ferric hMb^{11+} as a function of laser irradiation time, showing first-order kinetics. By acquiring the first-order rate constants at different laser intensities, plotting of the natural logarithm of the rate constant versus the natural logarithm of the laser intensity yields an activation energy (E_a) for the photodissociation process according to the equation[29]

$$E_a^{\text{laser}} = sk_B \frac{d \ln k_d}{d \ln I_{\text{laser}}}, \tag{3}$$

where k_d is the experimentally determined dissociation rate constant, k_B is the Boltzmann constant, and I_{laser} is the laser intensity. Due to the complexity of the IRMPD mechanism involved in such a large protein ion, we treat s here as a scaling factor by reference to the BIRD measurement. This scaling is deemed justified because a quasi-cw light source is used in this experiment.

Figure 6(b) displays the result of the activation energy measurement for pure ferric hMb^{n+} ions with $n=10-12$. The measured value for hMb^{10+} is slightly higher than those of hMb^{11+} and hMb^{12+}. However, compared with BIRD results (0.9 ± 0.1 eV for hMb^{10+} and hMb^{11+} and 0.8 ± 0.1 eV for hMb^{12+}),[5] these values suggest and averaged scaling factor of $s=4.6\pm0.5\times10^3$ K for all three ions. It should be noted that the scaling factor so derived is twice as large as that (2369 K) reported by Paech *et al.*[30] for four peptide ions using a cw CO_2 laser as the excitation source. This discrepancy, clearly, is associated with the size (mass $>16\,000$ Da versus mass <3000 Da) of the biomolecules studied, the wavelength

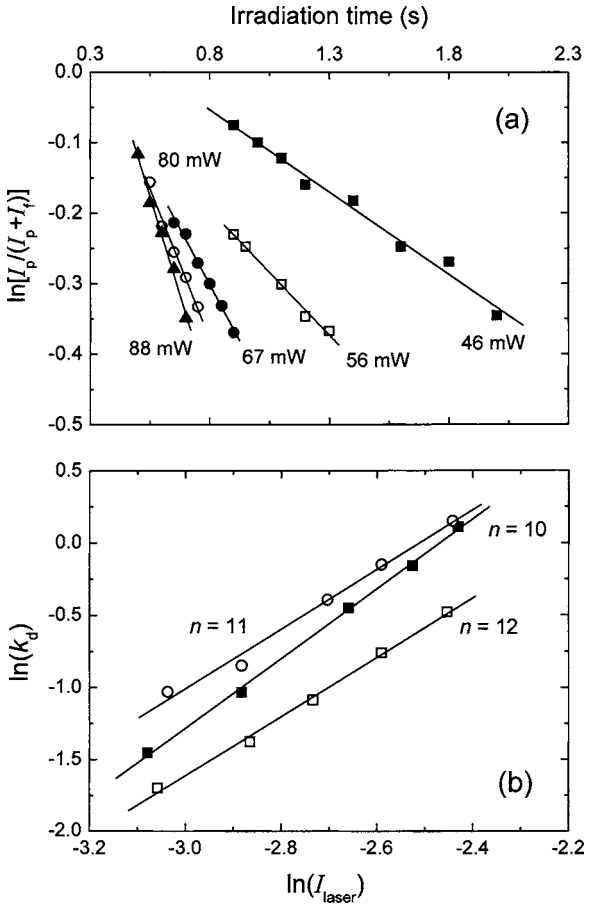

FIG. 6. IRMPD of pure ferric hMb^{n+}. (a) Time trace of charged heme loss at each of five laser intensities for the $n=11$ ion only. (b) Plot of the natural logarithm of the first-order unimolecular dissociation rate constant, k_d (s^{-1}), vs the natural logarithm of the laser intensity I_{laser} (in units of W cm^{-2}). The fitted slopes are 2.42 ± 0.05, 2.06 ± 0.12, and 2.05 ± 0.07 at $n=10$, 11, and 12, respectively. The frequency of the OPO laser excitation was fixed at 3333 cm^{-1}.

(~3 μm vs ~10 μm) of the infrared photons used, and the mechanism of the dissociation involved in these two types of measurements.

Based on this measured scaling factor, $s=4.6\times10^3$ K, we determined the dissociation energy for the neutral heme loss with IRMPD. In this measurement, both ferric and ferrous hMb^{n+} ions produced from ESI of a myoglobin solution pretreated with reducing reagents were first isolated by rf sweeps. The relative abundance of these two components was then determined with SORI-CID as depicted earlier. By exciting the NH stretches at 3333 cm^{-1} and monitoring the charged and the neutral heme loss channels simultaneously, the respective activation energies were determined by fitting two sets of experimental data separately to Eq. (3). Figure 7 shows the plots of $\ln(k_d)$ vs $\ln(I_{\text{laser}})$ for both ions. For the detachment of charged heme from the ferric components, we determined two slopes 2.39 ± 0.10 and 2.21 ± 0.20 for the $n=9$ and $n=10$ ions, respectively. The latter agrees well with that shown in Fig. 6(b) for ferric hMb^{10+} within our experimental accuracy, which serves as an independent validation for this IRMPD method. With the use of the same scaling factor as before, we obtained an average activation energy of $E_a=1.1\pm0.1$ eV for the detachment of the neutral heme

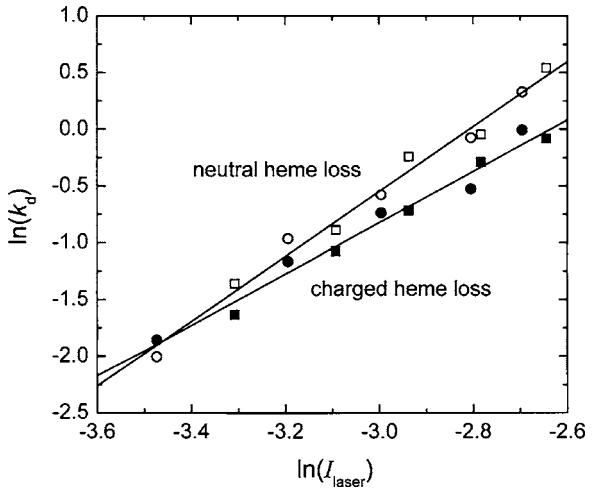

FIG. 7. Plot of the natural logarithm of the dissociation rate constants k_d (s^{-1}) of charged vs neutral heme loss of hMb^{9+} (■,□) and hMb^{10+} (●,○) as a function of the natural logarithm of the laser intensity I_{laser} (in units of W cm^{-2}). The laser excitation frequency was fixed at 3333 cm^{-1}. The fitted slope of charged vs neutral heme loss is 2.26±0.12 vs 2.86±0.12, respectively.

group from the ferrous hMb^{9+} and hMb^{10+} components. In line with the conclusion reached earlier by SORI-CID, this IRMPD measurement indicates that the difference in dissociation activation energy between these two channels is small, ~27% of the total energy (cf. caption of Fig. 7) or 6±1 kcal mol^{-1}.

IV. CONCLUSION

We have demonstrated that it is possible to determine fairly accurately the relative dissociation activation energies of charged versus neutral heme loss from a mixture of ferric and ferrous hMb^{n+} protein ions using IRMPD assisted by SORI-CID in a FTICR mass spectrometer. The excitation was made specifically through the high-frequency vibrational modes, such as the NH stretches, which have similar absorption cross sections among proteins with different charge numbers and oxidation states. By monitoring the dissociation kinetics of these two heme loss channels simultaneously, we conclude that the barrier for neutral heme release in ferrous hMb^{n+} is significantly higher than that for charged heme release in ferric hMb^{n+} by ~27% at both $n=9$ and $n=10$.

Infrared photodissociation spectra at the 3 μm region were obtained for the first time for gaseous myoglobin ions in this work. With the availability of lasers (such as the free electron lasers) covering a wider range of excitation wavelength,[44] employment of the approaches as presently illustrated is expected to provide additional insight into the structure and binding characteristics of this and other noncovalent protein complexes in the gas phase. Further elucidation of these complex systems may come from quantum chemistry calculations, which have been shown to be accurate enough to predict the electronic structures of unligated or ligated ferroporphyrins.[45]

ACKNOWLEDGMENTS

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