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Electrospray Mass Spectrometry Studies of Non-Heme Iron-Containing Proteins

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The oligomeric state and the metal atom stoichiometry of a series of non-heme iron-containing, multimeric proteins have been measured using electrospray ionization (ESI) in a time-of-flight (TOF) mass spectrometer. The proteins were obtained both from natural sources and by overexpression of recombinant DNA in *Escherichia coli.* ESI-TOF mass spectra of the metalloproteins present in nondenaturing solutions exhibit peaks corresponding to the multimeric forms of the holoproteins containing the expected number of metal atoms. Capillary−skimmer dissociation of the holoproteins produces a series of ions, which allows an exact count of the number of metal atoms present in each subunit, and also provides an indication of the oxidation state of the metal atoms. Two recombinant proteins, *Phascolopsis gouldii* hemerythrin (Pg-Hr) and *Desulfovibrio vulgaris* rubrerythrin (Dv-Rr), have been examined as well as hemerythrin isolated from *Lingula reevii* (Lr-Hr). ESI-TOF measurements of the aqueous solution of Pg-Hr at pH 6 yields ions of mass 108 783 Da, in close agreement with the calculated average molecular mass of an intact octameric holoprotein. Capillary−skimmer dissociation of the ions of the holoprotein produces a mass spectrum that contains peaks corresponding to a low *m*/*z* monomer and a high *m*/*z* heptamer. The masses of the monomer ions produced in this manner are assigned to the aposubunit, [subunit + Fe − 3H]$^+$, and [subunit + 2Fe − 6H]$^+$. Naturally occurring Lr-Hr is composed of two subunits with average molecular masses measured under denaturing conditions by ESI-TOF to be 13 877.0 Da for the α-subunit and 13 517.5 Da for the *β*-subunit. Under nondenaturing conditions, a multimeric species with a molecular weight of 110 663 Da is measured by ESI-TOF, corresponding to an $\alpha_4\beta_4$ octamer. Capillary−skimmer dissociation of the $\alpha_4\beta_4$ oligomer produces ions corresponding to both types of monomers (α and *β*) and the corresponding heptamers ($\alpha_3\beta_4$ and $\alpha_4\beta_3$). In ESI-TOF measurements of recombinant rubrerythrin Dv-Rr using nondenaturing conditions, the principal ion observed corresponds to a homotetramer with an average molecular mass of 86 844 Da. Capillary−skimmer dissociation of the rubrerythrin tetramer leads to formation of a series of peaks corresponding to the subunit of the apoprotein and to subunits containing from one to three specifically bound iron atoms.

Metalloproteins are an important class of biomolecules that play a central role in many biochemical processes, including respiration, metabolism, nitrogen fixation, photosynthesis, nerve transmission, muscle contraction, signal transduction, and protection against toxic and mutagenic agents.[1] The role of metal atoms in metalloproteins is both functional and structural, as the interactions between proteins and metal ions have been found to be critical to their catalytic function as well as to their structural stability.[2] Although a variety of spectroscopic techniques has been used for many years to probe the metal centers in metal-containing proteins, it is only recently that mass spectrometry has been able to probe metal binding by proteins, including measurements of stoichiometry,[3,4] cooperativity of multiple binding,[4] and relative binding affinities.[4−12]

Electrospray ionization mass spectrometry (ESI-MS)[13] is increasingly being applied to measurements of weakly bound

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species, including metal−protein complexes and noncovalent complexes,[4−12,14−36] and the potential of using ESI-MS to determine the stoichiometry of metal atoms in proteins in a rapid and sensitive fashion is promising. ESI-MS has been used to show that zinc finger proteins bind Cu^{2+} and Zn^{2+} in the gas phase.[6,10,12,37−39] Metallothionein is found to bind Zn^{2+} and Cd^{2+} at neutral pH but to release the metals at low pH by ESI-MS.[40,41] Rubredoxin has been studied in both positive[22] and negative[42] modes by ESI-MS, and complexes with iron and zinc have been observed at neutral pH in the negative ion mass spectrum.[42] Cooperativity in the binding of two Ca^{2+} to parvalbumin and calmodulin has been observed by ESI-MS.[3,4] The stoichiometry of Ca^{2+} binding to several proteins measured by ESI-MS is found to agree with previously reported results obtained by other physical methods.[3] These and other studies suggest that, with appropriate sample introduction conditions, ESI mass spectra reflect specific association of metals to peptides and that such association can be distinguished from nonspecific interactions.

We report here the use of ESI time-of-flight (TOF) mass spectrometry to examine structural details of multimeric, non-heme iron-containing proteins. ESI-TOF is shown to provide accurate measurements of the oligomeric stoichiometry of the subunits of a holoprotein, the number of metal atoms in a holoprotein, and the distribution of metal atoms in each subunit. As mentioned above, mass spectrometry studies have previously been reported for the small, monomeric, non-heme metal-containing proteins rubredoxin (6 kDa)[22,42] and ferredoxin (11 kDa).[22] Most previous experiments of this type have used quadrupole mass spectrometers, but the limited m/z range of most of these instruments (usually $m/z < 3000$) does not allow detection of many noncovalent complexes, which may only exist near physiological pH values. Under such conditions, protein ions often have a much lower average charge than those observed from denaturing solutions. Time-of-flight mass spectrometers have an effectively unlimited m/z range, and ESI-TOF has been recently shown to be particularly useful for examining noncovalent complexes of high-molecular-weight proteins.[28−30] This is the first reported application of ESI-MS to large (80−120 kDa), multimeric, metal-containing proteins. Despite the complexity of the target molecules, details of the stoichiometry of subunit association and metal atom binding are readily obtained.

Non-heme iron-containing proteins are of interest as models to examine the process of metal atom insertion into proteins and to delineate the effects of metal atoms on protein folding, function, and stability.[1,2] These proteins serve also as models to study the manner in which metals affect the intersubunit noncovalent interactions responsible for protein quaternary structure, as many metalloproteins exist as multimers in their active forms. One challenge for studies of metal-containing proteins is that the material produced by overexpression of recombinant DNA is often missing the metal atoms that are present in the native protein. The reconstitution and refolding of recombinant proteins with the appropriate metal atoms is an important step in structure−function studies of these biomolecules. Here we show that mass spectrometry can be used to monitor the reconstitution process by providing an accurate measurement of the number of metal atoms that are incorporated into a protein and the number of subunits that are bound through noncovalent interactions in the holoprotein.

The non-heme iron-containing metalloproteins examined here are hemerythrin and rubrerythrin. Hemerythrin is a dioxygen transporter found in some marine invertebrates.[43] Hemerythrins are typically composed of eight subunits, each of which contains an oxo-bridged diiron site that is coordinated by seven amino acid residues.[44] Examined in this work are examples of the two principal types of hemerythrin that have been reported, a heterooctamer composed of two subunits with a proposed stoichiometry of $\alpha_4\beta_4$, isolated here from *Lingula reevii* (Lr-Hr),[45,46] and a homooctamer, produced for this study by overexpression of a gene

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encoding *Phascolopsis gouldii* hemerythrin (Pg-Hr).[47] Also examined in this study is rubrerythrin, a non-heme iron containing protein found in the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* (Dv-Rr).[48−51] This protein contains a mononuclear rubredoxin-like FeS_4 center as well as a diiron cluster similar to that found in hemerythrin. Overexpression of Dv-Rr in *Escherichia coli* has previously been shown to lead to the formation of a protein that is deficient in iron.[51] The methods presented here demonstrate the capability to rapidly evaluate the success of metal atom insertion during the reconstitution of recombinant proteins such as Dv-Rr as well as to determine the stoichiometry of metal atoms in proteins isolated from natural sources. Moreover, these methods provide a means to measure the oligomeric state of both reconstituted and as-isolated metal-containing proteins.

EXPERIMENTAL SECTION

The ESI-TOF measurements were made with an instrument that was designed and constructed at the University of Manitoba.[52] The difficulties in performing these experiments are not particular to the nature of these metal-containing proteins but are generally the same as for observing noncovalent complexes by ESI, as has been reported previously.[28] In this device, ions produced in the ESI source pass through three stages of differential pumping into a storage region, and from there into a reflecting TOF spectrometer via orthogonal injection. After reflection in the ion mirror, the ions are detected by a pair of microchannel plates. The detector signal is registered by an Orsay time-to-digital converter (model CTM-M2). Mass resolution for this instrument is approximately 5000. In the present work, peak width is limited by the isotope distribution of the high-molecular-weight samples and not by the mass resolution of the instrument. Mass accuracy for the instrument is 0.01% for external calibration but is somewhat lower when unresolved adducts are present. Deconvolution of the mass spectra was accomplished using the method proposed by Fenn and co-workers.[53] This method can produce artifactual peaks. Thus, all peaks in the deconvoluted mass spectra were confirmed by checking for their existence in the raw mass spectra.

Samples were electrosprayed from a stainless steel capillary (id 0.12 mm) at a flow rate 0.25 μL/min. Some of the ions produced enter the first stage of the ESI interface through a heated stainless steel capillary (length 12 cm, id 0.5 mm); the first stage operates at a pressure of approximately 3 Torr. On leaving the first stage of the interface, the ions pass through an aperture (the "skimmer") into the second stage. The voltage between the capillary and skimmer controls collision-induced dissociation of ions in this relatively high pressure region. Recently, an rf-only quadrupole ion guide was installed in the second stage of the ESI interface.[54] It increases the instrument sensitivity and minimizes mass discrimination.

To obtain independent measurements of the monomer masses, ESI−Fourier transform ion cyclotron resonance mass spectrometry (FTICR) measurements were made with a Bruker BioApex 4.7-T FTICR spectrometer at the Billerica, MA laboratory of Bruker Analytical Instruments. The FTICR spectrometer is equipped with an external electrospray ionization source and a 4.7-T superconducting magnet. The samples were examined using conventional electrospray conditions as well as pneumatically assisted electrospray.

For all the metalloproteins studied here, the best quality results are obtained for protein solutions of low ionic strength and in which sodium and potassium ions have been rigorously excluded. Purified proteins are desalted by filtration using a membrane with a 10-kDa cutoff (Millipore Corp.), followed by resuspension of the protein in 5 mM ammonium acetate. The solubility of hemerythrin at this low ionic strength is less than that in the original solution used for its isolation and reconstitution, and some of this protein was found to precipitate. Rubrerythrin is found to be soluble at this ionic strength. The solutions used for ESI-MS are estimated to have a protein monomer concentration of 1−10 μM.

Recombinant proteins were obtained by using published molecular biology procedures.[55,56] The gene for *P. gouldii* hemerythrin was obtained by reverse transcription of *P. gouldii* RNA and polymerase chain reaction (PCR) of the resulting cDNA using degenerate primers coding for the N- and C-terminal nucleotide sequences inferred from the published amino acid sequence[57,58] and containing *Nde*I and *Hin*dIII restriction sites, respectively. The PCR product was inserted into the *Nde*I/*Hin*dE restriction sites of plasmid pT7−7,[59] generating pDKl-10. Nucleotide sequencing of pDK1−10 showed that the inferred amino acid sequence was that of a major variant of *P. gouldii* Hr, which contains a threonine in place of glycine at position 79.[57] The recombinant hemerythrin was overexpressed as the insoluble apoprotein from pDKI-10 in *E. coli* BL21(DE3) (Novagen, Inc.) according to the supplier's recommended conditions. Iron was inserted into the recombinant hemerythrin by a published procedure[47] using the pellet from the lysed cells of *E. coli* BL21(DE3)[pDK1-10]. *L. reevii* Hr was obtained as described previously.[45] The Hrs used for mass spectrometry were determined to be in the met [Fe(III),Fe(III)] form on the basis of the characteristic absorption spectrum.[60]

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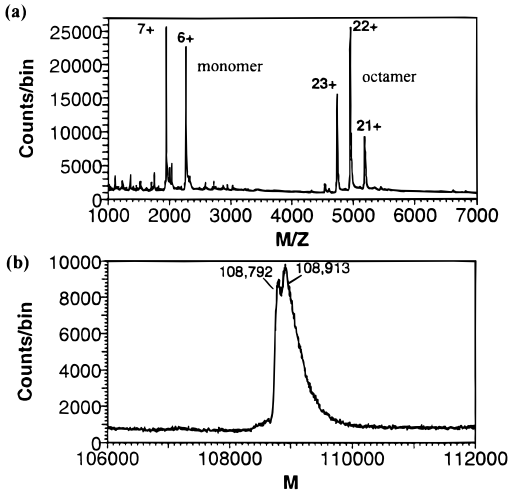

Figure 1. (a) ESI-TOF mass spectrum of recombinant Pg-Hr, obtained under nondenaturing conditions and at a capillary−skimmer potential of 100 V. The octameric holoprotein produces the peaks from *m*/*z* 4000 to 6000. The subunit of the protein produces the peaks between *m*/*z* 1000 and 3000. (b) Deconvoluted mass spectrum obtained from peaks with $z = 21-23$ in the *m*/*z* spectrum shown in (a).

Recombinant *D. vulgaris* Rr was prepared as described previously.[51]

The molecular weights of the subunits of each of the proteins were confirmed by ESI−FTICR measurements. The average molecular masses measured for the subunits of the proteins were found to agree within less than 1 Da of the values predicted on the basis of their elemental composition. The molecular masses of the subunits that were measured and their predicted values (shown in parentheses) are Pg-Hr, 13 474.3 ± 0.2 Da (13 474.1 Da); Lr-Hr, α-subunit 13 878.2 ± 0.3 Da (13 877.9 Da), *β*-subunit, 13 518.7 ± 0.3 Da (13 518.3 Da); Dv-Rr, 21 543.5 ± 1.5 Da (21 544.2 Da).

RESULTS AND DISCUSSION

***Phascolopsis gouldii* Homo-Octamer Hemerythrin (Pg-Hr)**. Oligomeric forms that contain iron have been observed in the mass spectra obtained for the aqueous solution of the Pg-Hr proteins at pH 6.0 with the ESI-TOF instrument. Figure 1a shows a mass spectrum obtained at a capillary−skimmer potential of 100 V. Two different charge state distributions are observed, both at much higher *m*/*z* values (*m*/*z* 2000 and 5000) than observed for ESI-MS of the denatured protein (*m*/*z* 700−1100) obtained by ESI-TOF or ESI−FTICR. It is well-known that the ESI mass spectra observed with nondenaturing conditions, i.e., an aqueous buffered solution of near neutral pH, exhibit narrower charge state distributions of lower average charge compared to the mass spectra of denatured proteins from low pH solutions which contain organic solvents.[14,15,19,28−30] It has been previously suggested that the shift in the distribution to lower charge states indicates that the protein is folded at the time of ionization and that fewer basic sites are accessible to be protonated.[13,35,36]

Figure 1b shows the mass spectrum obtained by deconvolution of the mass-to-charge scale to a mass scale for the three abundant peaks near *m*/*z* 5000, with 21−23 charges. The peak in the deconvoluted mass spectrum exhibits splitting. The same splitting is found in each of the peaks corresponding to the three charge states from which the transformed mass spectrum is derived. The centroid of the lower mass peak of the tranformed mass spectrum corresponds to an ion with an average molecular mass of 108 792 Da, in good agreement with the molecular mass that is calculated for the holoprotein. The holoprotein molecular mass is calculated by adding the average molecular weight of the eight subunits (8 × 13 474.2) to the average atomic masses of 16 iron atoms (16 × 55.8) and 8 oxygen atoms (8 × 16.0) (bridging oxygens of the diiron group), minus 16 protons (each diiron site is coordinated by two carboxyl groups from amino acid side chains) (−16 × l0.0), minus 16 additional protons (−16 × 1.0) to balance the charge carried by the iron atoms (3+), to give an average molecular mass of 108 783 Da. Based on the changes in the peak shape observed at different capillary−skimmer voltages, the higher mass peak in the transformed mass spectrum (108 913 Da) appears to result from the association of the holoprotein with solvent molecules or, perhaps, other small organic molecules to which the protein may have been exposed during its isolation or purification. It is reasonable to expect that an organized protein complex might retain solvent molecules during the electrospray process. This presents a limitation to the mass accuracy of these measurements that reduces the confidence in assigning the total number of metals that are present in the complex. However, the dissociation of the complex into its subunits, as shown below, allows a more precise measurement of the number of metal atoms per subunit.

At lower capillary−skimmer voltages, the splitting of the peak corresponding to the octameric holoprotein disappears, and the centroid shifts to higher mass, suggesting noncovalent associations of small species, perhaps water, to the holoprotein. Increasing the capillary−skimmer voltage causes the splitting of this peak to sharpen but ultimately leads to a reduction of the octamer signal as the molecule undergoes collisional dissociation. As the capillary−skimmer voltage is increased, peaks near *m*/*z* 2000 become more abundant, as shown in Figure 2. These peaks correspond to an ion with a molecular weight equal to that of a subunit of the protein. At 150 V, peaks corresponding to a heptamer appear at *m*/*z* 5500−8000. These peaks and the monomer peaks between *m*/*z* 1000 and 3000 continue to increase at the expense of the octamer peak as the capillary−skimmer voltage is increased. At 250 V capillary−skimmer potential, peaks between *m*/*z* 8000 and 11 000 appear, corresponding to a hexamer, while the heptamer peaks have decreased in intensity. This pattern suggests a stepwise loss of a monomer subunit with 5−7 charges from the octamer complex to yield first a heptamer and then a hexamer. It is an interesting observation that the holoprotein ion, with approximately 3 charges per subunit, splits the charge asymmetrically upon dissociation of the ion, with proportionally more charge going to the monomer (6 per subunit) than to the heptamer (2 per subunit) or the hexamer (1.5 per subunit). This behavior has been observed previously in the dissociation of multimeric proteins,[28,61,62] but no explanation has been advanced.

A closer examination of the region of the mass spectrum which contains the monomer subunit produced by capillary−skimmer

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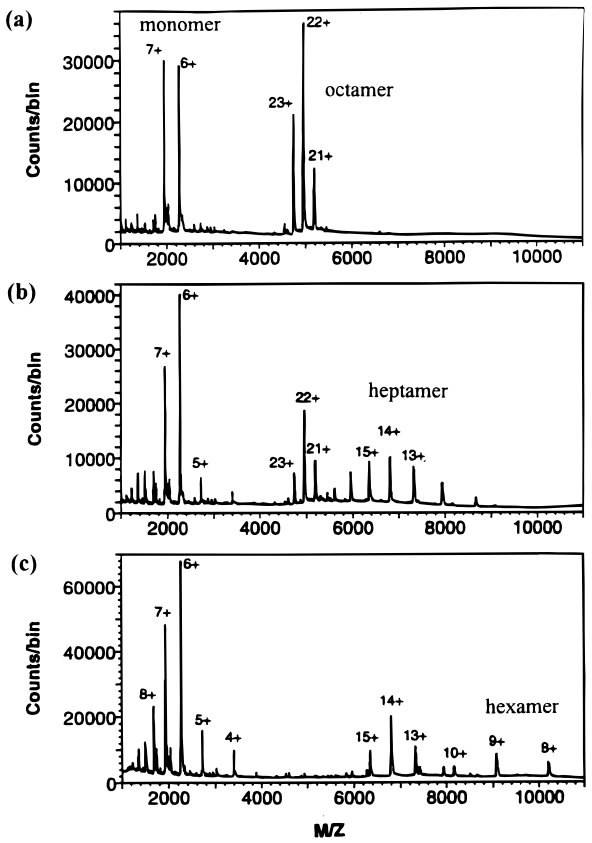

Figure 2. ESI-TOF mass spectra of recombinant Pg-Hr, obtained under nondenaturing conditions and at capillary−skimmer potentials of (a) 100, (b) 200, and (c) 250 V. Peaks corresponding to the octameric holoprotein (*m*/*z* 400−6000) decrease in intensity, while peaks corresponding to a monomer (*m*/*z* 1000−3000) and to heptamer (*m*/*z* 6000−8000) and hexamer (*m*/*z* 8000−10000) complexes increase in intensity as the capillary−skimmer potential is increased.

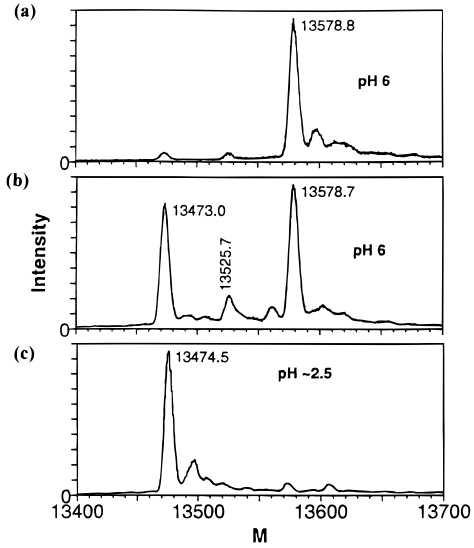

Figure 3. Deconvoluted ESI-TOF mass spectrum of the monomer region of the mass spectrum of Pg-Hr ($z = 5-8$), showing peaks corresponding to the mass of a subunit with 0−2 iron atoms. (a) At 150 V declustering potential, a peak corresponding to the aposubunit plus two iron atoms (13 578.8 Da) is observed. (b) At 250 V declustering potential, the aposubunit with two (13 578.7 Da) and with no iron atoms (13 473.0 Da) produces the most intense peaks. The peak at lower abundance (13 525.7 Da) corresponds to the aposubunit plus one iron atom. (c) Under denaturing conditions, the only component present in the mass spectrum is the aposubunit (13 474.5 Da).

dissociation of the holoprotein reveals details of the metal binding. Figure 3 shows a deconvolution of the region around *m*/*z* 1000−3000, corresponding to monomer ions with 5−8 charges. At a declustering potential of 150 V, one principal peak is observed, with a mass of 13 578.8 Da, shown in Figure 3a. At a higher declustering potential, 250 V, two major peaks are observed that correspond to ions with average molecular masses of 13 473.0 and 13 578.7 Da, and a minor contribution is found at 13 525.7 Da, as shown in Figure 3b. The lowest mass ion of this group has the same average molecular mass as that obtained by either ESI−FTICR or by ESI-TOF of the subunit using denaturing conditions, shown in Figure 3c, corresponding to the monomeric apoprotein. The peaks in Figure 3b are equally spaced, with a 53 mass unit separation. This pattern suggests that the higher mass peaks contain one and two iron atoms. The mass separation between peaks that differ in the number of iron atoms that are present is 53 Da in the deconvoluted mass spectrum. That the mass difference is not 56 Da, corresponding to the atomic mass of iron, may seem surprising. However, the smaller mass difference is an artifact of the manner in which the deconvolution process is performed. The deconvolution procedure assumes that all the excess charge present on an ion is due to protons. The masses of the excess protons are subtracted from the product of the charge times the mass-to-charge ratio in determining the deconvoluted mass of the ions. Because this procedure ignores the fact that the metal ions also carry charge, the deconvolution procedure produces a mass that corresponds to an elemental composition with fewer hydrogens for metal-containing proteins than that of the apoprotein. The number of fewer hydrogens is equal to the sum of the oxidation states of all the metal atoms. The mass difference between the metal-containing peaks and that of the apoprotein can, therefore, be used to determine the oxidation state of the metals. For example, the data in Figure 3 suggest that the oxidation state of both irons in a subunit of this protein is 3+, i.e., Fe(III), as expected for the net oxidation level of hemerythrin.[43,47] This pattern of metal binding accompanied by proton loss has been observed by others in the ESI mass spectra of small, monomeric metalloproteins. Fenselau and coworkers have reported that the peaks in the mass spectra of metallothioneins correspond to the replacement of two protons by each Zn^{2+} and Cd^{2+} that is bound.[40] A number of researchers have studied Cu^{2+}, Zn^{2+}, and Cd^{2+} binding by zinc finger proteins and have reported these metals displace a number of protons that corresponds to the coordination numbers of the metals that have been introduced.[10,38] Similar phenomena have been observed by Loo et al. for the angiotensin peptides with zinc[21]and by Van Dorsselaer et al. for the binding of Fe ions by a synthetic protein and for the monomeric metalloproteins rubredoxin and ferredoxin.[22] Unlike these earlier studies, we observe a complete series of peaks corresponding to the mass of the subunit with all possible numbers of metal atoms (i.e., 0−2) up to the maximum value that is known to be bound by the subunit of the protein in solution. This series of ions makes it easy to count the exact number of metal atoms associated with a subunit and, furthermore, seems to indicate the oxidation state of each metal atom. This pattern is found for all the metalloproteins analyzed in this study. Some

caution must be taken in relating the oxidation state observed for the metal as a gas-phase ion to the oxidation state of the metal in the solution-phase protein. It is possible that the oxidation state of the metal can change during the electrospray ionization process.

It is notable that there is no evidence of the presence of the bridging oxo ion in the mass spectrum of the hemerythrin subunit, even though it appears to be present in the holoprotein mass spectrum. It is possible that the capillary−skimmer dissociation conditions used to dissociate the subunit from the holoprotein were vigorous enough to remove the bridging oxo ion. The complementary heptamer produces a deconvoluted mass spectrum (not shown) with a low-abundance component at 95 101 Da and a more abundant component at 95 202 Da, compared to the calculated mass of 95 185 Da for a heptamer with bridging oxygens present at the diiron site. From these data, it is difficult to conclude whether the bridging oxygen is present in each diiron site of the heptamer. Of the three peaks corresponding to different numbers of irons bound by the subunit, the peak corresponding to the subunit plus one iron atom has the lowest abundance. This is consistent with the apparent "all or none" iron atom affinity of hemerythrin. Hemerythrin has never been reported in a stable form containing only a single iron per subunit. Previous reports of cooperative metal-binding by monomeric proteins have utilized ESI titration studies, in which changes in the metal distribution are measured for mixtures of apoproteins with several concentrations of metal salts.[3,4] Here, evidence of cooperative metal-binding is obtained directly from the ESI mass spectrum of the protein, without the addition of metal salts. The results presented for hemerythrin show that cooperative metal-binding by the gas-phase ions of hemerythrin does not require oxygen bridging of the metal atoms.

***Lingula reevii* Hetero-Octamer hemerythrin (Lr-Hr).** We have examined a naturally occurring hemerythrin protein isolated from *L. reevii*. Previous work had shown that this hemerythrin has a hetero-octameric structure with two types of subunits, a heavy subunit, α, and a lighter subunit, β, present in approximately equal proportions.[45,46] High-performance liquid chromatography was used in prior work to determine the proportion of the subunits in the holoprotein. The present study is the first to use mass spectrometry to accurately determine the oligomer stoichiometry of this protein. Both subunits have been sequenced and are known to consist of 117 residues and to contain a diiron site esentially identical to that in *P. gouldii* hemerythrin.[46]

This protein was dissolved in the same manner as the recombinant Pg-Hr for ESI analysis. The protein was found to have a low solubility in the 5 mM ammonium acetate buffer that was used for ESI studies, and some of the Lr-Hr was found to precipitate from a 5 μM (protein concentration) solution. The monomer concentration of the Lr-Hr solution used for these studies is estimated to be between 1 and 5 μM. Mass spectra acquired for the intact holoprotein by ESI-TOF at capillary−skimmer potentials of 100, 140, and 180 V are shown in Figure 4a−c, respectively. At 100 V capillary−skimmer voltage, Figure 4a, peaks corresponding to the octameric holoprotein are observed between m/z 4500 and 5500. The 23+ charge state gives rise to the most intense peak in the series. When the capillary−skimmer voltage is increased to 140 V, the most intense peak in the mass spectrum results from the 22+ charge state, as shown in Figure 4b. The trend of decreasing charge states at higher capillary−skimmer voltages has been reported for ESI previously by others.[34,35,47] We observe, at the same time, that the molecular weight of the peak corresponding to the holoprotein decreases, asymptotically approaching a final value, when the capillary−skimmer voltage is increased, and this can be rationalized as more effective desolvation at higher capillary−skimmer voltages. The heptamer peaks and the monomer peaks become much stronger at higher capillary−skimmer voltages. Deconvoluted mass spectra of the octamer peaks at capillary−skimmer voltages of 140 and 180 V, as well as the heptamer peak at 180 V, are shown in Figure 5. The measured average molecular weight is 110 596 (at 180 V declustering potential). This may be compared with a calculated mass of 110 581 Da based on a subunit stoichiometry of $\alpha_4\beta_4$, with iron, oxygen, and hydrogen accounted for as in the manner used for the homo-octamer Pg-Hr hemerythrin. Thus, the mass measurement error is 135 ppm for the holoprotein and appears to be largely due to incomplete desolvation of the holoprotein. Consistent with this interpretation is that the mass spectrum exhibits an asymmetric peak with a long tail on the high mass side, as seen in Figure 5. Other stoichiometries of the subunits would produce much larger deviations (>2500 ppm) from the measured values. We conclude that the principal multimeric state of this protein is $\alpha_4\beta_4$, as suggested, but never conclusively demonstrated, in prior work.[45,46] There is not sufficient resolution to completely separate octamer peaks of other stoichiometries if they were present, although other stoichiometries would be expected to produce shoulders to the observed peak, if they had a substantial abundance. The slight shoulder at 111 000 Da might indicate a minor component of $\alpha_5\beta_3$, but a higher resolution

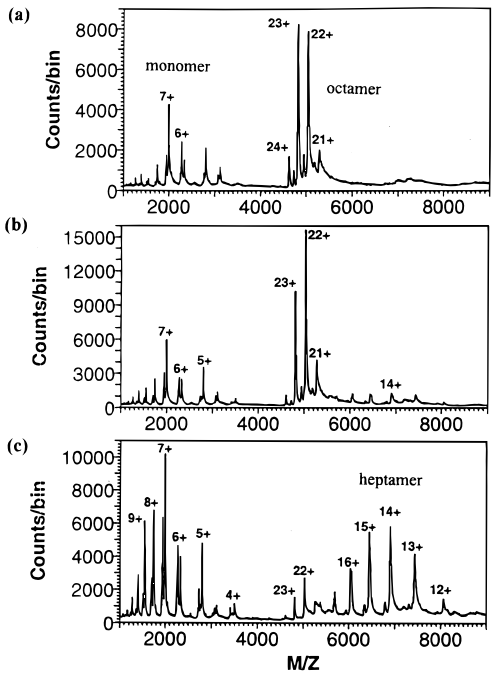

Figure 4. ESI-TOF mass spectra of naturally occurring Lr-Hr, obtained under nondenaturing conditions and at capillary skimmer potentials of (a) 100, (b) 140, and (c) 180 V. Peaks corresponding to an octameric holoprotein are found from m/z 4000−6000. Peaks corresponding to the subunits of the protein appear from m/z 1000−4000. At high capillary−skimmer potentials, peaks corresponding to heptameric complexes of the protein are found from m/z 6000 to 8000.

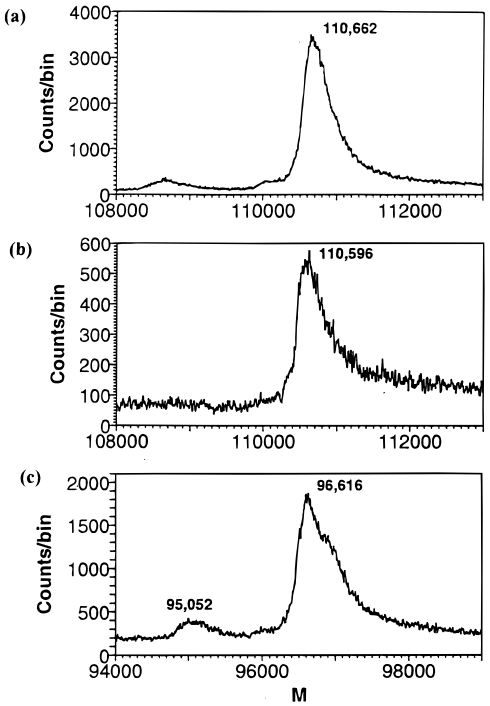

Figure 5. (a) Mass spectrum produced by deconvolution of the peaks between *m*/*z* 4000 and 6000 (*z* = 21−23) in the mass spectrum of Figure 4b, corresponding to the octameric holoprotein of Lr-Hr at a 140 V declustering potential. The large width of the octamer peak is caused by the heterogeneity shown in Figure 6. (b) Mass spectrum produced by deconvolution of the peaks between *m*/*z* 4000 and 6000 (*z* = 21−23) in the mass spectrum of Figure 4c, corresponding to the octameric holoprotein of Lr-Hr at a 180 V declustering potential. (c) Mass spectrum produced by deconvolution of the peaks between *m*/*z* 6000 and 8000 in Figure 4c, corresponding to the heptameric complexes (*z* = 14−16) $\alpha_3\beta_4$ and $\alpha_4\beta_3$.

measurement would be required to discern this possibility. At high capillary−skimmer voltages, a very broad peak centered around 96 616 Da is observed, with a shoulder near 97 000 Da, corresponding to the formation of a heptamer by collisional dissociation of the octameric holoprotein. The mass difference between the center of the main heptamer peak and its shoulder corresponds to the calculated mass difference between ions of stoichiometries $\alpha_3\beta_4$ and $\alpha_4\beta_3$, indicating that either subunit is lost by collisional dissociation of the holoprotein, although loss of the heavier chain seems favored.

The details of metal binding in the subunits of this protein are easily discerned from an examination of the monomer region of the mass spectrum, i.e., *m*/*z* 1000−4000. Figure 6 compares the deconvoluted mass spectrum of the monomers obtained at a capillary−skimmer potential of 180 V with the mass spectrum obtained for the denatured protein by ESI-TOF using a 3% acetic acid−methanol−water solution. Iron-free α (13 877.0 Da) and β-subunits (13 517.5 Da) are observed in the mass spectrum of the denatured protein shown in Figure 6a, with additional peaks corresponding to a low concentration of two modified components (M + 16, M + 41), but no iron-containing states are observed. When the protein is dissolved in aqueous solution at pH 6, the monomer peaks observed in Figure 6b are obtained at a capillary−skimmer potential of 180 V. Both the aposubunits and iron-containing α and β subunits are observed. The additional peaks (M + 16, M + 41) observed in the mass spectrum of the

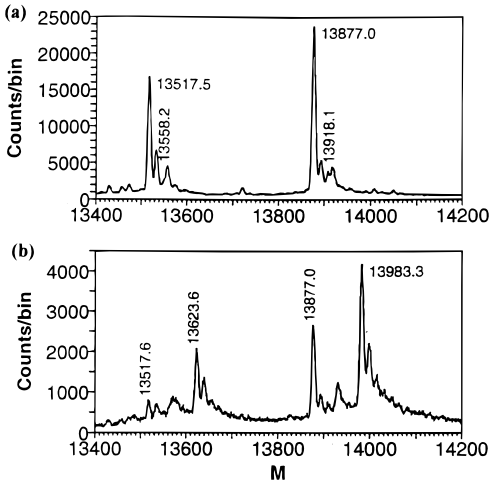

Figure 6. (a) ESI-TOF mass spectrum of Lr-Hr obtained under denaturing conditions, showing peaks corresponding to the α and β subunits of the protein. There is no evidence of iron attachment in this mass spectrum. (b) In contrast, the mass spectrum produced by deconvolution of the peaks between *m*/*z* 1000 and 4000 in Figure 6c, corresponding to the monomeric subunits of Lr-Hr, shows evidence of the presence of 0−2 iron atoms.

denatured protein are also observed in the mass spectrum of the metal-containing subunits. The average molecular masses of the aposubunits are measured to be 13 877.0 and 13 517.5 Da. Compared with the average molecular weights derived from the amino acid sequences, 13 878.9 and 13 519.3, the experimental data are low by about 2 amu, an error of 125−150 ppm. This is not surprising in view of the poorer quality of the spectra here. The two subunits bind up to two iron atoms, as seen in the mass spectrum of Figure 6b. Di-iron-containing states are found at 13 983 and 13 624, 106 amu above the peaks for the aposubunit α and β chains. As in the case of Pg-Hr, this pattern suggests an oxidation state of 3+ for both iron atoms. As was observed for Pg-Hr, the peaks corresponding to the attachment of a single iron atom to the subunit have much lower abundance in the mass spectrum than do the subunits either with no metal atoms or with two metal atoms, consistent with cooperative binding of the metal atoms by the subunits.

***Desulfovibrio vulgaris* Rubrerythrin (Dv-Rr).** Rubrerythrin has three metal atoms per subunit. One is located in a rubredoxin-like FeS_4 group and the other two in a hemerythrin-like oxo-bridged di-iron center.[48−51] The apoprotein of wild-type Dv-Rr has been sequenced and found to have 191 amino acids per subunit, with an average molecular mass of 21 544.2 Da.[50] The oligomeric nature of the rubrerythrin has been studied by both gel filtration and X-ray crystallography prior to this work. The results obtained in earlier studies are in conflict with each other. The gel filtration experiments indicate a homodimer for the holoprotein,[48,51] while the crystal structure suggests a tetramer.[63] Iron-containing, multimeric forms of Dv-Rr are observed by ESI-TOF mass spectrometry of the protein dissolved in 5 mM ammonium acetate solution at pH 6. Three mass spectra of Rr recorded at capillary−skimmer potentials of 150, 220, and 300 V are shown in Figure 7a−c, respectively. The mass spectrum from

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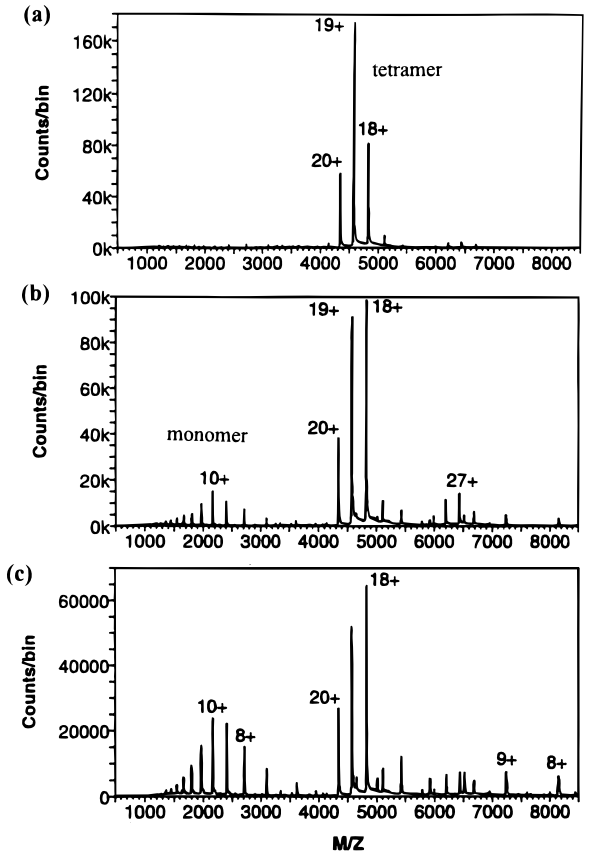

Figure 7. ESI-TOF mass spectra of recombinant Dv-Rr, obtained under nondenaturing conditions, at capillary−skimmer potentials of (a) 150 V, (b) 220 V, and (c) 300 V. Peaks corresponding to a tetrameric holoprotein are found at *m*/*z* 4000−5000. At higher capillary−skimmer potentials, peaks corresponding to a subunit appear at *m*/*z* 1000−4000 and to a trimeric complex at *m*/*z* 6000−9000.

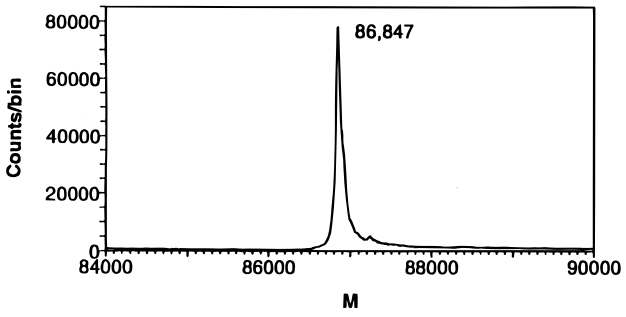

Figure 8. Mass spectrum produced by deconvolution of the peaks from *m*/*z* 4000−6000 (*z* = 18−20) in the mass spectrum of Figure 7b. The mass of the observed peak corresponds to a tetrameric complex.

the deconvolution of the data collected at 220 V capillary−skimmer potential is shown in Figure 8. A tetrameric protein with an average molecular mass of 86 847 Da is observed, without any evidence of a dimer structure. As expected, the molecular mass of the tetramer ion is observed to decrease (and to asymptotically approach the expected value) as the capillary−skimmer potential is increased from 150 to 300 V, consistent with the dependence of the observed holoprotein mass on capillary−skimmer potential that was observed for the two hemerythrin proteins. A molecular mass of 86 874 Da is calculated for tetrameric Dv-Rr, assuming

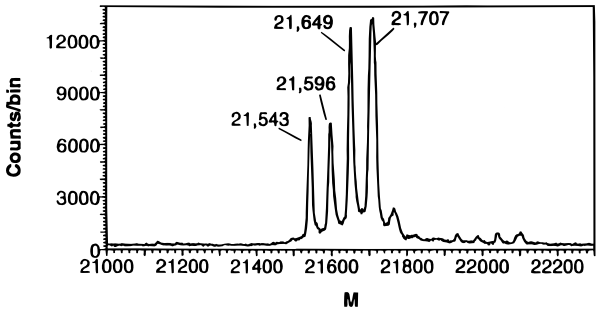

Figure 9. Mass spectrum produced by deconvolution of the peaks from *m*/*z* 1500−3000 (*z* = 8−12) in the mass spectrum of Figure 7c. The masses of the peaks correspond to the mass of a subunit that contains from 0 to 3 iron atoms.

that each subunit binds three iron atoms, that each of the FeS_4 clusters is formed by the loss of four hydrogen atoms from cysteine residues, that each di-iron site has a bridging oxygen group, and that each di-iron cluster displaces five hydrogen atoms from the side chains of glutamic acid residues.[63] The close agreement between the measured and calculated values (0.04%) for Dv-Rr tetramer supports our assignment of the composition of the holoprotein. A small amount of octameric protein is observed at *m*/*z* 6000−7000 at the lowest capillary−skimmer potential, Figure 7a. Curiously, the relative abundance of the octamer increases slightly at a capillary−skimmer potential of 220 V, Figure 7b. Trimer and monomer states are observed at higher capillary−skimmer potentials, but no dimer state is observed.

The tetrameric complex observed by ESI mass spectrometry in this study is in agreement with the crystal structure that has been obtained for this molecule.[51] However, it should be noted that the ionic strength of the solution that has been examined in the mass spectrometry experiment (5 mM ammonium acetate) is much lower than the ionic strength of the solution that was used for gel filtration measurements.[51] It is possible that the low ionic strength of the solution used for these mass spectrometry measurements causes the aggregation of the dimeric holoprotein into a tetramer. However, there is little evidence in the mass spectrum that the tetramer consists of a dimer of dimers, since collisional dissociation of such a complex would be expected to yield dimers, and these are not observed at any capillary−skimmer potential.

As expected, peaks corresponding to the monomer subunit increase in abundance as the capillary−skimmer potential is raised. A broad distribution of charge states is observed for the monomer, spanning *z* = 6−16, at capillary−skimmer potentials greater than 200 V. Unlike the previous hemerythrin examples, in which peaks corresponding to the monomer appeared even at low capillary−skimmer voltages, there are no significant peaks corresponding to the monomer of rubrerythrin at capillary−skimmer potentials less than 150 V. The deconvoluted mass spectrum of the monomer region (*m*/*z* 1500−3000, *z* = 8−12) obtained at a capillary−skimmer potential of 300 V is shown in Figure 9. Four peaks are observed at 21 543, 21 596, 21 649, and 21 707 Da, with spacings consistent with the presence of three iron atoms in each subunit, in agreement with the previously reported composition of the subunits of this protein. The three peaks of lowest mass are separated by 53 amu, as was seen for the two hemerythrin proteins, suggesting the presence of two Fe-

(III) ions. The three lower mass peaks show a pattern similar to that observed for the hemerythrin proteins; that is, the abundance of the middle peak is lower than that of the other two peaks. The highest mass peak of the four peaks that appear in the deconvoluted mass spectrum is separated by 58 amu from the adjacent peak This separation is somewhat greater than the 53 amu difference observed for the other two iron atoms. We have noticed that the peak corresponding to the subunit that contains three irons is always wider than the others in the mass spectrum. This suggests a superposition of a peak with the normal spacing of 53 and a peak at higher mass. Recent high-resolution data obtained by electrospray Fourier transform ion cyclotron resonance spectrometry of this protein suggest that this peak is composed of ions that are 53 and 71 amu higher than the peak that contains two iron atoms, corresponding to $[\text{subunit} + 3\text{Fe} - 9\text{H}]^+$ and $[\text{subunit} + 3\text{Fe} + \text{H}_2\text{O} - 9\text{H}]^+$.[64] In addition to the four principal peaks present in this mass spectrum, there is a minor peak at 21 765 Da that would suggest the attachment of a fourth iron atom to a small fraction of the protein sample. This observation is consistent with previous electron spin resonance studies of rubrerythrin that have detected a minor component of "adventitiously" bound iron.[51]

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CONCLUSIONS

The data presented here demonstrate the capability of ESI-TOF mass spectrometry to provide important structural details for metal-containing, multimeric proteins. Not only do these measurements provide accurate determinations of the molecular weights of the subunits and the holoproteins, they also yield information about subunit stoichiometry, metal atom stoichiometry, cooperativity of metal attachment, and, perhaps, the oxidation state of the metal atoms. This information can be obtained rapidly with small quantities (tens of picomoles) of protein. The capabilities presented here will provide a significant complement to the other spectroscopic techniques that are used to characterize metalloproteins.

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