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Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry of multimeric metalloproteins

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Abstract

Five multimeric metalloprotein species are examined by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Isotopically resolved mass spectra are obtained for noncovalent complexes with masses ranging from 25 to 86 kDa. Samples must be rigorously desalted, as these large molecules offer more sites for proton-sodium exchange, and the heterogeneity of multiply sodiated species causes a decrease in the duration of the beat signal in the acquired transient, and thus a decrease in signal to noise. Desalting methods reported by others for multimeric proteins, for example buffer exchange with citrate, cause loss of the metal center in the proteins studied here. Extensive dialysis was found to be necessary in order to observe isotopically resolved mass spectra. With these methods, multimeric complexes are observed for iron–sulfur proteins as well as heme proteins. (Int J Mass Spectrom 210/211 (2001) 651–663) © 2001 Elsevier Science B.V.

Keywords: FTICR; Metalloproteins; Electrospray; Ferredoxin; Nigerythrin; Hemoglobin

1. Introduction

Noncovalent interactions between protein and peptides play an important role in the chemistry of biomolecules for processes such as molecular recognition, as well as enzyme-substrate, protein-ligand, and antibody-antigen interactions [1]. These interactions are structurally specific in nature. Many physiologically active proteins are multimeric and possess active sites at the interface of the subunits [1]. Noncovalent interactions between the subunits of the folded protein lead to aggregation into a specific quaternary structure. These interactions are relatively weak and can be disrupted in protein solutions by altering the pH, the percentage of organic solvent, or by using a denaturing agent [2–4].

The detection of specific noncovalent interactions by electrospray ionization (ESI) mass spectrometry has been demonstrated by a number of researchers [4–38]. Until recently, mass spectrometry was not suitable for examining noncovalent complexes because these interactions were disrupted during the ionization process prior to mass measurement. With the appropriate experimental conditions, ESI can be used to examine a solution in or near its physiological state, thereby maintaining the oligomeric stoichiometry of multimeric proteins during ionization [5,12–14,18,21,28,31,39–42]. For example, the tetrameric protein soy bean agglutinin [molecular weight (MW)~116 kDa], one of the first examples of the measurement of a multimeric protein complex by

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mass spectrometry, was observed by ESI-time-of-flight (ESI-TOF) mass spectrometry to produce ions between *m/z* 4000 and 5000 that correspond to the tetrameric holoprotein [31]. Octamers and dodecamers were also detected at lower abundance and higher mass to charge. The oligomeric stoichiometry of 4-oxalocrotonate tautomerase has been observed by ESI-TOF [12,13], and by ESI Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry using in-trap cleanup methods to remove adduction [43]. ESI measurements of a sequence specific protein-DNA complex have been reported [9]. Noncovalent dimers of leucine zipper peptides have been detected by ESI, ionspray, liquid beam desorption, and laser desorption mass spectrometry [44–47]. ESI quadrupole mass spectrometry has been used to examine the avidin-biotin complex [27]. These and other results support the claim that higher order structures are retained in the gas phase by using the appropriate sample handling procedures.

ESI-TOF mass spectrometry is useful for examining noncovalent interactions because of the wide mass range of this instrument. However, the ESI-TOF mass spectrometer has limited mass resolution. ESI-quadrupole mass spectrometry has also been used to examine noncovalent interactions under physiological conditions, but the mass resolution and mass-to-charge range is limited with this instrument. The mass to charge of multimeric protein ions produced by ESI under nondenaturing conditions often exceeds the mass range of standard quadrupole mass spectrometers, and only those instruments with an extended mass range can be used for such studies [10,18,19,27,48]. ESI-FTICR mass spectrometry combines high resolution with a wide mass range, and therefore offers additional capabilities for the study of noncovalent interactions [6,8,17,28,32,43].

Although Fourier transform ion cyclotron resonance (FTICR) mass spectrometry seems well suited for the analysis of protein multimers, a few issues are worth consideration. First is the time scale of the ESI-FTICR experiment. A high resolution ESI-FTICR experiment requires that a noncovalent complex is stable for 1–10 s, five orders of magnitude longer than the 100 μs required for an ESI-TOF measurement. Although several examples of FTICR analyses of noncovalent protein multimers have been published [6,8,9,17,28,32,43,44,49–51], only a few show isotopic resolution [43,50]. These examples have served to establish that the lifetime of multiply charged noncovalent complexes is suitable for FTICR analysis. Another consideration is that in the FTICR experiment, all species in the cell are detected at the same time. The constructive and destructive interferences of the observed frequencies of the closely spaced isotope species in the cell produce a beat pattern in the transient that is recorded [52,53]. As the mass of the molecule increases, the beats in the transient become shorter in duration and more widely spaced in time [54]. In order to achieve isotopic resolution, it is necessary to sample at least two beats in the transient [54,55]. For high mass molecules such as noncovalent complexes, the challenge is maintaining the coherence of the ion packet for the long time necessary (several seconds) to observe the second beat in the transient.

Here we present a study of five metalloprotein noncovalent complexes with molecular weights from 25 to 86 kDa that illustrate the capabilities and challenges of using ESI-FTICR for such measurements. Metalloproteins are more difficult to analyze as holoproteins than standard proteins. The purification and desalting methods that have been successful for other proteins often result in loss of the metal in the metalloprotein active site, thus limiting the types of procedures that can be used [43,56,57]. Also, the metal centers of some metalloproteins are unstable toward ionization, leading to heterogeneity in the detected species. These and other issues are illustrated in the present work.

2. Experimental

Clostridium pasteurianum [2Fe–2S] ferredoxin was isolated as the native protein from bacterial culture [58]. Desulfovibrio vulgaris nigerythrin and desulfovibrio vulgaris rubrerythrin were obtained by overexpression in escherichia coli [59]. Bovine hemoglobin was obtained from Sigma (St. Louis, MO,

USA). Ferredoxin was desalted by repeated ultrafiltration with a 10 kDa MWCO membrane (Millipore, Bedford, MA, USA) followed by resuspension in 10 mM ammonium acetate. Final ferredoxin dimer concentration was approximately 10 μM. Nigerythrin and rubrerythrin were desalted by 16 h of dialysis in a bath of 10 mM ammonium acetate in a 10 kDa Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA). Final concentration was 40 μM for nigerythrin and hemoglobin and 80 μM for rubrerythrin based on the mass of the subunits of the proteins. Concentration for bacterial proteins was determined by UV-visible spectrophotometry. Hemoglobin heterotetramer was desalted by repeated centrifugation through a 30 kDa MWCO ultrafiltration tube (Millipore, Bedford, MA, USA) followed by resuspension in 10 mM ammonium acetate. The protein concentration was 80 μM (for the subunit). All proteins except the hemoglobin heterodimer were sprayed out of 10 mM ammonium acetate. The hemoglobin heterodimer was sprayed out of 10% methanol to decrease the abundance of the tetrameric and increase the abundance of the dimer. Without methanol, monomer, dimer, and tetramer are all observed in the mass spectrum of hemoglobin. With 10% methanol, only the monomer and dimer are observed.

Mass spectrometry was performed with a 7 T FTICR mass spectrometer (Bruker BioApex, Billerica, MA, USA) equipped with an electrospray ion source (Analytica, Branford, CT, USA). We were unable to observe any multimers with the standard glass capillary, and so it was replaced with a heated metal capillary inlet [60]. The heated metal capillary provides gentler desolvation and allows the noncovalent complex to be more easily observed. A capillary temperature of 150 °C to 200 °C was used for these experiments. Samples were ionized via nanospray, through tips fabricated in house from 100 μm i.d. fused silica (Polymicro Technologies, Phoenix, AZ, USA; Supelco, Bellefonte, PA, USA). Pumping was provided through a syringe pump at 6 μL/h, and the nanospray voltage was applied at the needle of the syringe. Space charge proved to be a major concern in the observation of multiple beats in the transient. The ion density within the cell was carefully controlled in order to minimize space charge effects. This was achieved by accumulating ions in the external hexapole for times no greater than 0.7 s. Trap voltages were 0.5 and 0.65 V for the front and back traps for ferredoxin and nigerythrin, and 0.4 and 0.55 V for hemoglobin dimer and tetramer and rubrerythrin. These low trap voltages reduced space charge, enabling the observation of isotopic structure for the high mass species presented here. 50 to 100 transients were coadded for each of the mass spectra presented here.

3. Results and discussion

Ferredoxin from clostridium pasteurianum (CpFd) forms a dimer containing one [2Fe–2S] cluster per subunit, with a dimer mass of approximately 23 kDa. Previous ESI-quadrupole mass spectrometric research on CpFd had shown a multiplicity of peaks within each charge state and were assigned as the dimer and alkali cationized dimer species, but the peaks were not resolved well enough to make an accurate assignment [61]. The ESI-FTICR mass spectrum obtained for CpFd dimer is shown in Fig. 1. At this relatively low molecular weight (for a protein multimer), isotopic resolution is readily achieved. The sample required more extensive desalting than monomeric proteins. Moderate capillary temperatures (150 °C) were used in order to accomplish adequate desolvation. Higher capillary temperatures caused degradation of the protein, whereas lower capillary temperatures resulted in loss of signal due to inadequate desolvation. Fig. 1(a) shows the mass spectrum, and the inset is an expansion of the 11+ charge state, demonstrating isotopic resolution. Three different isotopic distributions are evident within the 11+ charge state. The most abundant of these corresponds to a MW of 232 22.2 Da, which is 30 Da higher than the theoretical dimer MW of 231 92.5 Da, calculated for the amino acid sequence plus one [2Fe–2S] cluster per subunit [62]. Previous research with iron–sulfur proteins has demonstrated that sulfur adducts often arise in the presence of free cysteines through the formation of a dithiol (Cys–S–SH) [60]. CpFd contains five cys-

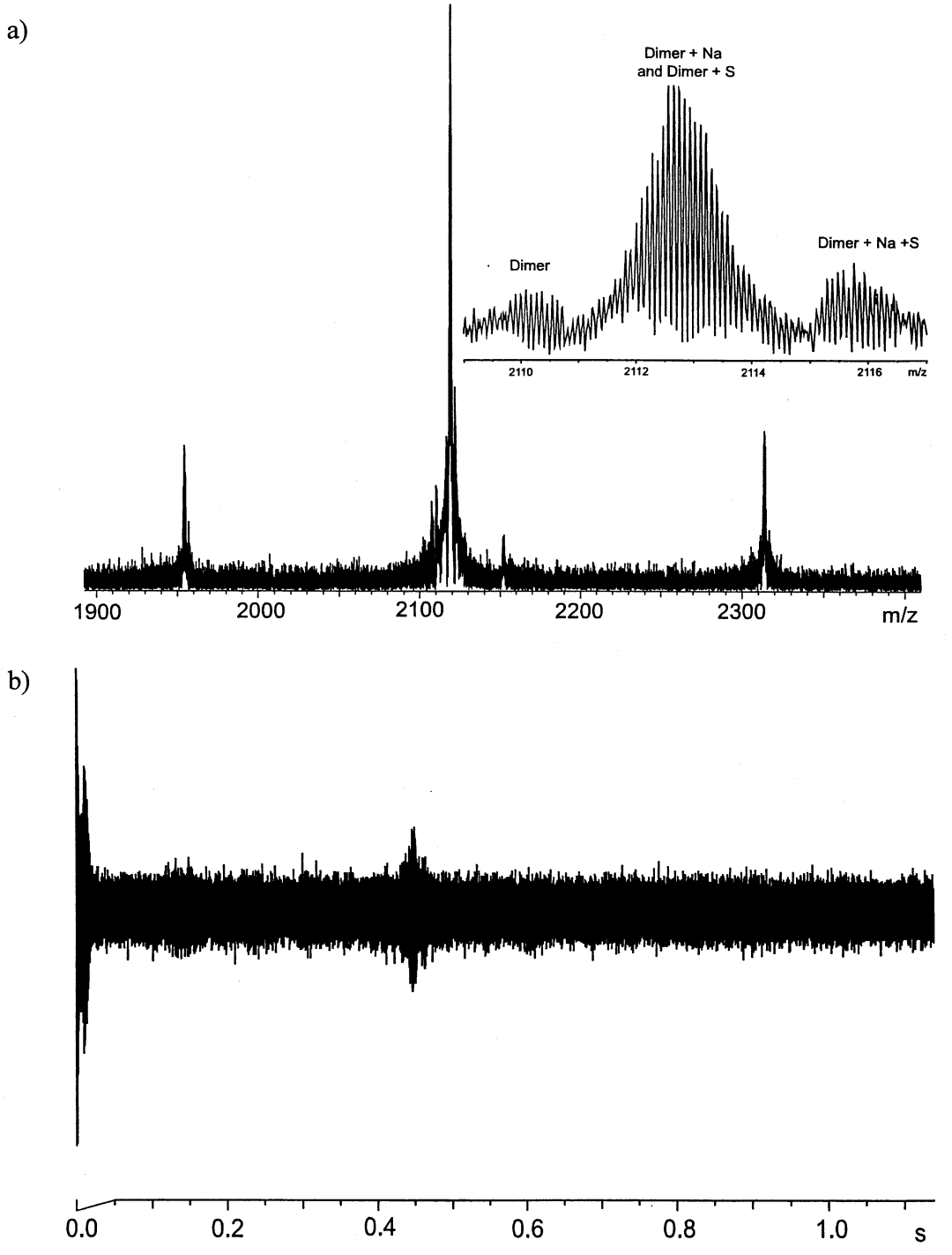

Fig. 1. ESI-FTICR mass spectrum of [2Fe–2S] ferredoxin from clostridium pasteurianum, a 23 kDa dimer. (a) Mass spectrum of the 12+ through 10+ charge states, with an expansion of the 11+ charge state showing isotopic resolution, binding of sodium and addition of sulfur to the dimer. 50 scans were coadded. (b) Transient for this mass spectrum, showing a second beat at 0.45 s.

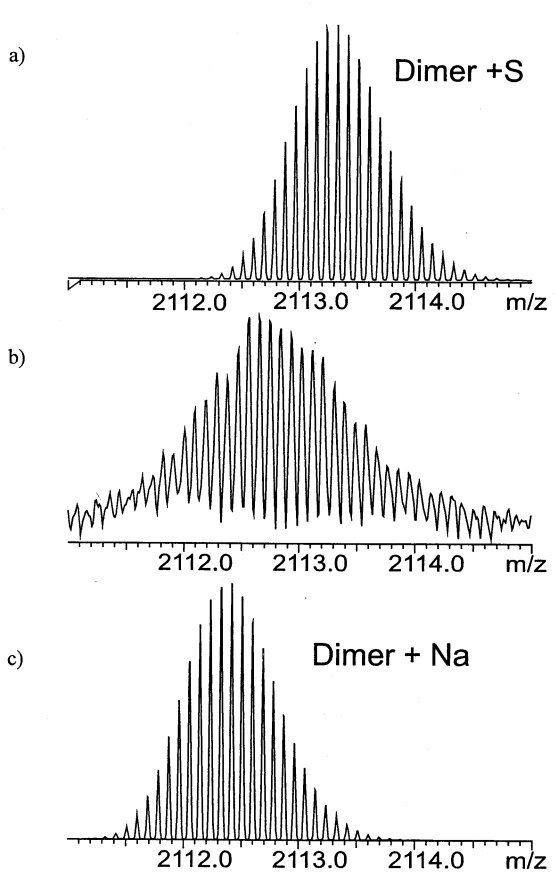

Fig. 2. Comparison of the isotopic distribution of the most abundant peak in the 11+ charge state for CpFd with theoretical isotopic distributions generated for the dimer plus sodium and for the dimer plus sulfur. (a) Theoretical isotopic distribution of CpFd dimer plus sulfur. (b) Experimental isotopic distribution of the most abundant peak in the 11+ charge state of CpFd. (c) Theoretical isotopic distribution of CpFd dimer with a single sodium-proton exchange.

teines, of which four are ligands to the iron–sulfur cluster. The fifth cysteine is free and capable of binding inorganic sulfide. The 30 Da mass difference between the theoretical dimer mass and the mass of the most abundant peak is too great for a sodium adduct, but too small for a sulfur adduct. Fig. 2 shows a comparison between the experimental isotopic distribution and theoretical isotopic distributions generated for the dimer plus a sodium adduct and the dimer plus a sulfur adduct. Careful examination of the width and shape of the experimental peak suggests that it is composed of a mixture of a dimer plus sodium with a dimer plus sulfur, an assignment only discernable through the high resolution of ESI-FTICR. This assignment is confirmed by the peaks centered around *m/z* 2116 in Fig. 1, which appear approximately 32 Da above the most abundant peak and are assigned as the addition of both a sulfur and a sodium to the dimer. Petillot and coworkers also observed three sets of peaks in the dimer mass spectrum of CpFd, which were assigned as the dimer plus multiple sodium and potassium adducts. The high resolution and high mass accuracy of the FTICR instrument shows that these higher mass species are a result of sodium binding and sulfur adduction.

The peaks centered around *m/z* 2110 appear in the correct range for the dimer, although poor isotope statistics do not allow this assignment to be made with high accuracy. To verify the mass of the dimer, the protein was placed in 25% MeOH, causing partial disruption of the noncovalent interactions. The apo-subunit of the monomer gave a monoisotopic mass of 114 17.3 Da, in good agreement with the mass calculated from the amino acid sequence, 114 18.6 Da, assuming the formation of two internal disulfide bonds between the cysteines that normally bind the [2Fe–2S] cluster. Accurate assignment of monoisotopic mass of the metal-containing protein requires that the oxidation state of the metal cluster be taken into account [60]. For the CpFd dimer, each [2Fe–2S] cluster carries a 2+ oxidation state, so that four charges are attributed to the metal clusters. For example, assigning the molecular weight from the mass-to-charge value of the 11+ charge state in the mass spectrum required the subtraction of only seven protons from the mass of the ion.

Identification of the peaks in the dimer mass spectrum arising from sodium binding and sulfur adduction requires high resolution. Fig. 1(b) shows the transient obtained for the dimer, exhibiting a second beat at 0.45 s. The beats in the transient are due to destructive interference of the cyclotron frequencies associated with the isotopes of the most abundant charge state. The duration between beats is given by

$$\Delta t \cong \frac{2\pi m^2}{zB} \times 10^{-8} \quad (1)$$

where Δt is the beat period, m is the molecular mass of the complex (in Da), B is magnetic field strength in Tesla, and z is the charge state of the principal peak [54]. For the 11+ charge state of this 23 kDa complex, the calculated beat period is 0.4 s, as observed for the experimental data. The charge state distribution falls within the range m/z 1900–2400. This narrow distribution of relatively low charge states is consistent with the expected mass spectrum of a folded protein, as many of the potential sites for protonation are not accessible with a compact protein structure. The dimer is the only type of multimer observed, indicating a specific noncovalent complex consistent with the known biologically relevant multimer stoichiometry of this protein.

Most biologically interesting noncovalent complexes have higher molecular weights than ferredoxin, and so we have worked to increase the mass range of the protein complexes that can be examined with isotopic resolution. The mass spectrum of nigerythrin from desulfovibrio vulgaris is shown in Fig. 3. Under native conditions, nigerythrin forms a dimer weighing approximately 40 kDa in which each subunit contains an FeS_4 rubredoxin-like site as well as an oxo-bridged diiron site [59]. Fig. 3(a) shows the mass spectrum of the nigerythrin dimer obtained at a capillary interface temperature of 200 °C, with the expansion showing the isotope peaks. Fig. 3(b) shows the transient for the nigerythrin mass spectrum, with four beats appearing in 5 s, exhibiting the expected increased spacing of the transient beats with increasing mass. The wide mass range of the isotope distribution can be attributed to the presence of sodium adducts. The opportunity for sodium-proton exchange appears to increase as the protein complexes become larger. Removing sodium requires increasingly rigorous desalting techniques. In this case, we were unable to remove all traces of sodium even after 16 h of dialysis. Attempts to remove sodium, using citrate, which has been successful for other protein multimers [43], resulted in loss of the metal from the protein. Although isotopic resolution is achieved, a reliable monoisotopic MW cannot be extracted from these data, as the isotopic composition of the peaks cannot be assigned. Nevertheless, an average mass of 447 12.2 Da can be extracted from the data, which was 180 Da higher than the average mass of 445 32.1 Da calculated by adding the mass of six irons to the published amino acid sequence (obtained from The Institute for Genomic Research website at http://www.tigr.org). To determine whether this 180 Da mass difference could be attributed to other factors besides the presence of multiple adducts, the protein was run under denaturing conditions (49:49:2 H_2O:MeOH:HOAc v:v) to determine the monoisotopic mass of the monomer aposubunit. The experimentally determined monoisotopic mass of the monomer was found to be 221 80.5 Da, a difference of 97 mass units from the sequence-derived mass of 220 83.9 Da, indicating a disparity in the actual amino acid sequence from the published sequence. The average mass of the subunit obtained from this measurement is 221 92 Da. Using the measured molecular weight of the subunit, the molecular weight for the dimer is calculated to be 447 20 Da [221 92×2+56 (Fe)×6], in close agreement to the measured value (180 ppm error).

Both dimeric and tetrameric hemoglobin was examined by ESI-FTICR. To examine the dimer, the protein was dissolved in 10% methanol, which was found to yield only monomer and dimer ions in the broadband mass spectrum. Fig. 4 shows the narrowband mass spectrum of the heterodimer of bovine hemoglobin, obtained at a capillary temperature of 200 °C. The dimer has a monoisotopic mass of 322 21.1 Da, in good agreement with the calculated mass of 322 20.6 Da for a complex of one alpha subunit, one beta subunit, and two iron-containing heme groups. Hemoglobin exists predominantly in solution as a heterotetramer with $\alpha_2\beta_2$ stoichiometry. No evidence is found in the mass spectrum of a trimer form of hemoglobin, or of a homodimer of either α_2 or β_2 stoichiometry, indicating the species observed result from a specific noncovalent complex. Although some heterodimer was in evidence in a pure aqueous solution, the strongest signal for dimer was obtained with a 10% MeOH solution. To avoid space charge-

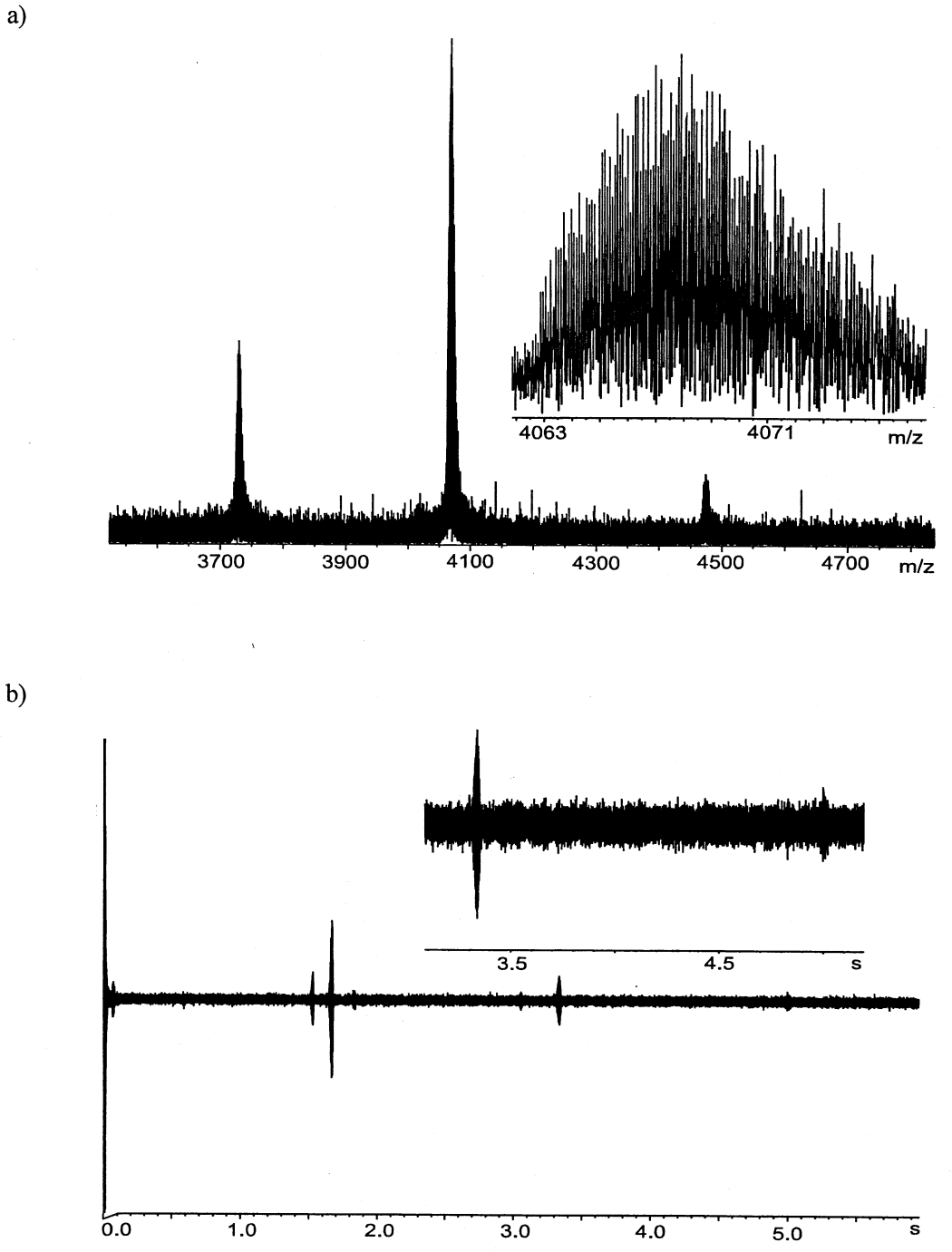

Fig. 3. ESI-FTICR mass spectrum of nigerythrin from desulfovibrio vulgaris, a 44 kDa dimer with two metal-containing active sites per subunit; one is an FeS_4 cluster, whereas the other is an oxo-bridged diiron site. (a) Mass spectrum of the 12+ through 10+ charge states, with an expansion of the 11+ charge state showing isotopic resolution. The broad isotopic distribution suggests sodium heterogeneity. 50 scans were coadded. (b) Transient for this mass spectrum, with an expansion showing the third and fourth beats at approximately 3.2 and 4.8 s, respectively.

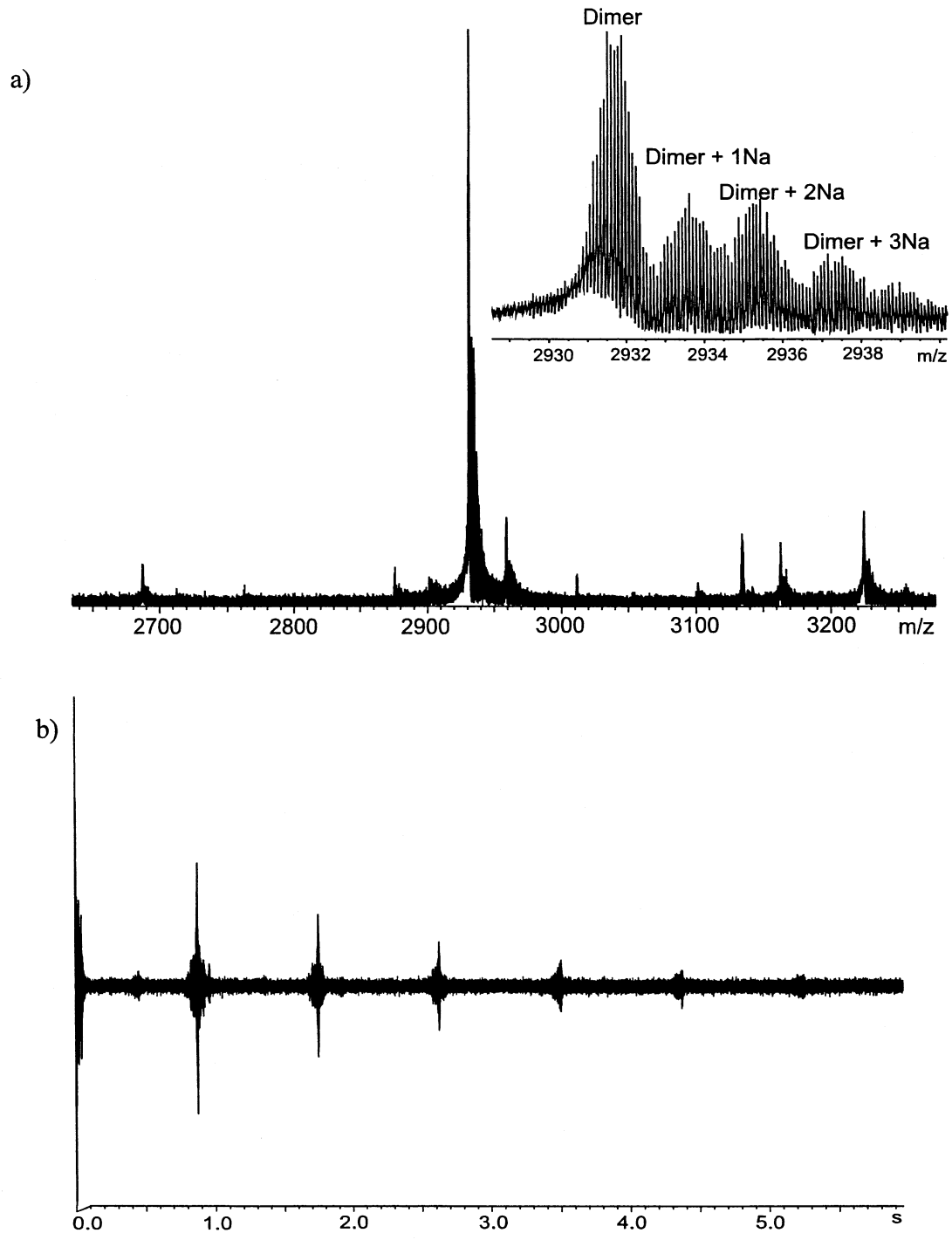

Fig. 4. ESI-FTICR mass spectrum of the dimer of bovine hemoglobin, with a mass of 32 kDa, corresponding to the alpha and beta subunits with their iron-containing heme groups. (a) Narrowband mass spectrum of hemoglobin dimer in 10% MeOH, with an expansion of the 11+ charge state showing isotopic resolution. 50 scans were coadded. (b) Transient for this mass spectrum, showing seven beats in 5.2 s.

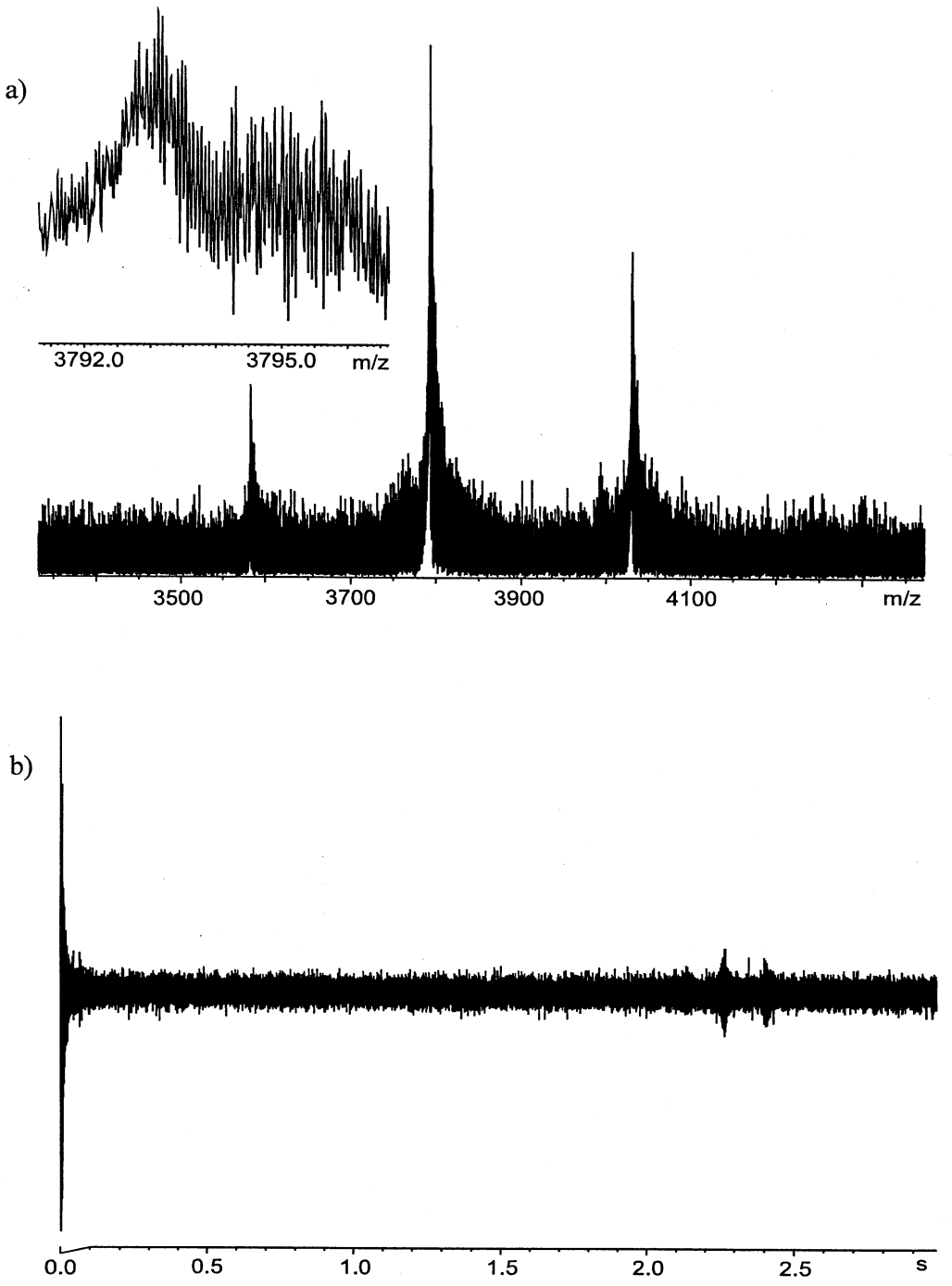

Fig. 5. ESI-FTICR mass spectrum of tetrameric bovine hemoglobin, under nondenaturing conditions. The 64 kDa tetramer has $\alpha_2\beta_2$ stoichiometry, with each subunit binding an iron-containing heme group. (a) Mass spectrum of the 18+ through 16+ charge states, with expansion of the 17+ charge state showing isotopic resolution. 100 scans were coadded. (b) Transient for this mass spectrum, showing a second beat at approximately 2.4 s.

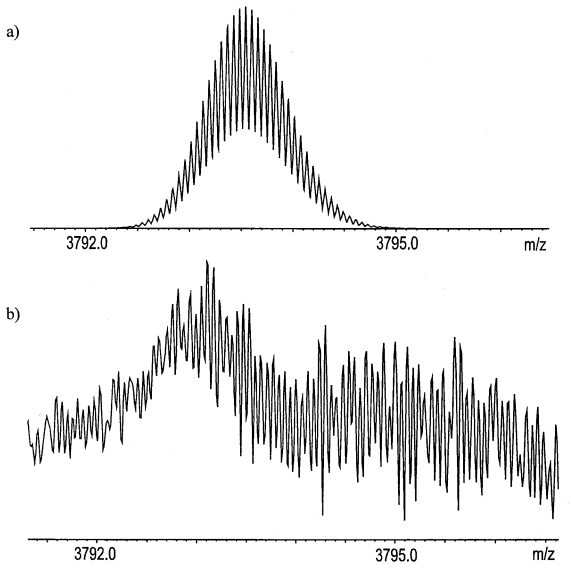

Fig. 6. Comparison of expected versus measured isotopic envelope for the 17+ charge state of heterotetrameric hemoglobin. (a) Expected isotopic distribution, calculated for the heterotetramer with $\alpha_2\beta_2$ stoichiometry. (b) Measured isotopic distribution, broader due to sodium adduction.

induced peak coalescence, it was necessary to use low trapping voltages, 0.5 and 0.65 V for the front and back trap plates, respectively. Fig. 4(a) shows a narrowband mass spectrum of the 11+ charge state, with the inset expansion showing the resulting isotopic structure. In addition to the dimer, higher mass peaks are observed corresponding to the substitution of up to three sodium cations for protons. In between the peaks corresponding to the substitution by one and two sodium cations, there is some signal in the region corresponding to the addition of dioxygen. We have been able to increase the abundance of this second oxygen molecule by saturating the protein solution with oxygen has prior to analysis. These results are the subject of a separate paper [63]. Fig. 4(b) shows the transient for this spectrum, which persists for over five seconds, with seven beats, ensuring isotopic resolution. At higher mass, beats in the transient are expected to become more widely spaced, as can be seen by comparison with Fig. 1(b). From equation 1, the beat period is predicted to be 0.85 s, in close agreement with the observation.

Fig. 5 shows the mass spectrum of the heterotetrameric form of hemoglobin, obtained by maintaining the analyte under nondenaturing conditions and heating the capillary interface temperature of 200 °C. Although the intact tetramer has been observed by both electrospray[19,64] and matrix assisted laser desorption/ionization [65], this is the first time that isotopic resolution has been achieved for this holoprotein noncovalent complex. The inset shows an expansion of the 17+ charge state, demonstrating isotopic resolution. Fig. 6 shows a comparison of expected versus measured isotopic envelope. The wide distribution of isotope peaks suggests heterogeneity induced by sodium-proton exchange. The most rigorous desalting techniques have not been able to remove all of the sodium from the sample, but has reduced the heterogeneity enough to allow observation of the second beat in the transient at approximately 2.3 s, Fig. 5(b). The breadth of the isotope distribution may also be due to oxygenation. Saturation of the protein solution allows us to observe a mass shift corresponding to the mass of four molecules of oxygen [63]. In addition to the problem of adduction, space charge has also proven to be a consideration. In order to achieve the second beat in the transient, it was necessary to further reduce the voltage on the front and back trap plates to 0.4 and 0.55 V, respectively. A shorter hexapole storage time (0.1 s) also seemed to aid the observation of isotopic resolution, presumably due to a reduction in the number of high-energy collisions in the hexapole, which might disrupt the noncovalent interactions. Because of the poor isotope statistics, the monoisotopic mass cannot be reliably assigned. Nevertheless, this is the highest mass metalloprotein complex for which an isotopically resolved mass spectrum has been reported. An average mass of 644 63 Da can be derived from these data, which compares favorably to the calculated value of 644 73 Da.

Fig. 7 shows a mass spectrum of desulfovibrio vulgaris rubrerythrin, a tetrameric protein with a MW of 86 kDa. Rubrerythrin contains an FeS_4 site and an oxo-bridged diiron site and has a high amino acid sequence homology to nigerythrin, but unlike nigerythrin, only the tetrameric form has been observed

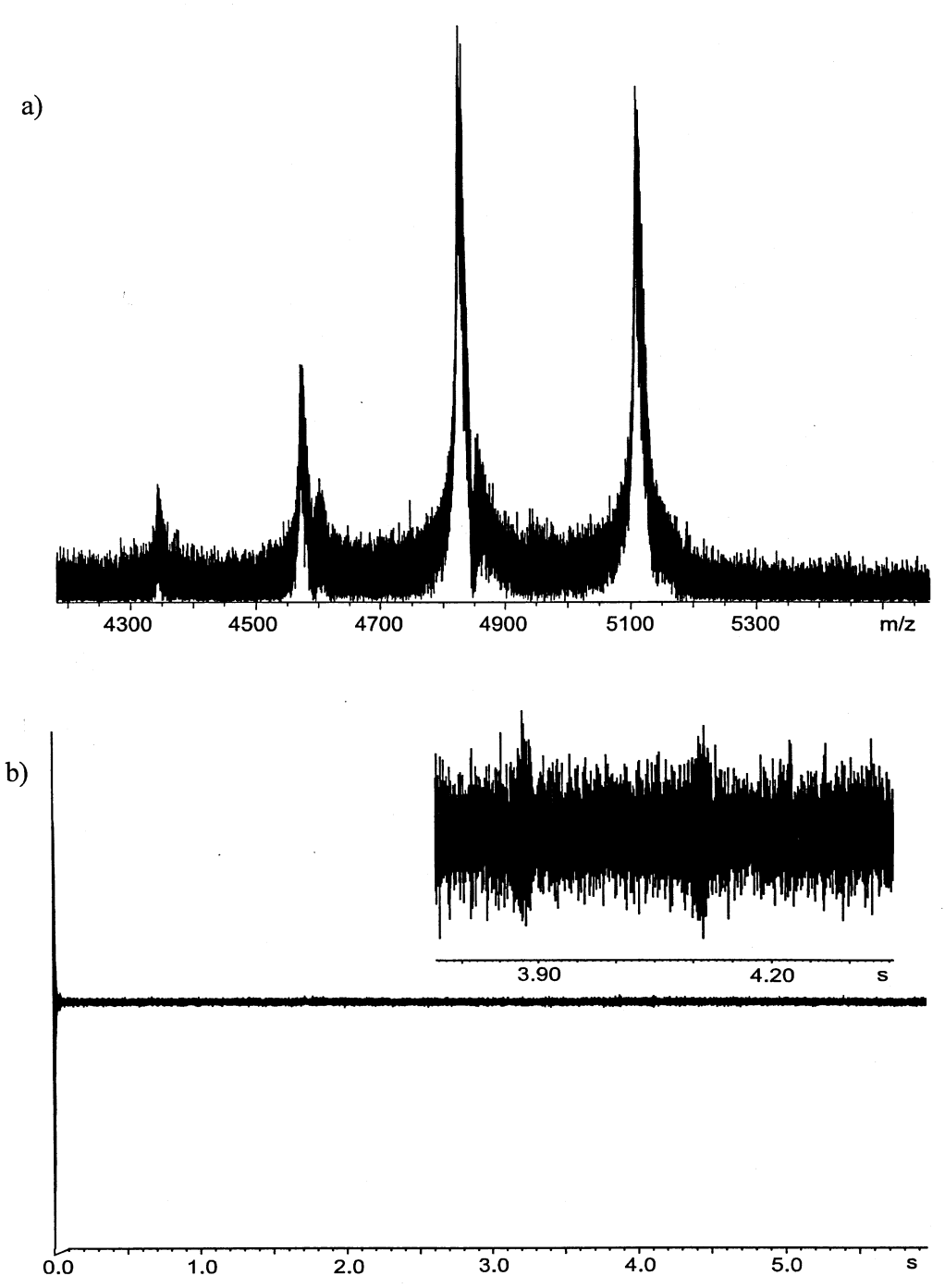

Fig. 7. ESI-FTICR mass spectrum of rubrerythrin from desulfovibrio vulgaris. Rubrerythrin has an FeS_4 active site as well as an oxo-bridged diiron site, and has a high sequence homology to nigerythrin. However, only an 86 kDa tetrameric form of rubrerythrin has been seen by mass spectrometry. (a) Mass spectrum of the 20+ through 17+ charge states. 100 scans were coadded. (b) Transient for this mass spectrum, with expansion showing a second beat at approximately 4.0 s. Although the second beat in the mass spectrum is discernable, the isotope peaks are indistinguishable from the noise in the mass spectrum.

by mass spectrometry [35]. At this high molecular weight, the second beat of the transient occurs at approximately 4 s. Rubrerythrin was not able to withstand 200 °C capillary interface temperature without degradation of the complex, so the temperature was reduced to 150 °C. Fig. 7(a) shows the mass spectrum of rubrerythrin, and Fig. 7(b) shows its accompanying transient. The inset is an expansion of the 3.8–4.3 s region of the transient, showing a very weak second beat. Although the second beat is apparent in the transient, isotopic structure is not evident in the mass spectrum. From these data, we can obtain an average molecular weight of 868 56.5 Da, which agrees well with the calculated value of 868 47.6 Da [59,66], consistent with a stoichiometry of Fe_{12}, as expected for the tetramer. Evidence of the bridging oxygens is not observed in these mass spectra, which would shift the mass of the complex by 64 Da. The absence of the bridging oxygens is consistent with high-resolution data for the monomer of this protein [17].

4. Conclusions

These data provide the first examples of isotopically resolved mass spectra of multimeric metalloproteins. Sample purity, particularly for material isolated from biological systems, is a primary concern. Samples obtained from bacterial media contain high quantities of salt as well as buffers, which are not compatible with mass spectrometry. Extensive desalting must be performed in order to remove as much of the adducting species as possible without denaturing the noncovalent complex. Larger metalloproteins are particularly challenging to desalt, as rigorous methods such as reverse phase high-performance liquid chromatography cause loss of the metal center. Sodium heterogeneity is found to be the principle limitation in extending the mass range of this technique. Space charge is a factor, as the closely spaced isotope peaks coalesce more readily as molecular weight increases. This necessitates a reduction in the trapping potentials as the molecular weight of the complexes increases. This decreases the number of ions and reduces the signal-to-noise ratio, necessitating extensive signal averaging. In order to increase mass resolution to examine even higher mass noncovalent complexes, it will be necessary to sustain the coherence of the ion packet for longer periods, and may require methods such as quadrupolar excitation [67,68], although this method can increase space charge effects such as isotope coalescence. Alternatively, higher magnetic field would aid such measurements.

Acknowledgements

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