Direct Observation of Nitrosylated Heme in Myoglobin and Hemoglobin by Electrospray Ionization Mass Spectrometry

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Abstract: Using electrospray ionization mass spectrometry (ESI-MS), we demonstrate the direct observation of NO attached to the heme moiety in horse heart myoglobin (Mb) and in the α- and β-chains of human hemoglobin (Hb). It was found that a narrow range of ESI-MS conditions conspire to make observation of Fe−NO interactions challenging, and this is presumably the reason why earlier attempts by other research groups to detect intact Fe−NO products by mass spectrometry were unsuccessful. For Mb and Hb, mass shifts are observed that are consistent with NO modification of the hemoproteins. ESI mass spectra of the apoprotein portions of Mb and Hb in the presence of NO demonstrated the absence of NO modification of the polypeptide backbones. UV/vis spectra of both Mb/NO and Hb/NO solutions, recorded at the time of ESI-MS analysis, demonstrated hemoprotein[II]−NO formation. To test the hypothesis that intact nitrosylated heme groups are observable by ESI-MS, a nitrosylated model metalloporphyrin was studied. The ESI mass spectrum of nitrosyl-α,β,γ,δ-tetraphenylporphinatoiron(II), [Fe(TPP)NO], showed peaks that were ascribed to [Fe(TPP)]$^+$ and [Fe(TPP)NO]$^+$. To test further our hypothesis that the hemoprotein−NO peaks are due to heme nitrosylation and contain no significant contributions from NO modification of the polypeptide backbone, we determined the ESI-MS conditions necessary for observing *S*-nitrosation of Cys residues in Hb. Human Hb contains one Cys residue in Hb$_α$ (Cys 104) and two Cys residues in Hb$_β$, but only Hb$_β$ Cys 93 is surface accessible. When metHb was incubated with *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), the ESI mass spectrum revealed a single SNAP modification in both Hb$_β$ and apoHb$_β$. The ESI-MS conditions used for analyzing the Hb/SNAP solution were too harsh for observing intact heme nitrosylation, and thus, we ascribe the SNAP-modified Hb$_β$ and apoHb$_β$ peaks to *S*-nitrosation of Cys 93 in Hb$_β$. Under appropriate denaturing sample conditions, it proved possible to *S*-nitrosate all three Cys residues in human apoHb. Our findings demonstrate that (once correct conditions are established) ESI-MS is a powerful tool for the detection of *intact* Fe−NO interactions in proteins and porphyrins.

Introduction

Nitric oxide (NO) has been implicated to play an important role in a range of biological processes although the mechanisms of its action are unclear.[1] Nevertheless, it has long been known that one specific target of NO modification is the heme moiety of hemoproteins (*e.g.*, myoglobin and hemoglobin).[2−4] Heme nitrosylation results in guanylate cyclase activation[5] and may lead to nitric oxide synthase inhibition.[6] NO may also exert its biological activity by the formation of *S*-nitrosothiols upon reaction of a thiol with NO$_x$.[7] Certain hemoproteins, such as hemoglobin[8] and prostaglandin H$_2$ synthase,[9] have the potential for undergoing both heme nitrosylation and *S*-nitrosation. A

number of techniques have been developed to measure heme nitrosylation and/or *S*-nitrosation. These include chemiluminescence,[8] X-ray crystallography,[10] and spectroscopies such as UV/vis,[11] infrared,[12] and electron spin resonance.[13] However, none of these techniques (with the possible exception of X-ray crystallography) can normally be used to measure directly both of these modifications, while simultaneously providing information regarding the molecular mass of the modified intact protein.

Electrospray ionization mass spectrometry (ESI-MS) is a gentle method for transferring protein ions from solution into the gas phase and for accurately determining their molecular masses.[14] A unique feature of ESI-MS is its ability for detecting noncovalent interactions between proteins and molecular partners,[15] as well as determining highly labile modifications of proteins.[16] We have previously reported the use of ESI-MS to monitor *S*-nitrosated cysteine derivatives of peptides and

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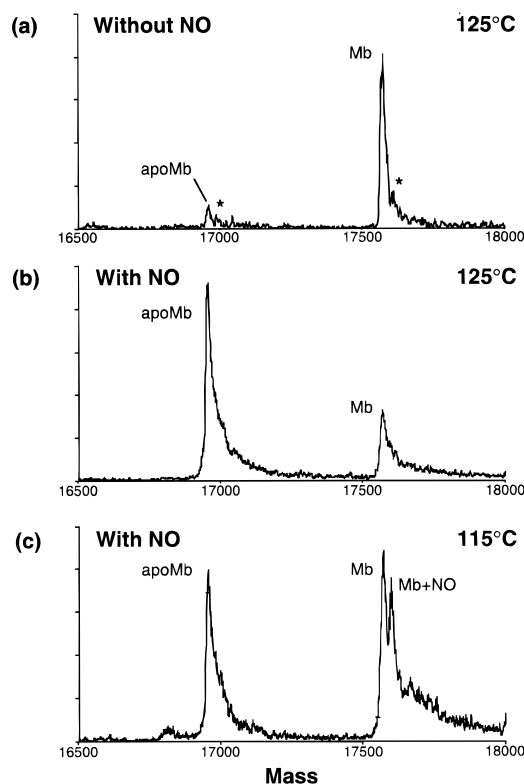
Figure 1. Comparison of deconvoluted ESI mass spectra of (a) Mb in the absence of NO with a transport capillary temperature of 125 °C, (b) Mb in the presence of NO with a transport capillary temperature of 125 °C, and (*c*) Mb in the presence of NO with a transport capillary temperature of 115 °C. Sample preparation conditions are given in the Experimental Section. The ESI-MS conditions: (a) declustering voltage 80 V, scan range 1800−2600 *m/z*, sample infusion rate 5 μL/min, number of scans 5 (3 s/scan); (b) declustering voltage 80 V, scan range 1800−2600 *m/z*, sample infusion rate 5 μL/min, number of scans 5 (3 s/scan); (*c*) declustering voltage 80 V, scan range 2000−2300 *m/z*, sample infusion rate 5 μL/min, number of scans 20 (3 s/scan). The asterisk in (a) denotes a potassium adduct of Mb.

proteins,[16,17] and more recently, others have reported the characterization of *S*-nitrosohemoglobin by ESI-MS.[18] However, earlier attempts by others to detect heme−NO adducts in proteins by ESI-MS proved unsuccessful.[19] Here, we demonstrate, for the first time, the direct ESI-MS observation of NO attached to the heme moiety in myoglobin (Mb) and in the α- and β-chains of hemoglobin (Hb). In addition, we demonstrate that ESI-MS can be used to detect *S*-nitrosation of Hb. By varying the ESI-MS conditions employed, it is possible to distinguish between heme nitrosylation (which can only be observed using extremely gentle ESI-MS conditions) and *S*-nitrosation (which still requires moderately gentle ESI-MS conditions).

Results

Heme Nitrosylation of Myoglobin. Figure 1a shows the deconvoluted[20] ESI mass spectrum of native Mb prior to

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treatment with NO. Two peaks are observed, corresponding to (i) native Mb (measured molecular mass, $M_r = 17\,568 \pm 2$ Da, calculated $M_r = 17\,568$ Da) and (ii) Mb that has undergone loss of the heme group during the MS measurement (apoMb; measured $M_r = 16\,950 \pm 2$ Da, calculated $M_r = 16\,951$ Da). The ESI mass spectrum in Figure 1a was obtained using a transport capillary temperature[21] of 125 °C and a declustering voltage[22] (the voltage difference between the transport capillary and tube lens) of 80 V. Under these ESI-MS conditions, Mb is the dominant species and very little apoMb is observed.

Figure 1b shows the deconvoluted ESI mass spectrum of Mb after incubation with NO under anaerobic conditions (*i.e.*, conditions under which NO is expected to react exclusively with the heme group of hemoproteins). The ESI-MS conditions are *exactly* identical to those used in Figure 1a. Again, two peaks that correspond to Mb and apoMb are observed, although with much different intensities to those seen in Figure 1a. No peak due to NO-modified Mb was observed under these ESI-MS conditions, even though UV/vis spectral analysis of the Mb/ NO solution indicated complete conversion to the heme-nitrosylated form of Mb (*vide infra*). Thus, we deduce that the presence of both unmodified apoMb and Mb in the mass spectrum shown in Figure 1b is the result of the decomposition of the heme-nitrosylated Mb during the ESI-MS measurement. In contrast to Figure 1a, the dominant species in Figure 1b is apoMb. This increased level of apoMb in Figure 1b provides evidence that the heme NO group in NO-modified Mb has a destabilizing effect on the heme−globin complex.

Figure 1c shows details of the deconvoluted ESI mass spectrum of the same Mb/NO solution used in Figure 1b, but under gentler ESI-MS conditions. A control ESI mass spectrum of Mb in the absence of NO under these new ESI-MS conditions showed a very similar spectrum to that given in Figure 1a (data not shown). Specifically, the transport capillary temperature was lowered by 10 °C from 125 °C (Figures 1a and 1b) to 115 °C (Figure 1c), while a declustering voltage of 80 V was maintained. Under these slightly gentler conditions, three peaks are observed for the Mb/NO solution, *i.e.*, the peaks ascribed to apoMb, native Mb, and a new peak at 17 599 Da. This latter peak is 31 ± 2 Da higher than the measured M_r of native Mb, consistent with NO modification of the protein. In addition to the observation of NO-modified Mb, the slightly gentler conditions used in Figure 1c lead to a lower production of apoMb than is observed in Figure 1b. These observations provide further evidence that the apoMb peak is to a large extent formed in the mass spectrometer and that heme nitrosylation destabilizes the heme globin interaction. It is important to notice that there is no evidence of a peak in Figure 1c that can be attributed to NO-modified apoMb, providing evidence that the polypeptide backbone is not a site of NO modification.

Heme Nitrosylation of Hemoglobin. Figure 2b shows a portion of the deconvoluted ESI mass spectrum of Hb after incubation with NO under anaerobic conditions (*i.e.*, under conditions that are expected to nitrosylate the heme group, but leave the globin thiols unmodified). Since it was not possible to measure the ESI mass spectrum of the intact Hb tetramer, we instead monitored those regions containing peaks corresponding to the α- and β-chain of Hb. The portion shown in Figure 2b includes the region encompassing the α-chain of Hb (Hb$_\alpha$; calculated $M_r = 15\,743$ Da). Under ESI-MS conditions in which the transport capillary temperature and declustering voltage were optimized for the observation of intact NO-

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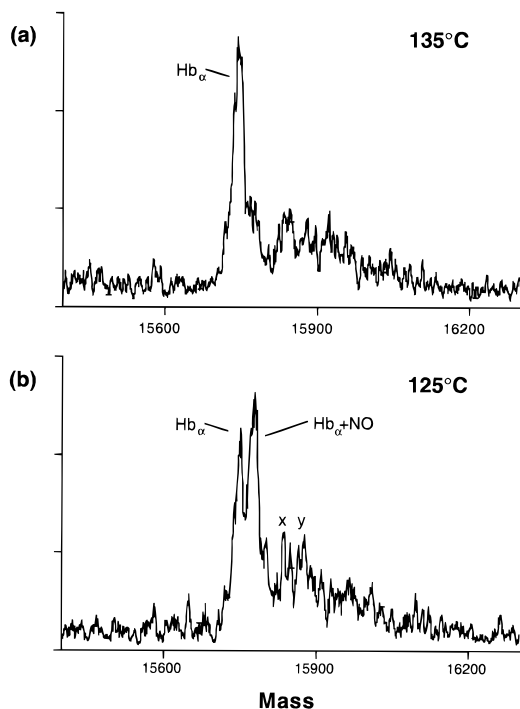
(a)

(b)

Figure 2. Comparison of deconvoluted ESI mass spectra of Hb in the presence of NO with a transport capillary temperature of (a) 135 °C versus (b) 125 °C. The portion of the spectrum containing the Hb_α peak is shown. The ESI-MS conditions: (a) declustering voltage 33 V, scan range 1900−2100 m/z, sample infusion rate 5 μL/min, number of scans 25 (3 s/scan); (b) declustering voltage 33 V, scan range 1900−2100 m/z, sample infusion rate 3 μL/min, number of scans 16 (3 s/scan). The x and y denote, respectively, an unidentified peak and a weak peak due to $apoHb_\beta$.
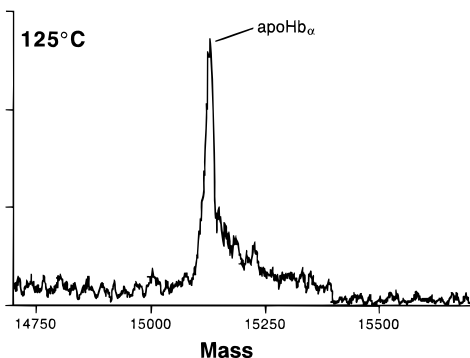

Figure 3. Deconvoluted ESI mass spectrum of Hb in the presence of NO, showing the region of the spectrum containing the $apoHb_\alpha$ peak. Transport capillary temperature 125 °C, declustering voltage 33 V, scan range 1800−2200 m/z, sample infusion rate 5 μL/min, number of scans 16 (3 s/scan).

modified Hb_α (see figure legend), two dominant peaks are observed (Figure 2b). The first peak corresponds to Hb_α (measured M_r = 15 746 ± 4 Da), while the second has a mass 29 ± 2 Da greater than that observed for Hb_α, indicative of NO modification of Hb_α. Comparison of Figures 2b and 2a demonstrate that an increase of the transport capillary temperature by as little as 10 °C leads to almost complete loss of the peak due to NO-modified Hb_α, a situation analogous to that observed for NO-modified Mb (*vide supra*).

Figure 3 displays a different portion of the deconvoluted ESI mass spectrum of NO-treated Hb shown in Figure 2b. The portion shown in Figure 3 includes the region encompassing Hb_α after loss of the heme group during the MS measurement ($apoHb_\alpha$; calculated M_r = 15 126 Da; measured M_r = 15 124 ± 2 Da). Significantly, only one peak due to $apoHb_\alpha$ is
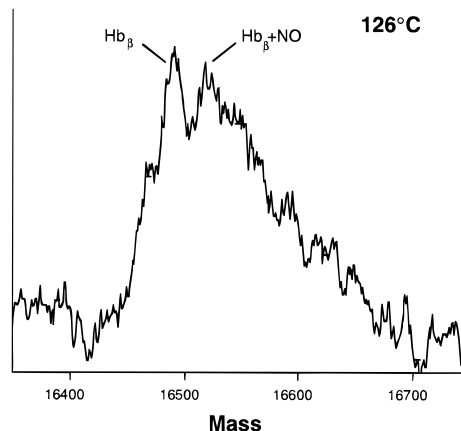

Figure 4. Details of the deconvoluted ESI mass spectrum of Hb in the presence of NO, showing the portion of the spectrum containing the Hb_β peak. Transport capillary temperature 126 °C, declustering voltage 116 V, scan range 2040−2100 m/z, sample infusion rate 3 μL/min, number of scans 124 (3 s/scan).

observed, which indicates that there is no obvious modification of the Hb_α polypeptide backbone and, hence, provides evidence that Hb_α Cys 104 has not undergone *S*-nitrosation under these conditions. It may be argued that the excitation introduced during the ESI process that causes loss of the heme group from Hb_α may also be sufficient to cause dissociation of NO from any *S*-nitrosated thiols that may be present in the protonated protein. However, we shall demonstrate below that dissociation of *S*-nitrosated thiols does not occur under the very gentle ESI conditions that were used to obtain the spectrum in Figure 3.

Figure 4 displays the portion of the deconvoluted ESI mass spectrum encompassing the β-chain of Hb (Hb_β), demonstrating NO modification of Hb_β (even though the peak corresponding to Hb_β is of very low intensity). The poor response of Hb_β compared to Hb_α has been reported previously but has not yet been explained.[23] The ESI-MS conditions used to obtain the spectrum in Figure 4 are different to those employed for the observation of NO-modified Hb_α (Figure 2b). More specifically, it proved necessary to increase the declustering voltage from 33 V (Figure 2b) to 116 V (Figure 4) in order to obtain discernible peaks. The two most intense peaks in Figure 4 are observed at 16 485 ± 6 and 16 513 ± 6 Da, and likely correspond to Hb_β (calculated M_r = 16 484 Da) and NO-modified Hb_β, respectively. A weak spectrum of $apoHb_\beta$ was also obtained in which no evidence of NO modification could be discerned (data not shown). We thus conclude that, under anaerobic conditions, the NO modification of Hb_β occurred exclusively on the heme group.

UV/Vis Spectroscopy of Hemoprotein/NO Solutions. UV/vis spectroscopy was used to monitor heme nitrosylation of both the Mb/NO and Hb/NO solutions prior to ESI-MS analysis. Figure 5 shows that in the presence of NO under anaerobic conditions, the characteristic absorption maxima for metmyoglobin (Mb^{III}) (410 and 500 nm) disappeared within 30 min, and new bands corresponding to an intermediate Mb^{III}−NO derivative (416, 539, and 575 nm) were formed. Within 2 h, the spectrum of Mb^{III}−NO gradually changed to that of Mb^{II}−NO (420, 547, and 577 nm).[2,11,24] Similarly, the UV/vis spectra monitoring Hb^{III} in the presence of NO showed initial Hb^{III}−NO production, followed by conversion to Hb^{II}−NO (data not shown). A mechanism for hemoprotein^{II}−NO formation from

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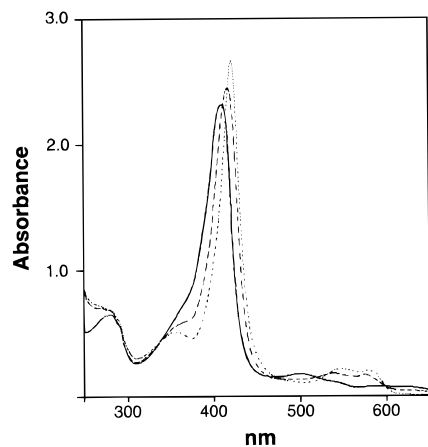
Figure 5. Time course of heme nitrosylation of Mb determined by UV/vis spectroscopy. (—) Mb in the absence of NO, (- - -) $Mb^{III}-NO$ formation after 30 min of incubation with NO, and (···) $Mb^{II}-NO$ formation after 2 h of incubation with NO.
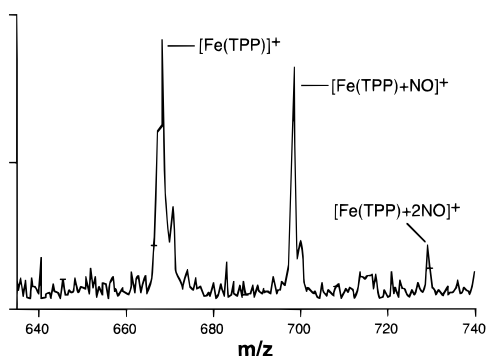

Figure 6. ESI mass spectrum of Fe(TPP)NO. Transport capillary temperature 70 °C, declustering voltage 79 V, scan range 650−800 *m/z*, sample infusion rate 5 μL/min, number of scans 16 (3 s/scan).

hemoprotein^{III}/NO solutions has recently been published.[24] In the present study, the hemoprotein/NO solutions were monitored by UV/vis spectroscopy until $Fe^{II}-NO$ formation was evident. These solutions were then analyzed by ESI-MS. UV/vis spectra of the Mb/NO and Hb/NO solutions at times following mass spectral analysis showed no additional changes (data not shown). We thus deduce that the hemoprotein−NO peaks observed in the mass spectra (Figures 1c, 2b, and 4) are due to hemoprotein^{II}−NO. Although the UV/vis spectra of both the Mb/NO and Hb/NO solutions showed complete conversion to the heme-nitrosylated derivatives, the mass spectra showed the presence of (i) apoprotein, (ii) nitrosylated protein, and (iii) native protein. We infer that the signals corresponding to the apo- and native protein arise from the decomposition of the protonated nitrosylated derivative under ESI-MS conditions.

Heme Nitrosylation of an Iron Tetraphenylporphyrin. To test further our hypothesis that the hemoprotein−NO peaks observed in Figures 1c, 2b, and 4 are due to heme nitrosylation, we studied a nitrosylated derivative of a heme model compound: nitrosyl-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(II), [Fe-(TPP)NO]. To the best of our knowledge, intact nitrosylated iron porphyrins have not previously been observed by ESI-MS. Electron impact ionization mass spectrometry was previously used to characterize Fe(TPP)NO, but only the NO^+ and $[Fe(TPP)]^+$ fragments were detected.[25] The ESI mass spectrum of Fe(TPP)NO obtained under the present conditions is shown in Figure 6. It is noteworthy that the conditions required to observe the intact Fe(TPP)NO are considerably gentler (transport
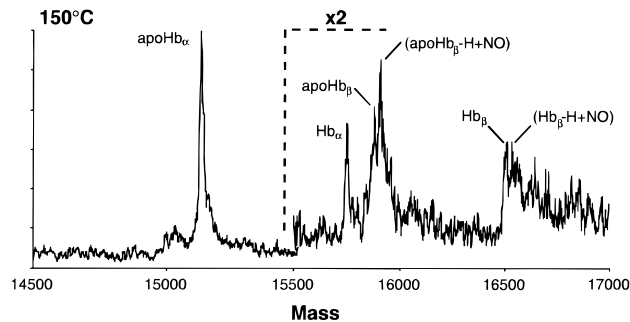

Figure 7. Deconvoluted ESI mass spectrum of Hb incubated with SNAP. Transport capillary temperature 150 °C, declustering voltage 94 V, scan range 1500−2400 *m/z*, sample infusion rate 3 μL/min, number of scans 100 (3 s/scan). The −H denotes loss of hydrogen from the β-chain of Hb.

capillary temperature 70 °C, declustering voltage 79 V) than those used to observe nitrosylation of intact heme-containing proteins. In addition to the peak at *m/z* 668.2, corresponding to $[Fe(TPP)]^+$ (calculated M_r = 668.6 Da), a second intense peak is observed at *m/z* 698.5 (*i.e.*, 30.3 Da higher than that measured for $[Fe(TPP)]^+$) corresponding to $[Fe(TPP)NO]^+$. Finally, a weak peak was observed at *m/z* 729.0 (*i.e.*, 60.8 Da higher than that measured for $[Fe(TPP)]^+$), which we tentatively attribute to the dinitrosyl species, $[Fe(TPP)(NO)_2]^+$.[26]

S-Nitrosation of Hemoglobin. Although NO by itself does not react with sulfhydryl groups under anaerobic conditions at neutral pH to produce *S*-nitrosothiols,[27] *S*-nitrosation may be possible in the presence of NO and oxygen.[28] To test further our hypothesis that the hemoprotein−NO peaks in Figures 1c, 2b, and 4 are due to heme nitrosylation and that the peaks contain no significant contributions from NO modification of the polypeptide backbone, we determined the ESI-MS conditions necessary for observing *S*-nitrosated Cys residues in Hb. Figure 7 shows the deconvoluted ESI mass spectrum of methemoglobin (metHb) after incubation with *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) under aerobic conditions. The labelled peaks shown in Figure 7 were ascribed to (i) apoHb$_\alpha$ (measured M_r = 15 130 ± 3 Da), (ii) Hb$_\alpha$ (measured M_r = 15 747 ± 2 Da), (iii) apoHb$_\beta$ (measured M_r = 15 875 ± 3 Da), (iv) a reaction product at 15 905 Da, which is 30 ± 2 Da higher than the measured M_r of apoHb$_\beta$, (v) Hb$_\beta$ (measured M_r = 16 503 Da), and (vi) another reaction product at M_r = 16 532 Da, which is 29 ± 2 Da higher than the measured M_r of Hb$_\beta$. Thus, Figure 7 shows that while apoHb$_\alpha$ and Hb$_\alpha$ remain unchanged in the presence of SNAP, both apoHb$_\beta$ and Hb$_\beta$ are modified by SNAP. On the basis of previous studies of *S*-nitrosation of Hb,[8,18] as well as solvent accessibility derived from X-ray crystallography data,[29] we ascribe the SNAP-modified apoHb$_\beta$ and Hb$_\beta$ peaks to *S*-nitrosation of Hb$_\beta$ Cys 93. Other high mass peaks that occur on the tail of the Hb$_\beta$ peaks are too weak to identify. Similar results were obtained on incubating Hb with *S*-nitrosated glutathione (GSNO; data not shown).[8] It is important to note that the ESI mass spectra shown in Figure 7 were recorded under ESI-MS conditions (transport capillary temperature 150 °C, declustering voltage 94 V) that were much too harsh for observing intact heme-nitrosylated proteins. In addition, the UV/vis spectrum of the Hb/SNAP solution showed

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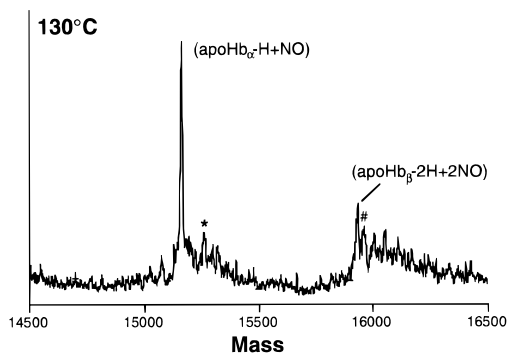
Figure 8. Deconvoluted ESI mass spectrum of Hb subsequent to incubation with NO under acidified conditions in the presence of air. Transport capillary temperature 130 °C, declustering voltage 55 V, scan range 600−1700 *m/z*, sample infusion rate 3 μL/min, number of scans 15 (3 s/scan). The asterisk denotes an adduct of H_3PO_4 and the pound sign (#) denotes a Na adduct. The −H denotes loss of hydrogen from the α- and β-chains of Hb.

no evidence of heme nitrosylation (data not shown). Thus, we deduce that (i) the SNAP-modified Hb_β peak shown in Figure 7 is solely due to SNAP modification of the polypeptide backbone and (ii) the *S*-nitrosated Hb_β derivative is considerably more stable than the heme−NO−globin complex.

Figure 8 shows that under appropriate denaturing sample conditions, it is possible to *S*-nitrosate apoHb completely and determine the resulting product. The sample was prepared by withdrawing an aliquot from an Hb/NO solution (prepared under anaerobic conditions) and acidifying the aliquot with acetic acid in the presence of air. Under these sample conditions, the heme group is lost from the protein, the polypeptide backbone is denatured, and all three Cys residues in human apoHb (Cys 104 in $apoHb_\alpha$, Cys 93 and Cys 112 in $apoHb_\beta$) are *S*-nitrosated by a higher oxide of NO.[30] The spectrum in Figure 8 displays two peaks at 15 159 and 15 931 Da, which are respectively 28 ± 2 and 62 ± 5 Da higher than that measured for $apoHb_\alpha$ (15 131 Da) and $apoHb_\beta$ (15 869 Da), consistent with *S*-nitrosation of Cys 104 in $apoHb_\alpha$ and of both Cys 93 and Cys 112 in $apoHb_\beta$.

Discussion

Observation of Heme−NO Interactions Requires Careful Adjustment of ESI-MS Parameters. In Figures 1a and 1b, we see that the measurement of intact Fe−NO interactions in proteins is challenging because (i) under normal ESI-MS conditions, heme loss from the protein occurs during the MS measurement and (ii) heme loss is greater in the presence of NO. Presumably, the enhanced MS loss of the heme group after treatment with NO is a result of the destabilizing effect of heme−NO on the heme−globin complex. In order to observe intact Fe−NO interactions in proteins and porphyrins by ESI-MS, it proved critical to control carefully the conditions used for sample handling and electrospray ionization (see figure legends). Thus, it was necessary to adjust the source parameters that control the amount of energy transmitted to the nitrosylated molecules during ESI. In the Finnigan MAT TSQ-700 electrospray ion source, this energy input into the solvated ion is controlled by the transport capillary temperature[21] and the declustering voltage.[22] A slight variation in these parameters had a large influence on whether species containing Fe−NO moieties were observed. If the capillary temperature and/or declustering voltage were set too high, the Fe−NO bond was broken and heme loss from the protein increased. Thus, a slight increase in the capillary temperature (*i.e.*, 10 °C higher than the temperature used to obtain the mass spectra shown in Figures 1c and 2b) resulted in the complete disappearance of the peaks assigned to Mb^{II}−NO and Hb^{II}−NO (see Figures 1b and 2a). If, on the other hand, the capillary temperature and/or declustering voltage were set too low, solvent molecules remained attached to the ionized protein and a broad tailing parent ion peak was observed in the spectrum. It is, therefore, evident that a narrow, but reproducible, range of ESI-MS conditions exist in which nitrosylated hemoproteins and nitrosylated Fe-containing porphyrins can be observed.

Appropriate Sample Handling Conditions Are Critical for the Observation of Heme−NO Interactions. In order to ensure the observation of NO-modified heme moieties, it was also necessary to control carefully the sample handling conditions. A mixture of methanol (10% v/v) and ammonium bicarbonate (5 mM) proved to be an excellent electrospray buffer for these measurements, because methanol is volatile and ammonium bicarbonate decomposes to volatile NH_3, CO_2, and H_2O under MS conditions.[31] It was essential to maintain anaerobic conditions to prevent the formation of nitrous acid which, if present, could potentially lower the pH and lead to (i) disruption of the coordinate bond between the heme and the apoprotein[32] and (ii) *S*-nitrosation of Cys residues.[33] The above-mentioned factors conspire to make observation of Fe−NO interactions in proteins and porphyrins challenging and are, presumably, the reason why earlier attempts to detect heme−NO products by mass spectrometry were unsuccessful.[19]

S-Nitrosated Proteins Can Be Readily Differentiated from Heme−Nitrosylated Proteins by ESI-MS. To exclude the possibility that NO may be modifying the polypeptide backbone, we investigated the ESI-MS conditions under which *S*-nitrosation of cysteine residues in Hb can be observed. Human Hb contains three Cys residues (Hb_α Cys 104, Hb_β Cys 93, and Hb_β Cys 112), but only Hb_β Cys 93 is surface accessible and available for *S*-nitrosation. Thus, treatment of Hb at neutral pH with a transnitrosating agent such as SNAP or GSNO should lead to modification of a single Cys residue in Hb_β, but no modification of Cys in Hb_α.[8,34] The results shown in Figure 7 confirm this hypothesis. The ESI-MS conditions under which we observe *S*-nitrosation of Hb_β (most likely Hb_β Cys 93)[8,18] by SNAP modification of Hb_β (Figure 7) are much harsher than those used to observe intact Fe−NO interactions in either Mb (Figure 1c) or Hb (Figures 2b and 4). Thus, the data presented here demonstrate that heme-nitrosylated proteins are much more prone to dissociation during ESI-MS than *S*-nitrosated proteins.

ESI-MS Can Be Used To Determine the Number of NO Modifications in Proteins. Under appropriate sample conditions, it proved possible to *S*-nitrosate all three Cys residues in human apoHb and to observe these modifications by ESI-MS. In Figure 8, we demonstrate that when an anaerobic Hb/NO solution is denatured by acidification and is exposed to air, all three Cys residues in human apoHb are observed to be *S*-nitrosated. Under physiological conditions, this ability to count the number of NO modifications has proved useful in studies of NO treatment of bovine cytochrome *c* (Cyt *c*) under anaerobic conditions. ESI-MS analysis of a Cyt *c*/NO solution revealed that two forms of Cyt *c* were produced during heme

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nitrosylation.[35] One form corresponds to native Cyt *c*, while the second form is a product that is 17 Da lighter in mass than Cyt *c*. By subjecting the NO-modified Cyt *c* proteins to enzyme digests and subsequent MS analysis, we have discovered that the Cyt *c* derivative arises from a deamination reaction (involving Lys 72) in the presence of NO (data not shown).

Conclusions

ESI-MS was found to be a versatile tool for Fe−NO detection in proteins and porphyrins. In the present study, we have established mass spectrometric conditions to observe such weak Fe−NO interactions in Mb, Hb, and Fe(TPP)NO. Limitations of this method include the following: (a) the unavoidable partial decomposition of the nitrosylated product under certain ESI-MS conditions, (b) the present inability to measure heme nitrosylation in intact tetrameric Hb, (c) the relative difficulty in measuring β-nitrosyl hemes, and (d) the present inability to measure concentrations <20 μM. Advantages of the present ESI-MS approach to the detection of Fe−NO interactions include the ability to obtain, (a) direct measurements of the intact Fe−NO-containing complex, (b) information concerning the exact number of NO moieties attached to the molecule, and (*c*) information concerning the formation of other byproducts (*e.g.*, dinitrosylation of heme in Fe(TPP) and deamination of a Lys residue in Cyt *c*),[35] as well as *S*-nitrosation of cysteines.

Experimental Section

Materials. Horse heart metmyoglobin and human methemoglobin were purchased from Sigma; nitric oxide (99.0% min) was obtained from Matheson; $\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(III) chloride was obtained from Aldrich. All were used as received. *S*-Nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitrosoglutathione (GSNO) were synthesized according to procedures described by Field *et al.*[36] and Hart,[37] respectively.

Preparation of Heme-Nitrosylated Hemoproteins. The nitrosylated hemoproteins were prepared by anaerobically adding NO (1 atm) to either horse heart metmyoglobin (Mb^{III}) or human methemoglobin (Hb^{III}) (20 μM) dissolved in a buffer (10% v/v methanol and 90% v/v 5 mM ammonium bicarbonate; pH 8) in a Thunberg cell. The samples were monitored by UV/vis spectroscopy until Fe^{II}−NO formation was evident. Samples were withdrawn from the cell and injected into the mass spectrometer *via* a gas-tight Hamilton syringe.

Preparation of a Heme-Nitrosylated Tetraphenylporphyrin. Nitrosyl-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(II), Fe(TPP)NO, was prepared according to a modified version of a preparation described by

Scheidt and Frisse.[25] Briefly, $\alpha,\beta,\gamma,\delta$-tetraphenylporphinatoiron(III) chloride, Fe(TPP)Cl, (0.2 g) in chloroform (60 mL) containing dry pyridine (1 mL) was degassed in a Schlenk tube. NO (1 atm) was passed above the stirred solution for 20 min. Dry MeOH was added until a precipitate of Fe(TPP)NO appeared. Filtration under N_2 with a cannula gave a purple powder. For the purposes of electrospray, the sample (400 μM) was prepared in deaerated chloroform and injected into the mass spectrometer *via* a gas-tight Hamilton syringe.

Preparation of *S*-Nitrosated Hemoglobin. A stock solution of SNAP (10 mM) was prepared by dissolving SNAP (2.2 mg) in dimethyl sulfoxide (DMSO, 10 μL) and deionized water (990 μL). A stock solution of human Hb^{III} (50 μM) was prepared by dissolving Hb^{III} in a buffer (10% v/v methanol and 90% v/v 5 mM ammonium bicarbonate; pH 8). The stock SNAP and Hb^{III} solutions were mixed in appropriate proportions to yield a 10:1 molar ratio of SNAP/Hb^{III}. The mixture was allowed to react for 10 min at room temperature under aerobic conditions prior to injection into the ESI mass spectrometer. A GSNO/Hb^{III} solution was prepared in a similar manner, except that no DMSO was necessary in the preparation of the stock GSNO solution.

Preparation of *S*-Nitrosated Apohemoglobin. A solution of Hb/NO (40 mM in 10% v/v MeOH and 90% v/v 5 mM ammonium bicarbonate) was prepared as above under anaerobic conditions. An aliquot was removed from this mixture and exposed to air, followed by a 4-fold dilution with 29% v/v H_2O/68% v/v acetonitrile/3% v/v acetic acid.

ESI-Mass Spectrometry. A Finnigan-MAT TSQ-700 triple quadrupole instrument was used to obtain all ESI mass spectra. The solutions were sprayed through a 100 μm (inner diameter) fused silica capillary into the mass spectrometer source using a Harvard syringe pump (model 24000-001). The ESI-MS conditions (capillary temperature, declustering voltage, scan range, sample infusion rate, and number of scans) are given in the figure legends. The ion signals were recorded by a Finnigan ICIS data system operated on a DEC station 500/120 system. The reconstructed molecular mass profiles were obtained by using a deconvolution algorithm (FinniganMAT).

UV/Vis Spectroscopy. UV/vis spectra were recorded using a Perkin Elmer Lambda-19 UV/vis/near IR spectrophotometer. All UV/vis spectra were recorded at room temperature.

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