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Infrared Spectroscopy Provides Insight into the Role of Dioxygen in the Nitrosylation Pathway of a [2Fe2S] Cluster Iron–Sulfur Protein

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Supporting Information

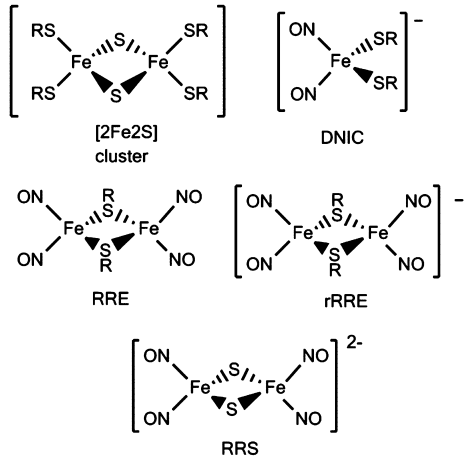
ABSTRACT: We use infrared spectroscopy to demonstrate the critical role that trace O_2 plays in determining the products formed when a [2Fe2S] cluster protein reacts with nitric oxide (NO). The observed importance of O_2 may have physiological relevance, as many pathogens sense NO using iron–sulfur proteins and will be exposed to NO in an aerobic environment during a mammalian immune response. We show that the [2Fe2S]-containing spinach ferredoxin I undergoes reaction with NO at pH 6.0, with the proportion of protein-bound Roussin's Red Ester compared to the dinitrosyl iron complex product favored by trace O_2. Roussin's Red Ester is also favored on nitrosylation in the presence of the thiolate scavenging reagent, iodoacetamide, suggesting that the role of O_2 is in oxidative sequestration of cysteine thiolates. Infrared spectroscopy has been overlooked as a tool for studying iron–sulfur protein nitrosylation despite the fact that there exists a wealth of infrared spectroscopic data on small-molecule nitrosyl clusters which serve as models for the identification of protein-bound nitrosyl clusters.

A range of biological roles have emerged for nitric oxide (NO), including neurotransmitter, signaling molecule, and part of the human immune response. A number of iron–sulfur proteins are targeted by NO, which typically causes significant rearrangements of their metal clusters.[1−3] These essentially irreversible reactions are not only a major contributor to the toxicity of NO to micro-organisms, but are also exploited by bacteria to sense and respond to NO,[4,5] such as that produced by a mammalian immune response.[6]

The nitrosylation of iron–sulfur clusters is known to result in the formation of various nitrosyl iron complexes (Chart 1).[7−12] The conditions which favor each product, and the pathways of interconversion between them, have been intensely studied for small-molecule systems,[13−18] where infrared (IR) spectroscopy has been used heavily to distinguish different nitrosylated products on the basis of their characteristic patterns of NO stretching (ν_{NO}) bands. IR methods have been extended to small oligopeptide-bound nitrosylated clusters,[16] and applied to the NO-sensing protein, Fur, which is not an iron–sulfur protein but has Fe sites reported to react with NO to form iron-dinitrosyl species.[19]

Features arising from the protein backbone and water overlap with the ν_{NO} region of the IR spectrum, meaning that IR spectroscopy is challenging for protein-bound systems. Instead,

Chart 1. Complexes Relevant to This Work[a]



[a]R = protein cysteine residue.

electron paramagnetic resonance (EPR) spectroscopy has been used to identify the presence of mononuclear dinitrosyl iron complexes (DNICs, Chart 1), but fails to detect the EPR-silent Roussin's Red Ester (RRE, Chart 1) or Roussin's Black Salt. Nuclear resonance vibrational spectroscopy (NRVS) has recently been used to distinguish between different products of nitrosylation for a Rieske protein[8] and a [4Fe4S] ferredoxin,[7] but this technique is more difficult to apply, requiring a synchrotron source and the proteins to be isolated from cells grown on ^{57}Fe media.

We use an attenuated total reflectance IR spectroscopic method to provide insight into iron–sulfur protein nitrosylation. The method has a small sample volume requirement (*ca* 7 μL) at submillimolar concentrations and there is no need to label the metals. We have screened a range of conditions in order to understand factors which favor formation of different protein-bound nitrosylation products. Furthermore, by performing NO exposure, IR and UV–visible spectroscopy and preparation of EPR samples in the same anaerobic chamber (<1 ppm of O_2), and using rigorously purified gases, we can control exposure to molecular oxygen, which has been shown to be important in the interconversion of small-molecule nitrosylated species.[20,21] Dinitrogen was passed through an Agilent gas-clean filter (outlet < 50 ppb O_2), and NO was passed through a

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column of Ascarite II (20−30 mesh, Sigma) to remove higher nitrogen oxide impurities. Gas exposure was performed on samples in sealed vials with precisely controlled gas concentrations introduced into the head space using a gas mass flow mixing manifold.

We demonstrate the application of IR spectroscopy to iron−sulfur protein nitrosylation reactions by carrying out experiments on a model iron−sulfur protein, recombinant [2Fe2S] spinach ferredoxin I (Fd). This protein was purified aerobically and contains a single cluster in the oxidized $[2Fe2S]^{2+}$ form bound to four cysteines of a C-X_4-C-X_2-C-X_{29}-C motif. It has been reported previously that this protein is unreactive toward NO on the basis of a study carried out at pH 8.1.[11] Consistent with this, we found that overnight exposure of Fd at pH 7.5 to 10% NO gas in N_2 at 1 bar (corresponding to approximately 0.19 mM NO in solution) resulted in no clear peaks in the ν_{NO} region of the IR spectrum recorded against native Fd as background in Figure 1a,i. However, exposure of the Fd sample at pH 7.5 to a gas environment comprising trace O_2 (0.05%, corresponding to approximately 0.6 μM O_2 in solution) together with NO (10%) led to the appearance of a pattern of three bands at 1815 (weak), 1783, and 1757 cm^{-1} in Figure 1a,ii, consistent with reports of small molecule RRE clusters.[22] The band positions correspond particularly well to those of oligopeptide-bound RRE clusters examined in water by Lin et al.,[16] Table 1. In contrast, the ν_{NO} bands for Roussin's Red Salt (RRS, Chart 1), in which the iron sites are linked by bridging sulfide rather than thiolates, appear at lower wavenumbers (Table 1),[23] providing strong evidence that in Fd, two thiolate cysteine ligands are retained in a bridging position to form RRE. The release of elemental sulfur has been detected upon the reaction of iron−sulfur proteins with NO.[9,24] The RRE product remained stable when transferred to a N_2 atmosphere. To confirm that the RRE cluster remains protein-bound, an IR spectrum was recorded after buffer exchange to remove low molecular mass species; the RRE features were still observed in the protein fraction. Exposure to O_2 levels above 0.1% was found to cause rapid precipitation of the RRE-modified Fd.

Formation of RRE requires loss of two of the thiolate groups coordinating the cluster. It has previously been suggested that O_2 favors conversion of small molecule DNIC species to RRE by oxidatively removing thiolate groups as disulfides,[20,21] as confirmed by mass spectrometry analysis.[20] To determine whether trapping out thiolates is important in RRE formation for Fd, we exposed Fd at pH 7.5 to 10% NO under anaerobic conditions but in the presence of excess iodoacetamide (IAm), a thiolate alkylating agent. The resulting IR spectrum (Figure 1a,iii) clearly shows substantial RRE formation. This is consistent with RRE being favored by thiolate sequestration, suggesting that the role of O_2 is in oxidative removal of thiolates.

It has been noted that Fd undergoes structural rearrangement at pH 7.0 and below, which may lead to the [2Fe2S] cluster becoming more exposed.[25] A recent study has shown that exposure of oxidized Fd to NO at pH 7.0 causes conversion of 5−8% of the cluster to a DNIC form, as quantified by EPR spin integration.[24] We therefore used IR spectroscopy to investigate the reaction of Fd with NO at lower pH values. IR spectra recorded after exposure of Fd to NO at pH 6.0 are shown in Figure 1b. Exposure to 10% NO in N_2 caused slow yellowing of the Fd sample and an IR spectrum recorded after overnight exposure to this gas mixture, spectrum (i) in Figure 1b, showed clear peaks in the ν_{NO} region. The

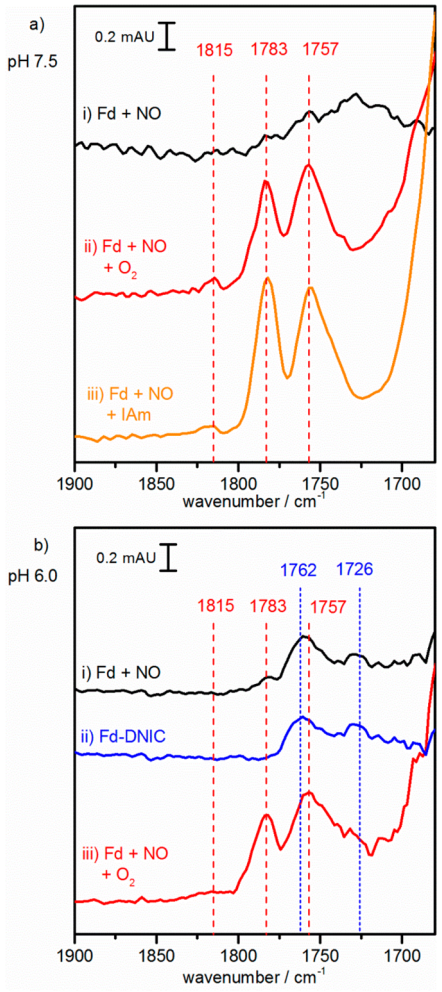

Figure 1. IR spectra of 0.8 mM Fd exposed to NO under different conditions. (a) Fd at pH 7.5 after overnight exposure to (i) 10% NO in N_2; (ii) 10% NO, 0.05% O_2 in N_2; and (iii) 10% NO with 24 mM iodoacetamide (IAm) in solution, all against a background of unexposed Fd at pH 7.5. (b) Fd at pH 6.0 (i) after exposure to 10% NO in N_2 overnight, with DNIC component shown (ii) by subtraction of 15% of spectrum a,ii from spectrum b,i; and (iii) after exposure to 10% NO in N_2 overnight followed by 2 h at 10% NO and 0.05% O_2. Spectra b,i and b,iii were recorded against a background of unexposed Fd at pH 6.0.

Table 1. Comparison of ν_{NO} Band Positions Described in This Work with Selected Literature Values

complex	IR ν_{NO}/cm^{-1}	ref
Fd-DNIC	1762, 1726	this work
oligopeptide[a]-DNIC	1767, 1722	16
Cys-DNIC	1772, 1727	16
Fd-RRE	1815, 1783, 1757	this work
oligopeptide[a]-RRE	1821, 1788, 1763	16
n-propyl-thiolate-RRE	1810, 1775, 1748	22
$[RRS]^{2-}$[b]	1658, 1617	23

[a]Lys-Cys-Ala-Cys-Lys. [b]$[AsPh_4]^+$ salt.

major component shows bands at 1762 and 1726 cm^{-1}. These are similar to peak positions reported for oligopeptide- and cysteine-bound DNIC clusters in water (Table 1).[16] An additional band at 1783 cm^{-1} suggests a contribution from RRE. Subtraction of 15% of the RRE spectrum (Figure 1a,ii)

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removes the 1783 cm^{-1} feature, giving spectrum b,ii which is typical of a DNIC. Similar IR spectral changes were observed at pH 6.5 and pH 7.0. DNIC formation at pH 6.0 was confirmed by EPR spectroscopy (Supporting Information, Figure S1), which showed a diagnostic signal at g_{av} = 2.03.[26] Spin integration suggested that the DNIC accounts for 8−9% of the total Fe content in the protein sample. An indirect route to quantification of the RRE component involves reduction of the sample using excess sodium dithionite and EPR spin quantitation of the EPR-active reduced RRE (rRRE, Chart 1) component. This indicated 10% of the total Fe was in the rRRE form (Figure S2). It was not possible to quantify unreacted $[2Fe2S]^{+}$ because the cluster in the reduced protein is unstable at pH 6.0 (Figure S3). UV−visible spectroscopy, Figure 2,

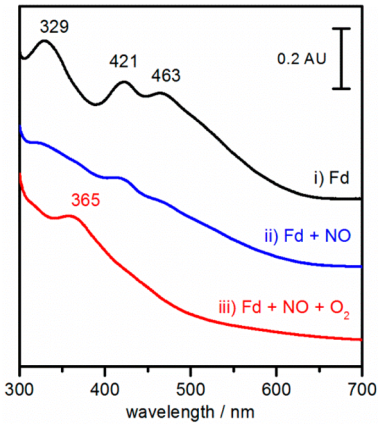

Figure 2. UV−visible spectra of 40 μM Fd (i) before NO exposure; (ii) after exposure to 10% NO in N_2 overnight; or (iii) after exposure to 10% NO in N_2 overnight followed by 2 h at 10% NO and 0.05% O_2.

confirms incomplete loss of the native Fd iron−sulfur cluster upon reaction with 10% NO. Although DNICs have been proposed as important products of iron sulfur protein nitrosylation,[27] conversion to these clusters is often incomplete.[9]

Addition of trace O_2 (0.05% O_2, 10% NO) to the Fd sample already exposed to NO at pH 6.0 resulted in rapid further yellowing of the sample, and the IR spectrum (ii) shown in Figure 1b exhibits peaks centered at 1783 and 1757 cm^{-1} consistent with conversion of the DNIC into RRE on contact with O_2. EPR spin integration confirmed that at least half of the DNIC was lost (Figure S4). Further confirmation of the formation of RRE as the major product of exposing Fd to trace O_2 and NO at pH 6.0 is provided by the UV−visible spectrum shown in Figure 2,iii, in which there is a single intense peak at 365 nm. This spectrum is similar to the UV−visible spectrum reported for nitrosylated Rieske protein, comprising a single peak at 367 nm, for which RRE was found to be the major product by NRVS.[8] RRE has also been proposed as the product of nitrosylation of [4Fe4S] WhiB-like response regulator proteins on the basis of the stoichiometry of NO requirement and sulfur release.[9] As expected, reduction with dithionite of the Fd sample exposed to NO and O_2 results in peaks in the UV−visible spectrum at 640 and 960 nm (Figure S5), which are characteristic of rRRE.[8] EPR spin quantitation of the rRRE showed it to account for 17% of the total iron (Figure S6). The addition of NO and O_2 simultaneously, or adding NO and iodoacetamide, to Fd at pH 6.0 also led to substantial RRE formation as confirmed by IR spectroscopy.

Bacterial NO-sensing proteins must undergo a conformational change upon NO exposure in order to transmit the appropriate signal.[28] It would be expected that a significant rearrangement of the protein backbone is needed to allow conversion of a [2Fe2S] cluster with terminal thiolate ligands into a RRE in which the thiolates are bridging. Examination of the second derivative of the amide region in the IR spectrum of Fd prior to NO exposure, and after exposure to NO and O_2 (Figure S7) shows a loss of sharp features at 1685, 1653, and 1635 cm^{-1}, which are associated with the amide I absorptions of specific protein secondary structures.[29] Two new features appear at 1690 and 1631 cm^{-1}, consistent with protein unfolding.[29] Nitrosylation-mediated iron−sulfur protein unfolding has previously been detected using NMR spectroscopy by Foster et al. for a high potential [4Fe4S] protein (HiPIP).[12]

Although Fd has been reported to be unreactive toward NO under anaerobic conditions at slightly alkaline pH,[11] we have shown that nitrosylation does occur at pH 7.5 in the presence of O_2 or iodoacetamide to generate RRE. We observed that Fd is more reactive toward NO at lower pH values, with a mixture of RRE and DNIC products formed under anaerobic conditions at mildly acidic pH values. Following the addition of O_2 or iodoacetamide at pH 6.0, the RRE component was enriched. It is likely that both of these reagents are scavenging thiolates which would favor conversion of DNIC species to RRE. This is consistent with the mechanisms proposed for RRE formation in small molecule[20,21] and oligopeptide-bound systems,[16] in which the conversion of DNIC to RRE clusters is favored by decreasing the thiolate to iron ratio.[13,16] In a phagolysosome, where bacteria are exposed to NO by the immune system, there will be multiple thiolate-reactive species, such as superoxide and peroxynitrite.[30] More specifically, the observed importance of O_2 may have physiological relevance to the effects of NO on microbial pathogens as they will be exposed to NO in an aerobic environment during a mammalian immune response. Additionally, this study highlights the need for rigorous gas purification and strict control over O_2 levels when studying reactions of NO with proteins. The presence of a small amount of RRE after anaerobic exposure to NO at pH 6.0 shows that there must also be an anoxic route to formation of lower levels of RRE from NO alone, perhaps relying upon structural change in the protein to move cysteine thiolates away from the cluster.

The reason for the effect of pH on Fd reactivity with NO is not clear. The structural change suggested for Fd below pH 7.0[24] did not have a substantial enough effect on secondary structure to be evident in the amide region of the IR spectrum. However, reduced Fd at pH 6.0 appears less stable than at pH 7.5 (see Figure S3). Future studies on the reactivity of other [2Fe2S] proteins with NO should reveal whether there is a fundamental role for pH in cluster nitrosylation.

In conclusion, we report the first IR study of nitrosylation of a protein-bound iron−sulfur cluster, show that spinach ferredoxin I reacts with NO under specific conditions, and reveal the importance of trace O_2 in determining reaction products. Comparison of nitrosylation in the presence of trace O_2 and iodoacetamide suggests that O_2 is needed for oxidative trapping of thiolates. We show that IR spectroscopy should be useful, alongside EPR, UV−visible spectroscopy, and NRVS in elucidating the reactions of other iron−sulfur proteins, particularly NO-sensing proteins.

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■ ASSOCIATED CONTENT

Supporting Information

Further details of experimental methods, EPR spectra quantifying Fd DNIC and Fd rRRE components, UV−visible spectra of reduced Fd at pH 6.0 showing cluster instability and of Fd rRRE species, and second derivative IR spectra of the amide I region of Fd before and after exposure to 10% NO/0.05% O_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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