Stopped-Flow Infrared Spectroscopy Reveals a Six-Coordinate Intermediate in the Formation of the Proximally Bound Five-Coordinate NO Adduct of Cytochrome *c′*

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In this communication we report the use of stopped-flow Fourier transform infrared (SF-FTIR) spectroscopy to monitor the binding chemistry of NO to cytochrome *c′* (cyt *c′*) from *Alcaligenes xylosoxidans.* NO plays a role in a diverse range of physiological processes in higher organisms such as the immune response to tumor cells, vasodilatation, and neuronal synaptic transmission.[1] An important element in these processes is the heme enzyme soluble guanylate cyclase (sGC) which responds to changes in [NO]. Cytochromes *c′* are small (27.2 kD per dimer) microbial proteins which are known to share unusual spectroscopic and ligand binding properties with sGC.[2,3] Unlike other high-spin hemoproteins, for example, both proteins form stable complexes with NO and CO but not O_2. In addition, the hemes are axially coordinated only to a single histidine ligand, resulting in nominally five-coordinate iron centers.[2,4] Interest in cyt *c′* has recently been stimulated by a crystallographic study of the *A. xylosoxidans* protein at 1.35 Å resolution which revealed a novel ability of CO and NO to bind to opposite faces of the heme.[4] CO forms a six-coordinate (6c-CO) adduct in which the CO binds to the Fe from the distal pocket side. By contrast, the crystal structure of the NO adduct shows a five-coordinate (5c-NO) complex with the NO adopting an unprecedented proximal Fe-coordination, displacing the proximal His-120 and leaving the distal side of the heme unligated. The mechanism of formation of this novel coordination is clearly of great interest, not least because it may account for ligand-discrimination and signal transduction in heme-based gas sensor proteins such as sGC.

The SF-FTIR experiments employed a two-syringe drive system. One syringe contained ferrous cyt *c′*, and the other contained buffer or a buffered solution of ^{14}NO or ^{15}NO.[5] The infrared spectrum of ferrous cyt *c′* in the absence of NO is dominated by a broad envelope of polypeptide amide-I vibrations centered at 1645 cm^{-1} (Figure 1a). Within 200 ms of mixing the protein (100 μM after mixing) with ^{14}NO (150 μM) a number of peaks and troughs are apparent in the "^{14}NO + cyt *c′ minus* cyt *c′*" difference spectrum (Figure 1b).[11] Only the major peak, at 1625 cm^{-1}, is sensitive to isotopic substitution with ^{15}NO, with a shift of 29 cm^{-1} to 1596 cm^{-1} (compare Figure 1, b and c), giving a single characteristic derivative in the "^{14}NO + cyt *c′ minus* ^{15}NO + cyt *c′*" difference spectrum (Figure 1d). We assign this band to a six-coordinate heme−NO complex (6c-NO), as its

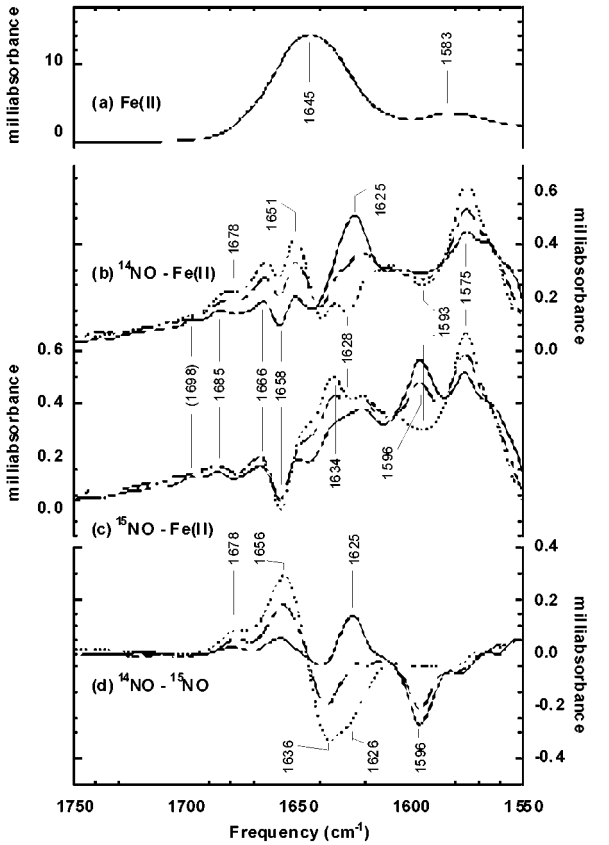

Figure 1. The reaction of ferrous *A. xylosoxidans* cyt *c′* with NO monitored by SF-FTIR spectroscopy. (a) Absorption spectrum of ferrous cyt *c′* in the absence of NO. (b) Time-dependent difference spectra of ^{14}NO + cyt *c′* − cyt *c′* with no NO. (c) As (b) but using ^{15}NO. (d) Time-dependent difference spectra of ^{14}NO + cyt *c′* − ^{15}NO + cyt *c′*. Data were recorded: 0.15−1.17 s (—); 1.79−2.81 s (— • — • —) and 17.3−70.7 s (•••) after mixing.

frequency is within the reported 1633−1607 cm^{-1} range,[12,13] the isotope shift is appropriate,[14] and its relatively sharp half-height line width of 8.0 cm^{-1} is consistent with other protein heme−NO complexes.[7,12,15] At longer times this band is replaced by a more complex spectrum (Figure, 1, b-d). The isotope difference data (Figure 1d) now reveal a complex line shape, which we assign to multiple conformers of 5c-NO. However, it is not possible to fit this spectrum with a simple combination of derivatives with the expected 30 cm^{-1} isotope shift. Since this line shape varied between experiments, we attribute this to

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(5) Cytochrome *c′* was isolated from *A. xylosoxidans* NCIMB 11015 as described.[6] Ferrous enzyme was generated by reducing 300 μl of 1 mM cytochrome *c′* with 10 mM sodium dithionite in a 0.5 ppm O_2 anaerobic glovebox (Belle Technology). Excess reductant was removed using a small Bio-Rad P-6 desalting column equilibrated with the D_2O buffer used for the FTIR experiments: 50 mM bis-tris propane 100 mM NaCl pD 9.4 (equivalent to pH 8.8). NO gas (98.5%) was purchased from Aldrich. ^{15}NO was manufactured from $Na^{15}NO_2$ as published,[7] and purity was confirmed to be greater than 98% by gas chromatography. NO solutions were made by shaking sealed 2.5 mL syringes containing approximately equal volumes of diluted NO gas and buffer and assuming saturated NO in buffer was 2 mM. Infrared spectra were measured at 4 cm^{-1} resolution as described.[8] The instrument setup gave a time resolution of 87 ms per spectrum. The wavenumber range was limited to 1800−1500 cm^{-1} by the optical filter fitted to enhance sensitivity.[8] The stopped-flow circuit was entirely contained within a 0.5 ppm O_2 anaerobic glovebox.[8,9] The stopped-flow cell had a path length of 48.2 μm and was thermostated at 25 °C. Time courses were obtained using a peak analysis method and kinetically fitted as described.[8,10] All results were reproduced. All concentrations are quoted after mixing.

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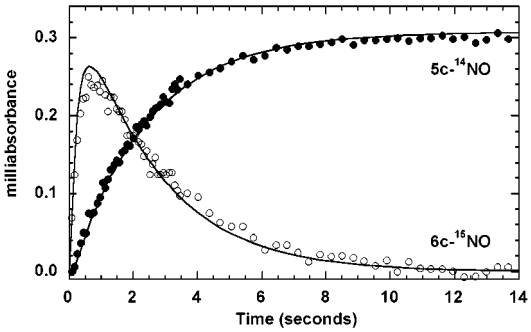

Figure 2. Time dependence of the cyt c' + NO isotope difference SF-FTIR spectra of Figure 1d, determined by integrating well-separated peaks: (○) 6c-^{15}NO (1596 cm^{-1}); (●) 5c-^{14}NO (1656 cm^{-1}). The solid lines are calculated time courses (see text).

apparently random differences in conformer distribution.[16] Given this premise, the simplest of many possible analyses comprise three 5c-NO conformers with ν(N═O) stretches in the region of 1678, 1666, and 1654 cm^{-1},[17] consistent with the 1703−1660 cm^{-1} range thus far reported for 5c-NO.[12,13] Our assignment of these bands to multiple conformers agrees with the crystal structure, which shows two distinct orientations of NO bound to the heme.[4]

Time courses measured for the ν(N═O) bands in Figure 1d at 1596 cm^{-1} (6c-^{15}NO) and 1656 cm^{-1} (5c-^{14}NO) clearly demonstrate the biphasic reaction (Figure 2). The transient 6c-NO reaches maximum intensity at ∼0.7 s and 5c-NO is fully formed within 12 s. No intermediates are apparent in the 6c-NO → 5c-NO reaction as the spectra show clear isosbestic points (Figure 1). Surprisingly, the rates of both phases increase on increasing [NO]. In initial experiments using 300 μM NO only the second phase was seen clearly and this was complete within 3.5 s, while with 1 mM NO both phases were too fast to readily observe.[18] Preliminary UV−vis stopped-flow measurements suggest a first-order [NO] dependence for both phases,[19] and consistent with this, the IR data can be fitted to [NO]-dependent rate constants of 36 ± 10 and 8.4 ± 0.6 mM^{-1} s^{-1} (solid line in Figure 2). We note a similar [NO] dependence, and a biphasic 6c-NO → 5c-NO reaction has been observed by kinetic studies on NO binding to sGC.[20] The implication is that both 6c-NO and 5c-NO separately require NO binding to form. We propose a mechanism in which NO first binds to the vacant heme distal site forming 6c-NO, thus weakening the bond between the heme iron and the proximal His-120. A second NO then binds to the heme iron on the proximal side, causing both the proximal histidine and distal NO to dissociate, leaving 5c-NO.[21]

The isotopically insensitive time-dependent features of Figure 1, b and c deserve some comment as these arise from the changes in the protein matrix and heme cofactor. Analyses show similar time dependencies to those in Figure 2. For example, a small band at 1698 cm^{-1} appears and disappears with the 6c-NO complex. That at 1685 cm^{-1} grows with 6c-NO and then remains during the conversion to 5c-NO. These occur in the polypeptide amide-I region (1700−1610 cm^{-1}) and probably reflect changes in the protein backbone. Most interesting are the peak at 1575 cm^{-1} and the troughs at 1593 and 1658 cm^{-1}, which track the formation of 5c-NO from 6c-NO.[22] These bands have a number of possible assignments. For example, infrared-active heme ring modes absorb at energies below ∼1625 cm^{-1}.[23] Alternatively, they could arise from deprotonation of a heme propionate, the 1658 cm^{-1} trough being the ν(C═O) of the protonated form and the 1575 cm^{-1} peak being a ν(COO$^-$)^{as} stretch,[23,24] but such chemistry seems unlikely at pD 9.4. Another possibility is Arg-124. Arginine in D_2O absorbs between 1608 and 1577 cm^{-1}, usually as a doublet.[25] The crystal structure data show that on formation of 5c-NO this residue rotates to stack against the heme plane, where it can hydrogen-bond to one conformer of the bound NO.[4] However, the most pronounced change in the 5c-NO structure is the displacement and dissociation from the heme of the proximal His-120. The ν(C═C) mode of histidine also absorbs in this region,[25] albeit with lower reported intensities than these bands.[26] In this interpretation, the trough at 1593 cm^{-1} arises from metal bound His-120, and the peak at 1575 cm^{-1} from the displaced form. The 1658 cm^{-1} band could then arise from associated amide-I changes. Clearly, a precise assignment requires a more complete study.

In summary, SF-FTIR has shown that for *A. xylosoxidans* cyt c', formation of the novel proximally bound 5c-NO complex proceeds via a 6c-NO intermediate. Preliminary kinetic data suggest that the formation of both species separately require NO binding. This novel chemistry may be the mechanistic key for NO-sensing heme enzymes.

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(11) Use of difference spectroscopy is essential for clarity, as the time-dependent changes are less than 2% of the absorption at 1645 cm^{-1} (Figure 1). Note that isotope differencing of time-dependent spectra assumes that any ^{14}N → ^{15}N kinetic isotope effect is negligible. Also note that free NO is not observed as it gives a very broad ill-defined band with ν(NO) ≈ 1876 cm^{-1}.

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(14) Modeling ν(NO) as a diatomic simple harmonic oscillator gives a ^{14}NO → ^{15}NO isotope shift of 29.2 cm^{-1} at 1625 cm^{-1}. Density functional theory calculations have suggested shifts 2−4 cm^{-1} larger.[13]

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(16) The origin of this effect is unclear. Any alternative interpretation requires the isotope shift to be unusually small (∼18 cm^{-1}) or for the conformational distribution to depend on the isotope, both of which are unprecedented. A recent resonance Raman study of cyt c' shows a weak broad band at 1661 cm^{-1} with the expected isotope shift.[3] Differences between infrared and Raman spectra could arise if the lower-energy NO conformers are hydrogen-bonded, inducing greater N−O polarization and concomitantly stronger IR and weaker Raman bands. X-ray crystallography shows two NO conformers, with one hydrogen bonding to Arg-124.[4]

(17) Spectra were fitted using least-squares minimization to a combination of appropriate Gaussian difference curves where the relative contributions of ^{14}NO and ^{15}NO conformers were allow to float. An example good fit is (i) λ_{14NO} = 1678.3 cm^{-1}, A_{14NO} = 0.090, A_{15NO} = −0.090, (ii) λ_{14NO} = 1666.0 cm^{-1}, A_{14NO} = 0.039, A_{15NO} = −0.26, (iii) λ_{14NO} = 1654.4 cm^{-1}, A_{14NO} = 0.38, A_{15NO} = −0.13 (absorbances quoted in milliabsorbance units). Bandwidths were fixed at 8.0 cm^{-1}.

(18) Note the maximum rate measurable with the spectrometer settings used is ∼10 s^{-1}. [NO] and pD were chosen to give relatively slow rates; however, recent preliminary UV−vis kinetic data demonstrate essentially identical kinetics at pH 7.0 and 9.0.[19]

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(21) This second step has two possible mechanisms. In the first, NO attacks 6c-NO to form a sterically crowded seven-coordinate intermediate. Alternatively, 6c-NO is in equilibrium with a small amount of five-coordinate heme with dissociated His-120, and NO binds to this minority species.

(22) A trough at 1628 cm^{-1} that is obscured by both ^{14}NO and ^{15}NO bands may also track 5c-NO formation. We note that these bands have no obvious counterparts in recent resonance Raman data.[3]

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(26) Barth[25] reports intensities up to 70 M^{-1} cm^{-1} from model data whereas the band at 1575 cm^{-1} is ∼600 M^{-1} cm^{-1}.