Nitric-oxide Reductase

We have applied resonance Raman spectroscopy to investigate the properties of the dinuclear center of oxidized, reduced, and NO-bound nitric-oxide reductase from Paracoccus denitrificans. The spectra of the oxidized enzyme show two distinct ν(Fe-O-Fe) modes at 815 and 833 cm⁻¹ of the heme/non-heme diiron center. The splitting of the Fe-O-Fe mode suggests that two different conformations (open and closed) are present in the catalytic site of the enzyme. We find evidence from deuteration experiments that in the dominant conformation (833 cm⁻¹ mode, closed), the Fe-O-Fe unit is hydrogen-bonded to a distal residue(s). The ferric nitrosyl complex of nitric-oxide reductase exhibits the ν(Fe³⁺-NO) and ν(N-O) at 594 and 1904 cm⁻¹, respectively. The nitrosyl species we detect is photolabile and can be photolyzed to generate a new form of oxidized nitrosyl complex of nitric-oxide reductase exhibiting the ν(Fe³⁺-NO) at 594 and 1904 cm⁻¹, respectively.

The bacterial nitric-oxide reductase (Nor) complex forms the N-N bond during denitrification (1-4). It is a membrane-bound cytochrome bc complex composed of two subunits, NorC and NorB, that catalyzes the reduction of NO to N₂O (5). The complex contains four known redox centers: three heme groups and one non-heme Fe atom (5-7). Heme c (six-coordinate, low-spin) is bound to NorC subunit and functions as the electron entry point of the enzyme. NorB contains one six-coordinate, low-spin heme b₃ and a five-coordinate, high-spin heme b₅, which, together with a non-heme iron atom, form the dinuclear NO reduction site. The sequence of NorB contains all six histidines that are the ligands of heme a, heme a₃, and Cu₅ in cytochrome c oxidase (4).

Resonance Raman (RR) scattering is a powerful technique for the study of heme proteins because the spectra are rich in information about the heme groups (8, 9). Moreover, vibrational modes of ligands bound to heme may be assigned by isotopic substitution measurements, and their properties, which reflect ligand structure, may be studied. It has been reported that in oxidized Nor, heme b₅ is not coordinated to the protein by its proximal histidine residue (6). In addition, the single oxygen isotope-sensitive ligand vibration observed at 811 cm⁻¹ in the RR spectrum was attributed to the ν(Fe-O-Fe) of the heme b₅-non-heme diiron center (7). However, the structural implications involved in the transition from oxidized to reduced enzyme were not reported.

The properties of oxidized Nor, when compared with those of the chemically generated deoxy (five-coordinate) and the nitrosyl complex, can be related to structural changes that take place in the protein upon ligand binding and release. It is these interactions between the heme and the protein that determine the biological properties of the heme protein. Moreover, in unraveling the NO protonation mechanism, it is necessary to establish sites in the enzyme that have exchangeable protons. If there are such sites near the high-spin heme b₅ or the non-heme Fe, then solvent exchange from protonated to deuterated buffers could lead to changes in the RR spectra because the vibrational frequencies are sensitive to effective mass.

In an effort to gain information concerning these observations, we have used 413.1 nm RR excitation to examine the spectra of oxidized Nor upon deuteration and H₂¹⁸O exchange and compare them to those of the fully reduced form, the NO-bound form, and the NO photoproduct. Our data indicate the presence of two oxygen-sensitive modes at 811 and 833 cm⁻¹ in the oxidized enzyme. The latter mode also shows deuteration sensitivity. With the identification of the two distinct ν(Fe³⁺-NO) modes at 815 and 833 cm⁻¹ of the heme/non-heme diiron center, its structural properties can be described now with more certainty. With the aid of isotopic substitution, we have also characterized the ferric-NO complex by its ν(Fe³⁺-NO) stretching frequency at 594 cm⁻¹ and ν(N-O) at 1904 cm⁻¹, and we postulate that this species is a catalytic intermediate in the NO reduction cycle. In addition, we have shown that the nitrosyl species we detect is photolabile and that the photoprotein has vibrational properties that are different from those of the resting enzyme. Based on our results, we propose a structure-function model of Nor for the transition from the oxidized to the reduced form and from the oxidized to the NO-bound form and its photoprotein.

EXPERIMENTAL PROCEDURES

Nitric-oxide reductase was purified as described elsewhere (5). The activity of the enzyme was measured according to Ref. 5 and was 40 μmol/mg/min (48°C). The samples were concentrated to 150 μM in 20 mM Tris, pH 7.4, containing 0.05% dodecyl β-D-maltoside and stored in liquid nitrogen until use. For hydrogen/deuteration and H₂¹⁸O isotope studies, solutions of the oxidized enzyme were exchanged three times in 99.9% D₂O (Aldrich) and 97% H₂O¹⁸ (Icon). The concentration
Fig. 1. Optical spectra of Nor in the oxidized (trace A), ferri-NO (trace B), and reduced (trace C) states.

The optical absorption spectrum of oxidized enzyme (Fig. 1, trace A) displays maxima at 411, 530, and 558 nm, which are indicative of low-spin hemes b and c and high-spin heme b₃. The shoulder at 595 nm is typical of the porphyrin-to-ferri charge transfer transition characteristic of ferric high-spin heme b₃. The spectrum of the NO-bound oxidized enzyme (Fig. 1, trace B) was obtained by the direct addition of gaseous NO to the resting enzyme and shows a Soret maximum at 416 nm and the visible transition at 562 nm along with the disappearance of the 595 nm band. For the reduced enzyme (Fig. 1, trace C) the Soret is at 420 nm, and the visible transitions are at 521, 551, and 558 nm.

The high-frequency RR spectra are used to assess the spin and ligation states of the hemes via the porphyrin marker band frequencies and the status of the proximal and distal environments for the resting, deoxy, NO-bound, and photoproduct form of heme b₃. The RR spectra of heme proteins in the high-frequency region contain several well-established porphyrin modes termed as the oxidation state ν₂ or the ligation state (ν₂, ν₃, and ν₅) marker modes. Fig. 2 shows the high-frequency RR spectra of oxidized (trace A) and fully reduced (trace B) Nor by using 413.1 nm excitation frequency, in which resonances from all hemes are enhanced, and the resulting assignments are summarized in Table I. The modes of the oxidized enzyme (trace A) at 1373 (ν₁), 1492 (ν₂), 1578 (ν₂), and 1630 (ν₁₀) cm⁻¹ indicate the presence of a five-coordinate high-spin heme b₃. This suggests that the heme b₃ iron is ligated by either a proximal or a distal ligand (see below). Also present in this spectrum are modes at 1505 (ν₂), 1584 (ν₂), and 1639 (ν₁₀) cm⁻¹, indicating the presence of six-coordinate low-spin heme c and heme b. The mode at 1597 cm⁻¹ originates from ν₂ of low-spin heme b or heme c. The ν₁ bands for the ferric hemes have coincident positions despite the large differences in their redox potentials and porphyrin substituents. This is unusual in view of the ν₁ dependence upon metal—porphyrin backboning (dp–dp, dp–ε₄,π). In the spectrum of the reduced enzyme (trace B), the ν₄ mode is located at 1362 cm⁻¹, establishing that all hemes are in the ferrous state. The ν₂ at 1472 cm⁻¹, ν₃ at 1560 cm⁻¹, and ν₁₀ at 1606 cm⁻¹ establish the presence of a ferrous, five-coordinate high-spin heme b₃. The presence of low-spin hemes c and b is shown by ν₂ intensity at 1494 cm⁻¹. The modes at 1584 and 1591 cm⁻¹ are assigned to ν₂ of the low-spin hemes b and c, respectively. The ν₁₀ vibrational mode for the low-spin hemes is expected around 1620 cm⁻¹. Overlap with the vinyl stretching vibration near 1625 cm⁻¹, however, hinders the assignment of the ν₁₀ vibration. The frequencies of all modes are in agreement with previous analyses of RR spectra of enzymes containing hemes b and c and with those of Fe protoporphyrin model compounds (10, 11).

Fig. 3 shows the low-frequency RR spectra in the 150–900 cm⁻¹ region of oxidized Nor in H₂O (trace A), H₂¹⁸O (trace B), and D₂O (trace C) and that of the fully reduced enzyme (trace D) obtained with 413.1 nm excitation. Raman bands in the low-frequency range do not only include modes from the porphyrin macrocycle and its substituents but also include Fe-ligand motions along the axis normal to the heme. The 250–750 cm⁻¹ region of the Nor spectra is crowded with bands, most of which can be assigned to porphyrin or substituent modes of ferric, six-coordinate low-spin hemes b and c. Intensity changes and frequency shifts upon H₂¹⁸O and D₂O exchanges are observed only in the 740–890 cm⁻¹ spectral region. This spectral region is displayed on an extended view in Fig. 4. With the...
was generated by the addition of dithionite to the oxidized enzyme.

The frequencies of the 207 cm\(^{-1}\) heme b histidine stretching modes in the 200–250 cm\(^{-1}\) (8). The frequency of the 207 cm\(^{-1}\) mode (trace D) is similar to that found for the \(\nu(\text{Fe}^{2+}\cdot\text{His})\) of other ferrous, five-coordinate high-spin hemes b (8).

In Fig. 4, the mode at 833 cm\(^{-1}\) shown in trace A has lost intensity in the \(\text{H}_2\text{O}\) spectrum (trace A), and the 813 cm\(^{-1}\) mode (trace A) has disappeared in the \(\text{H}_2\text{O}\) spectrum (trace B). Trace B shows a new mode at 796 cm\(^{-1}\) and increased RR intensity at 776 cm\(^{-1}\). By subtracting the \(\text{H}_2\text{O}\) spectrum (trace B) from that recorded in \(\text{H}_2\text{O}\), we obtain the difference spectrum A-B shown in the inset. This difference spectrum shows two oxygen isotope-sensitive band pairs at 815/776 and 833/796 cm\(^{-1}\). To determine if there is any evidence for hydrogen bonding to the oxygen-sensitive modes, we measured the RR spectrum in D\(_2\)O buffer. Trace C shows the spectrum of oxidized Nor in D\(_2\)O. Comparison of trace C with trace A shows that the 833 cm\(^{-1}\) band is upshifted to 836 cm\(^{-1}\) in D\(_2\)O. Further evidence for the upshift of the 833 cm\(^{-1}\) mode in the D\(_2\)O spectrum and of the two oxygen isotope-sensitive band pairs at 815/776 and 833/796 cm\(^{-1}\) for \(\text{H}_2\text{O}/\text{H}_2\text{O}\) is shown in the difference spectrum (inset C-B). The observed shift upon deuteration is consistent with hydrogen bonding interactions. The difference spectrum C-B also shows that the 815 cm\(^{-1}\) band appears to be somewhat broader in D\(_2\)O than in \(\text{H}_2\text{O}\). If the bandwidth depends on conformational inhomogeneity, then the 815 cm\(^{-1}\) band suggests packing of amino acid residues to the bridged oxygen. No other oxygen isotope-sensitive modes are detected in the 160–900 cm\(^{-1}\) region, suggesting that neither the symmetric stretch nor the Fe-O-Fe bend is enhanced with 413.1 nm excitation. In addition, we do not detect the 815 and 833 cm\(^{-1}\) modes in the RR spectrum of the fully reduced enzyme (Fig. 3, trace D), as would be expected from the transition from the oxidized to reduced state. The data demonstrate that both the 815 and 833 cm\(^{-1}\) modes can be assigned to the \(\nu(\text{NO})\) of Nor because the 38–39 cm\(^{-1}\) shift is in agreement with that expected from the harmonic oscillator approximation for Fe-O-Fe.

The spectral perturbations in the high-frequency region of the RR spectra caused by the addition of NO to the resting enzyme (Fig. 5 trace A) are limited except for the disappearance of \(\nu_3\) and \(\nu_3\) signals at 1492 and 1578 cm\(^{-1}\) from the ferric five-coordinate high-spin heme \(b_3\). Coordination of NO to heme \(b_3\) of the oxidized enzyme shifts the \(\nu_3\) and \(\nu_3\) modes to higher frequency, such that they coincide with those of the low-spin hemes c and b at 1505 and 1584 cm\(^{-1}\), respectively (trace B). This indicates that heme \(b_3\) is ferric six-coordinate low-spin in the NO complex. The marked differences we observe in the NO-bound spectra as a function of laser power (trace C) indicate the occurrence of a photolabile species. Identification of \(\nu_3\) and \(\nu_3\) at 1492 and 1572 cm\(^{-1}\) (trace C) indicates that photodissociation of the NO ligand is producing a five-coordinate high-spin heme \(b_3\). The core-size band \(\nu_3\) has lost intensity and is downshifted to 1572 cm\(^{-1}\), indicating an expanded core in the photoproduc, as compared with the oxidized form. Direct confirmation of the heme \(b_3\) nitrosyl complex requires detection of the heme Fe\(^{3+}\)–NO stretching vibration.

### Table I

Frequencies of several resonance Raman marker modes (cm\(^{-1}\)) in Nor

| Mode | Oxidized | Reduced | Fe(III)-NO |
|------|----------|---------|------------|
| \(\nu_2\) | 1578 | 1584 | 1560 |
| \(\nu_3\) | 1492 | 1505 | 1472 |
| \(\nu_4\) | 1373 | 1373 | 1362 |
| \(\nu_{10}\) | 1630 | 1639 | 1606 |
| \(\nu_{11}\) | 1597 | | 1550 |
| \(\nu_{37}\) | | | 1624 |
| \(\nu_{C-C}\) | | | 1624 |

*Fig. 3. Low-frequency RR spectra of oxidized Nor in \(\text{H}_2\text{O}\) (trace A), \(\text{H}_2\text{O}\) (trace B), and D\(_2\)O (trace C). The fully reduced spectrum (trace D) was generated by the addition of dithionite to the oxidized enzyme.*

*Fig. 4. Same as Fig. 3, but in the spectral region 740–890 cm\(^{-1}\). The spectra in the inset represent difference spectra of \(\text{H}_2\text{O}/\text{H}_2\text{O}\) (trace A-B) and D\(_2\)O-\(\text{H}_2\text{O}\) (trace C-B).*
Two Conformations of the Catalytic Site in Oxidized Nor—The most reasonable assignment for the two oxygen-sensitive modes present in the spectra of oxidized Nor at 815 and 833 cm\(^{-1}\) is that they arise from two different conformations of a heme Fe(III)-O-Fe(III) complex. The conformer with the weaker hydrogen bond to the bridged oxo is expected to have the weaker Fe-O-Fe bond and thus the lower frequency vibration. According to this interpretation, the more strongly hydrogen-bonded conformer in Nor contributes to the 833 cm\(^{-1}\) mode and has an Fe-O-Fe bond strength comparable to that found in the model compound \([\text{L} \text{Fe(III)-O-Fe(III)-Cl}]\) (11) observed at 818 cm\(^{-1}\) (7). The 815 cm\(^{-1}\) mode, however, is 26 cm\(^{-1}\) lower than that of the \([\text{L} \text{Fe(III)-O-Fe(III)-Cl}]\), heme/non-heme complex, despite the fact that the model compound reproduces the immediate coordination spheres expected to occur around the irons in the protein environment (7). The Nor species we detect has a frequency that is close to the 818–829 cm\(^{-1}\) bands observed for the six-coordinate ferryl complexes (11) and the five coordinate oxoferrylporphyrin \(\pi\)-cation radical (11) observed at 818 cm\(^{-1}\). Despite the similarity in these frequencies, we nevertheless favor a Fe-O-Fe structure for the species we detect.

We attribute the conformer with the high-frequency of \(v_{\text{Fe-O-Fe}}\) at 833 cm\(^{-1}\) as one with a strong positive polar interaction (including hydrogen bonding) between the oxygen atom and the distal residues. This way, a rigid (closed) structure is formed, in which the O atom is stabilized by hydrogen bonding. The conformer with the low-frequency of \(v_{\text{Fe-O-Fe}}\) at 815 cm\(^{-1}\) is assigned as one with an open structure. The \(I_{833}\) cm\(^{-1}\) indicates that the H-bonded conformer is the major species in the resting form of the enzyme. Because we can eliminate an explanation involving the heme Fe\(_2\) proximal ligation for rationalizing the open versus closed conformation behavior, we consider as an attractive model one that invokes variation in the extent to which formation of a hydrogen bond between the bridged oxo moiety and distal residues, including the axial ligands of non-heme iron and/or a \(\text{H}_2\text{O}\) molecule, can occur. This indicates that hydrogen bonding to the oxo moiety by distal residues may be the difference between the two conformations of the Fe-O-Fe unit in Nor. It will be interesting to determine whether there are associated variations in the catalytic function between these two forms because the interaction of the non-heme iron with the heme-bound NO intermediates...
in the dinuclear site could be quite different for these two forms of the enzyme. Additional studies are necessary to determine whether the two forms of the resting enzyme are a reflection of activity regulation.

**Properties of the Fe^{3+}-NO Adduct of Nor**—Table II summarizes ω(Fe^{3+}-NO), δ(Fe^{3+}-NO), and ω(NO) frequencies for several heme proteins and model compounds. In the histidine-containing proteins, using Soret excitation RR spectroscopy, only the Fe-NO stretching and Fe-N-O bending vibrations have been detected (13, 19, 20). This observation was attributed to the little orbital conjugation that exists between the NO group and the heme in the His-Fe(NO) complexes. In the cysteine-containing enzyme P-450Nor, however, in addition to the Fe-NO stretching vibration, the NO has been detected using Soret excitation RR spectroscopy, the Fe(NO) stretching vibration, the N-O has been detected using Soret excitation RR spectroscopy, and the Fe(NO) frequencies for several heme proteins and model compounds.

### Table II

| Molecules | ω(Fe-NO) | δ(Fe-N-O) | δ(NO) | ω(NO) | Ref. no. |
|-----------|----------|-----------|-------|-------|---------|
| Mb        | 595      | 573       | 1922  | 13–16 |         |
| HbA       | 594      | 574       | 1925  | 13    |         |
| HRP       | 604      | 574       | 1903  | 13, 14|         |
| CPO       | 538      | 558       | 1853  | 17    |         |
| P-450Nor  | 520      | 528       | 1853  | 18    |         |
| P-450     | 520      | 528       | 1853  | 18    |         |
| NOS(−)    | 540      | 570       | 1853  | 12    |         |
| Nhsae     | 592      | 570       | 1853  | 12    |         |
| Nor       | 594      | 570       | 1904  | This work |

The unusual low frequency of the Fe-CO stretching frequency found in Nor has been attributed to a negatively charged distal pocket (6). A negative charge in the dinuclear center of Nor will stabilize the Fe − N = O’ resonance form and raise ω(NO). Our results, however, suggest that the Fe^{3+} = N = O resonance form is stabilized. Taken together, these observations strongly suggest that the negative polarity of the dinuclear center, suggested previously (6), has no direct control on the strength of the Fe-NO and N-O bonds in our case. The present RR and FTIR results provide clear evidence that there do not appear to be any unusual stereochemical influences in the dinuclear site of Nor resulting in the modification of the Fe-NO bonding, and even though two conformers have been detected in the ferric form of the enzyme, NO binds in a single conformation to heme \( b_i \). If the NO ligand was bound in two distinct conformations, a second pair of ω(Fe-NO) and δ(NO) modes should appear in the RR and FTIR spectra. However, neither of these modes was observed. The single conformation detected in Nor-NO is essentially that observed in nitrosyl-Mb and Hb.

**Structural Concept of the Catalytic Site**—In an effort to understand the structural implications involved in the transition from oxidized to reduced Nor and from oxidized to the NO-bound form and its photoprotein, we propose a model that is illustrated in Fig. 7. In the oxidized form, the two different conformations of the dinuclear center represent the open and closed structures in which the high-spin heme \( b_2 \) is five-coordinate, and the histidine is not the proximal ligand. Two electrons enter the binding site, resulting in the rupture of the Fe-O-Fe bond and the concomitant reduction of the dinuclear center. In this form of the enzyme, histidine occupies the fifth coordination site on heme \( b_2 \) and the Nδ is hydrogen-bonded as in essentially all heme proteins (24).

The data from the ferric and ferrous forms of the enzyme clearly demonstrate a transition from a five-coordinate high-spin heme Fe(III)-O-Fe(III) configuration in the oxidized form to a five-coordinate high-spin configuration His-Fe(II) in the reduced form. The small distance (3.5 Å) between the heme Fe and non-heme Fe implies that disposition of aromatic rings (imidazoles) in the distal pocket can cause steric crowding (4). Thus, although individual residues may supply the dominant interaction for the presence of two conformers, multiple effects can exist, and heme pocket reorganization is also likely to happen. We propose that reduction of the dinuclear center causes a conformation change in heme \( b_{3} \). This would induce a heme reorientation and a movement of the heme Fe toward the proximal site, resulting in its ligation to the proximal histidine. Thus, the heme Fe/non-heme Fe distance is expected to be larger in the reduced form. In addition, a heme \( b_2 \)-non-heme Fe interaction, which is mediated by the position of the non-heme Fe with respect to the NO bound to heme \( b_3 \), may be very important in the physiological mechanism of NO reduction. One of the most critical features in the structure of Nor is the positioning of the metal centers, especially the relative distance between the non-heme Fe and heme \( b_3 \). These metal centers are known to be close to each other, and in all proposed enzymic mechanisms, the NO or its reduction products have been proposed to interact directly with the heme \( b_2 \) and the non-heme Fe (6, 25). Therefore, any change in the relative positions of these metal centers is of great significance because it modu-

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**TABLE II**

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|-----------|----------|-----------|-------|-------|---------|
| Mb        | 595      | 573       | 1922  | 13–16 |         |
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| HRP       | 604      | 574       | 1903  | 13, 14|         |
| CPO       | 538      | 558       | 1853  | 17    |         |
| P-450Nor  | 520      | 528       | 1853  | 18    |         |
| P-450     | 520      | 528       | 1853  | 18    |         |
| NOS(−)    | 540      | 570       | 1853  | 12    |         |
| Nhsae     | 592      | 570       | 1853  | 12    |         |
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Addition of an electron to the radical NO, which has one electron in the \( \pi^* \) orbital, weakens the N-O bond and yields NO−, whereas the removal of an electron strengthens the N-O bond and yields NO+. The frequencies of NO−, NO+, and NO are located at 1284, 1876, and 2345 cm−1, respectively (21, 22). The frequencies of ω(NO) in Mb (acidic form), Mb (neutral form), and horseradish peroxidase are at 1910, 1922, and 1903 cm−1, respectively, suggesting that the electronic states of bound NO to these heme proteins are close to that of NO− and that the NO moiety is electron-deficient. As suggested by Tomita et al. (15), the ω(NO) in a variety of Fe^{3+}-NO heme proteins, which all have a neutral His as the trans ligand, is electron-deficient, and the effect of π donation from the proximal His is small. Accordingly, we suggest that the NO moiety in Nor adopts the resonance structure Fe^{3+} = N = O, which is the same as that of the acidic form of metMb-NO (ω(NO) = 1910 cm−1) rather than the Fe − N = O + of the neutral form (ω(NO) = 1922 cm−1). The latter structure in Mb is stabilized by the lone pair electrons on Nδ of the distal histidine, which is located adjacent to the O atom of the bound NO, raising ω(NO). It also appears that the degree of backbonding in the Fe^{3+}-NO of Nor is the same as that found in nitrosyl-Hb and Mb, and because it has been established that the frequency of the Fe^{3+}-NO mode is sensitive to the type of the proximal ligand, its frequency is consistent with a neutral imidazole rather than imidazolate coordination, as occurs in peroxidases (15).
lates the reaction kinetics and even determines the possible reaction products.

Addition of NO to the resting enzyme causes the rupturing of the Fe-O-Fe bond and the concomitant ligation of the proximal histidine to the heme iron, producing the His-Fe$^{3+}$-NO species. It appears that there is a communication linkage between the distal and proximal sites through bond networks. Therefore, a structural change in the distal site upon NO binding can potentially be communicated to the proximal site through a polypeptide backbone. In the NO association process, we cannot exclude the possibility that the non-heme iron is a way-stop for NO on its route to the heme $b_3$ binding site, causing the rupture of the Fe-O-Fe bond and, concomitantly, the ligation of the proximal His to the Fe heme $b_3$. However, we can exclude with certainty the binding of two NO molecules to the ferric dinuclear center and thus conclude that the heme $b_3$ has higher affinity for NO compared with the non-heme Fe. The photodissociation of the ferric NO species is problematic because ferric-heme complexes have not been amenable to detailed study due to their chemical reactivity. The experimental data presented here, however, show that the ferric-NO species has high photo-dissociation quantum yield and that achieving complete photodissociation is not difficult. The photodissociated species has a structure in which the proximal histidine is intact, and the photodissociated NO is not bound to the non-heme Fe.

It is important to note that both proposed mechanisms for the NO reduction by Nor involve two NO molecules in the dinuclear center. In a model proposed by Moenne-Loccoz and de Vries (6), the reaction is initiated when the dinuclear center is fully reduced and one molecule of NO binds to each metal center. They postulated that binding of NO to the ferrous heme $b_3$ results in the dissociation of the proximal histidine. After dimerization, the bound NO molecules are reduced to N$_2$O by using the available electrons on both metal centers. The reduction of 2NO to N$_2$O leaves the ferric five-coordinate heme $b_3$ bridged to the non-heme Fe. Upon reduction of the heme $b_3$ by electron transfer from the other cofactors, the bridging ligand is lost in favor of the original histidine ligand. Alternatively, it has been proposed by Grönberg et al. (25) that the dinuclear site exists in a mixed valence form (heme $b_3^{3+}$/non-heme Fe$^{2+}$) before NO binding and that two NO molecules bind sequentially to the non-heme iron, leaving the heme $b_3$ essentially as a spectator in the catalytic cycle.

The midpoint redox potentials of each of the metal centers in the enzyme have been measured, and from the unexpectedly low midpoint redox potential of heme $b_3$ ($E_m = 60$ mV), it was suggested that full reduction of the dinuclear center is thermodynamically unfavorable (25). The redox potentials of the other redox centers were reported at 310 mV for Fe(II) heme c, 345 mV for Fe(II) heme $b$, and 320 mV for the non-heme Fe(II) (25). Thus, it is possible that under physiological conditions, NO activation in Nor occurs with a mixed valence form of the enzyme in which the low-spin hemes $b$ and $c$ and the non-heme Fe are reduced, and heme $b_3$ is in the oxidized form. This way,
The initial step in the reduction process is the formation of the reduced species Fe\(^{2+}\) needed for the reduction of NO are directly transferred from ponitrite (HONNO\(^{-}\)) to transiently yield hy-

The N-O bond produces the ferric enzyme, N\(_2\)O, and H\(_2\)O. Such a process occurs in P-450Nor (26), where the electrons needed for the reduction of NO are directly transferred from NADH (2NO + NADH + H\(^+\) → N\(_2\)O + H\(_2\)O + NAD\(^+\) + H\(_2\)O).

The initial step in the reduction process is the formation of the Fe\(^{3+}\) -NO complex, and reduction of the ferric nitrosyl complex by NADH yields a transient species that is spontaneously de-

composed to the Fe\(^{3+}\) state. In other enzymes, however, such as cytochrome c oxidase, where the binuclear center is easily reduced, it has been shown that binding of two molecules of NO in the heme pocket of the fully reduced enzyme (4e\(^-\)) leads to oxidation of the heme as well as the nearby copper atom (27). This process involves the uptake of two protons and the generation of N\(_2\)O and H\(_2\)O. The molecular mechanism of NO reduction to N\(_2\)O appears to be complex, inasmuch as our results demonstrate the formation of a photolabile nitrosyl heme b\(_3\) species and that NO coordinates only to heme b\(_3\). Additional studies of NO coordination to mixed valence Nor are necessary to formulate a complete mechanism for the reduction of NO to N\(_2\)O under physiological conditions.

Resonance Raman spectroscopy was used to characterize the coordination structure and properties of Nor. The measurements that we have reported here unequivocally demonstrate the presence of two distinct modes at 815 and 833 cm\(^{-1}\) that we assigned to open and closed states of the heme b\(_3\) Fe-O- Fe dinuclear center. The observation of the heme b\(_3\) Fe\(^{2+}\) -NO and N-O stretching modes lays the foundation for identifying other NO modes such as those associated with activated NO intermediates and thereby unraveling the catalytic mechanism of this fascinating enzyme. Further characterization of the heme b\(_3\) Fe\(^{3+}\) -NO photoproduct should allow for a clear determination of the molecular relaxation pathway of photodissociated Nor.

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