Spectral Properties of Bacterial Nitric-oxide Reductase

RESOLUTION OF pH-DEPENDENT FORMS OF THE ACTIVE SITE HEME $b_3$*

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Sarah J. Field‡‡, Louise Prior§, M. Dolores Roldán‡‡‡, Myles R. Cheesman, Andrew J. Thomson§, Stephen Spiro‡‡, Julea N. Butt§, Nicholas J. Watmough‡‡, and David J. Richardson‡‡**

From the Centre for Metalloprotein Spectroscopy and Biology, Schools of ‡‡ Biological Sciences and ‡‡‡ Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom

Bacterial nitric-oxide reductase catalyzes the two electron reduction of nitric oxide to nitrous oxide. In the oxidized form the active site non-heme Fe$\alpha$ and high spin heme $b_3$ are $\mu$-oxo bridged. The heme $b_3$ has a ligand-to-metal charge transfer band centered at 595 nm, which is insensitive to pH over the range of 6.0–8.5. Partial reduction of nitric-oxide reductase yields a three electron-reduced state where only the heme $b_3$ remains oxidized. This results in a shift of the heme $b_3$ charge transfer band $\lambda_{\text{max}}$ to longer wavelengths. At pH 6.0 the charge transfer band $\lambda_{\text{max}}$ is 605 nm, whereas at pH 8.5 it is 635 nm. At pH 6.5 and 7.5 the nitric-oxide reductase ferric heme $b_3$ population is a mixture of both 605- and 635-nm forms. Magnetic circular dichroism spectroscopy suggests that at all pH values examined the proximal ligand to the ferric heme $b_3$ in the three electron-reduced form is histidine. At pH 8.5 the distal ligand is hydroxide, whereas at pH 6.0, when the enzyme is most active, it is water.

Bacterial nitric-oxide reductases (NOR)† catalyze shown in the following Reaction 1.

$$2\text{NO} + 2e^- + 2H^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$$

**Reaction 1**

This reaction serves either as a key step in the pathway of denitrification that uses N-oxoanions and N-oxides as respiratory electron acceptors or as a way of removing cytotoxic NO (1). The capacity for NO reduction is now recognized in a phylogenetically diverse range of bacteria, which includes soil denitrifying bacteria such as Paracoccus denitrificans and pathogenic bacteria such as Neisseria meningitidis (2–4). Three classes of NOR have been identified. The two-subunit (NorCB)-dependent class from P. denitrificans, Pseudomonas stutzeri, and Rhodobacter sphaeroides, which use cytochromes $c$ or cupredoxins as an electron donor, the single subunit (NorB) quinol-oxidizing class from Ralstonia eutropha and N. meningitidis, and the Cu$_A$ containing quinol and cytochrome $c$ oxidizing enzyme of Bacillus azotoformans (2, 3, 5).

Primary structure analysis, in combination with spectroscopic studies, has clearly established NORs as divergent members of the family of respiratory heme-copper oxidases. Characterization of P. denitrificans NorBC has established that the catalytic NorB subunit binds a bis-histidine-coordinated heme $b$ that is functionally equivalent to heme $a$ in cytochrome-c oxidase. It also binds a high spin heme $b$ (termed $b_3$) that is equivalent to heme $a_3$ in cytochrome-c oxidase and heme $o_9$ of Escherichia coli cytochrome-b$_3$ quinol oxidase. In cytochrome-c oxidase and quinol oxidases this heme is magnetically coupled to a copper ion ($\text{Cu}_{b}$) to form a dinuclear center, which is the site of oxygen binding and reduction. By contrast, in NOR this Cu$_b$ is replaced with a non-heme iron, Fe$\beta$ (6–8), which is probably ligated by the three conserved histidine residues that serve as ligands to Cu$_b$ in cytochrome-c oxidase. A fourth metal center, a covalently bound low spin heme $c$ with histidine and methionine axial ligands, is bound by the NorC subunit. This site has no structural counterpart in cytochrome-c oxidase but is functionally equivalent to Cu$_A$ in that it serves as a site of electron input for the respiratory complex.

Recent NOR studies have addressed the nature of the catalytic site through site-directed mutagenesis (9), redox potentiometry (8), and ligand binding studies (6). The redox potentiometry revealed that the high spin heme $b_3$ of the dinuclear center had a surprisingly low midpoint redox potential ($E_{\text{m}(\text{pH } 7.6)} = +60$ mV) (8), which imposes a large thermodynamic barrier to reduction by the low spin electron transferring heme $c$ ($E_{\text{m}(\text{pH } 7.6)} = +310$ mV) and heme $b$ ($E_{\text{m}(\text{pH } 7.6)} = +345$ mV). This may be a means by which reduction of the heme $b_3$ is avoided to prevent forming a potentially dead-end ferrous heme-nitrosyl species with NO (8). It has also been noted that reduction of the Fe$\beta$ in the dinuclear center, which occurs when three electrons are introduced to the enzyme, results in a shift in the absorption maximum of the ligand to metal charge-transfer (CT) band associated with the high spin ferric heme $b_3$ from ~595 to 605 nm (8). Taken together with recent resonance Raman studies of NOR (10), this observation can be accounted for by a change in the ligation from a $\mu$-oxo bridged dinuclear center in which there is no proximal ligand to the ferric heme $b_3$ to a form of the ferric heme $b_3$ with a proximal histidine ligand and an anionic distal ligand (8).

There is currently no agreement on a model for the catalytic cycle of NOR. However, it must involve the transfer of two protons and two electrons to the active site as these are re-
required for the reduction of two NO molecules to N₂O and H₂O (Reaction 1). It is now generally agreed that these protons are moved to the active site from the periplasm (3). In membranes this overall process is non-electrogenic because the electrons are also derived from donors located in the periplasm. A possible proton-conducting pathway that involves one or more conserved glutamate residues has emerged from site-specific mutagenesis of NOR (9).

Two recent NOR studies have reported that the spectroscopic properties of the fully oxidized enzyme are not significantly affected by pH (10, 11). These observations are perhaps surprising given the requirement of proton uptake to the dinuclear center for NO reduction. This work reports an electronic absorption and magnetic circular dichroism (MCD) spectroscopic study of NorCB from P. denitrificans at a range of pH and redox states. This has led to the identification of three spectrally distinct forms of the ferric heme b₃, which may reflect μ-oxo bridged, hydroxide-bound, and water-bound species. The latter two species are only observed in the three electron-reduced enzyme and are pH-dependent. The results may help us to elucidate the catalytic cycle of NOR.

**EXPERIMENTAL PROCEDURES**

The source of the NOR used in this study was P. denitrificans strain 93.11 (ΔactD, ΔactDII quoxB::kan) (12) grown in batch culture in minimal medium under anaerobic denitrifying conditions. The two-subunit form of the enzyme was purified essentially as described by Grönberg et al. (8).

Electronic absorbance spectra were recorded either on an Aminco DW2000 spectrophotometer or a Hitachi U3000 spectrophotometer. Room temperature magnetic circular dichroism (RT-MCD) spectra were recorded on a Jasco J-500D circular dichrograph. An Oxford Instruments super-conducting solenoid with a 25-mm room temperature bore was used to generate magnetic fields of up to 6 tesla. MCD spectral intensities depend linearly on the magnetic field at room temperature and are expressed per unit magnetic field as Δε/H (m⁻¹ cm⁻¹ tesla⁻¹).

Mediated equilibrium redox titrations of NOR were done at 20 °C in 20 mM bis-Tris propane (BTP) supplemented with 0.05% (w/v) dodecyl maltoside, 0.5 mM EDTA, and 340 mM NaCl and adjusted to the required pH. The methodology was essentially as that described by Dutton (13). Dithionite was used as the reductant and potassium ferricyanide as the oxidant. The redox mediators, each at a final concentration of 10 μM, were phenazine methosulfate (PMS), phenazine ethosulfate (PES), 5-anthraquinone 2-sulfonate, 6-anthraquinone 2,6-disulfonate, and benzylviologen. A solution of saturated quinhydrone at pH 7 was used as a redox standard (E° = +295 mV). All potentials quoted are with respect to the standard hydrogen electrode.

Preparation of three electron-reduced NOR under the control of a potentiostat was achieved using a three-electrode cell configuration with a closed sample compartment thermostated at 4 °C. All manipulations were performed in an anaerobic chamber (N₂ atmosphere with O₂ at less than 2 ppm). A 200-μl sample of 25 μM NOR in 20 mM BTP, 0.05% (w/v) dodecyl maltoside, 0.34 mM NaCl, and 0.5 mM EDTA at the desired pH and supplemented with a mixture of redox mediators, which included ferricyanide, PES, PMS, and 2,6-dimethylbenzazquinone, was placed in a glassy carbon pot that provided the working electrode. A Ag/AgCl reference electrode and a platinum foil counter electrode contacted the solution through a Luggin tip and Vycor frit, respectively.

**FIG. 1. Effects of pH and redox state on visible absorption spectra of NOR.** Spectra were recorded during mediated potentiometric titrations. Panels A–D, dashed line, fully oxidized sample; dotted line, three electron-reduced sample; solid line, fully reduced sample. Panel A, recorded at pH 6.5. Panel B, recorded at pH 7. Panel C, recorded at pH 7.5. Panel D, recorded at pH 8.5. Spectra were recorded on samples of NOR at concentrations of between 10 and 30 μM, buffered in BTP supplemented with 0.34 mM NaCl, 0.5 mM EDTA, and 0.05% dodecyl maltoside.

**FIG. 2. Effects of pH on the CT band of NOR in the three electron-reduced enzyme.** Difference spectra were taken of the (three electron-reduced) – (fully reduced) samples. Panel A, recorded at pH 6.5. Panel B, recorded at pH 7. Panel C, recorded at pH 7.5. Panel D, recorded at pH 8.5. Spectra were recorded on samples of NOR at concentrations of between 10 and 30 μM, buffered in BTP supplemented with 0.34 mM NaCl, 0.5 mM EDTA, and 0.05% dodecyl maltoside.
Spectrally Distinct Forms of Bacterial NOR

| Ligand set          | Protein derivative* | Absorption peak | RT-MCD trough | References |
|---------------------|---------------------|-----------------|---------------|------------|
| Histidine/H₂O       | HH & SW Mb, Lb      | 626–631         | 635–646       | 17–22      |
| Histidine/HO⁻        | HH & SW Mb          | 605–620         | 618–622       | 17, 19, 24 |
| Histidine/F⁻         | HRP, SW & HH Mb,    | 600             | 618           | 17         |
| Histidine/Tyr⁻       | SW Mb H₆Y           | 600             | 634–645       | 26, 27     |
| Histidine/RCOO⁻      | Mb + HCOO⁻ + H₂COO⁻ | 638–645         | 658–663       | 17, 24, 28, 29 |
| Histidine/N₃⁻        | HRP, c', BrCN-Mb    | ~630            | ~640          | 19, 23     |
| Histidine/N₅⁻        | SW & HH Mb          | ~630            | ~640          |            |

* Mb, myoglobin; HH, horse heart; SW, sperm whale; Lb, soybean leghemoglobin; HRP, horse radish peroxidase; c', cytochrome c'.

In multiheme proteins the positive part of this MCD feature is often obscured by adjacent low spin heme bands; the wavelength of the intensity minimum to longer wavelengths has been quoted.

RESULTS AND DISCUSSION

Samples of *P. denitrificans* NOR were equilibrated at pH 6.5, 7.0, 7.5, 8.0, and 8.5 in BTP buffer. Samples were poised at a range of potentials between +400 and 0 mV and absorbance spectra were collected in the range of 500 to 700 nm. At each pH value the oxidized (E = +400 mV) spectrum showed the characteristic α/β band absorption features of low spin ferric hemes in the region 520–570 nm (Fig. 1). These features have previously been assigned to the histidine/methionine-ligated α-heme of the NorC subunit and the bis-histidine-ligated β heme of the NorB subunit (14). Each sample also exhibited a clearly resolved absorption shoulder at ~595 nm. The λ_max and intensity of this band was not significantly affected by the pH of the bulk phase. This 595-nm feature arises from a CT band associated with ferric high spin heme b₃, which has no proximal ligand but has a distal μ-oxo bridge to the Fe₆ (10, 14). Complete reduction (E = −0 mV) of NOR at each pH led to the appearance of the intense absorption peaks at 550 and 560 nm that arise from reduction of the low spin heme α (550 nm) and heme b (560 nm) (Fig. 1). The heme b₃ 595 nm CT band disappears on reduction. Again, analysis of the reduced spectrum at each pH value revealed no substantial differences in the λ_max and intensity of the absorption peaks.

In an earlier study of NOR (8) carried out in Tris-HCl buffer at pH 7.6, it was demonstrated that lowering the potential of the NOR sample buffer from approximately +400 to +150 mV resulted in the reduction of the low spin hemes (heme b and heme c) and the non-heme iron (Fe₆). In the resulting three-electron-reduced species the λ_max of the heme b₃ CT band shifts from 595 to −605 nm, and the extinction coefficient decreases. Thus, reduction of the Fe₆ in the dinuclear center resulted in a chiange in the coordination environment of the heme b₃. On the basis of MCD analysis and taking into account recent resonance Raman studies (10), this coordination change is likely to be a rebinding of the proximal histidine and the breaking of the μ-oxo bridge to form a His/anion species.

In the present study the three-electron-reduced state of the enzyme has been investigated at a range of pH values in BTP buffer, which allows examination over a broad range of pH values and temperatures. Chemical poising of samples at +150 mV led to the reduction of the two low spin hemes and the Fe₆, whereas the high spin heme b₃ remained oxidized. Analysis of these samples revealed that the absorption intensity of the red-shifted CT band is strongly affected by pH. The CT band is least intense at pH 6.5 and most intense at pH 8.5 (Fig. 1, A and D). This suggests that the form of the heme b₃ present at pH 8.5 is different from that present at pH 6.5. Examination of (three-electron-reduced) – (fully reduced) difference spectra (Fig. 2) shows that at pH 8.5 the CT band is positioned at 605 nm. At pH 7.5 the heme b₃ appears to in a mixed form, with one population exhibiting a 605-nm CT band and a second population exhibiting a 635-nm CT band. At pH 6.5 the 635-nm form dominates the spectrum. From these data, molar extinction coefficients of ε₆00–700 or ε₇00–850 as 3.66 mm⁻¹ cm⁻¹ and 1.18 mm⁻¹ cm⁻¹ were calculated for the 605 and 635-nm bands, respectively. These data suggest that at different pH values the ferric heme b₃ in the three-electron-reduced enzyme has a different distal ligand.

High spin ferric hemes give two characteristic CT bands that produce derivative-shaped features in the MCD spectrum (15, 16). The higher energy band is seen in the 600–700-nm region, and its precise wavelength is sensitive to the nature of the axial ligands (Table 1). RT-MCD studies have been successfully used to assign the exogenous-bound distal ligand of histidine-ligated
high spin ferric heme of *E. coli* cytochrome *bo*₃ (30). Thus, this approach was exploited to determine the likely nature of the distal ligand to NOR heme *b*₃ samples buffered at pH 6.0 and 8.5. Samples were poised electrochemically at +150 mV and examined by electronic absorption and RT-MCD spectroscopy. Electrochemical poising allowed the preparation in a small volume of the concentrated samples required for MCD analysis. The electronic absorption spectra clearly show that at pH 6.0 the heme *b*₃ is in a pure 635-nm form and at pH 8.5 in a pure 605-nm form (Fig. 3A, inset). The RT-MCD spectra show peaks in the α, β, and Soret regions dominated by signals from low spin ferrous heme. CT bands associated with the high spin ferric heme *b*₃ are seen in the 600–650-nm region of the RT-MCD spectrum. Analysis of this region shows a band at 620 nm in the pH 8.5 sample, which is characteristic of high spin heme with a proximal histidine and a distal hydroxide (His/OH⁻). In the pH 6.0 sample this band is red-shifted to 640 nm, a position characteristic of His/H₂O-ligated high spin ferric heme (Table I). This analysis is in good agreement with the UV-visible spectroscopic analysis of the three electron-reduced NOR generated by chemical reduction and confirms that the ligand change is not an artifact of reduction with sodium dithionite. Moreover, these data suggest that direct ligation of the heme *b*₃ is dependent on both the redox state of the dinuclear center and on pH. In the oxidized form the heme *b*₃ and non-heme Fe₇₉ are bridged by a μ-oxo group. As the non-heme Fe₇₉ is reduced, the heme *b*₃ becomes ligated by hydroxide at high pH levels (~8.5) and by water at low pH levels (~6). Recent evidence from a study of carbon monoxide binding to the fully reduced (four electron-reduced) NOR suggests that these coordination states are retained by ferrous heme *b*₄ (31).

Quantitative analysis of the dependence of the 605- and 635-nm bands on the potential at pH 7 to obtain *E°* for each redox species is made difficult by the low intensities and overlapping nature of these bands. In the fully oxidized enzyme (Fig. 4, —) the CT band is clearly positioned at 596 nm. As the dinuclear center starts to become reduced, the 595-nm band begins to decrease in intensity. At +250 mV (Fig. 4, —–) it has decreased to approximately half its original intensity, and a new band has appeared centered at 635 nm. This movement in the CT band represents a change from a μ-oxo bridged species to one where the heme *b*₃ is ligated by His/H₂O. The next phase of reduction from +250 mV to +140 mV (Fig. 4, ——) causes the 595-nm band to further decrease in intensity. However, this further decrease does not correspond to a further increase in the 635-nm band but to the appearance of another new band at 605 nm. This represents the formation of a His/OH⁻-ligated heme *b*₃ from the μ-oxo bridged species. During the remainder of the reduction of the dinuclear center the 605 and 635-nm bands titrate at similar potentials and have completely disappeared by +22 mV with the full reduction of the heme *b*₃ (Fig. 4, ———). This three-phase reduction of the dinuclear center can be clearly seen when fitting the change in absorbance of the 595-nm band against potential (Fig. 5B). The data can be fitted to three *n* = 1 Nernst components. The first phase corresponds to the breaking of the μ-oxo bridge, between the Fe₇₉ and the heme *b*₃ and the associated shift of the 595-nm band to 635 nm, to form the His/H₂O-ligated heme *b*₃. This rearrangement of ligation within the dinuclear center has an *E°* of +325 mV. The second phase represents a change in the dinuclear center from the μ-oxo bridged species to one where the heme *b*₃ is His/OH⁻-ligated and is associated with the shift in the 595-nm band to 605 nm. This phase has an *E°* of +240 mV. The final phase corresponds to the full reduction of both species from a ferric heme *b*₃ to a ferrous heme *b*₃, where the shoulder of the 605 and 635 bands detected at 595 nm disappear. Both processes occur at isopotentials where the *E°* is +50 mV (Fig. 6). By contrast, at pH 8.5, where only a single species is present (His/OH⁻) in the three electron-reduced form, a simple two-phase titer is seen (Fig. 5B). The first phase represents the...
**Spectrally Distinct Forms of Bacterial NOR**

Having identified three spectral forms of the ferric heme \( b_3 \), it is possible to forward tentative suggestions as to both their origin and their role in possible catalytic cycles of the enzyme. At present there is no agreement on the mechanism of NO reduction by NOR. Arguments have been put forward for the two substrate NO molecules binding to FeB in the so-called cis model (2). An alternative trans mechanism where one NO molecule is bound by each of FeB and FeC has also been forwarded (2). However, in both cases it is likely that at some point in the catalytic cycle a ferric heme \( b_3 \) is bound by a water molecule. The identification of the 635-nm spectral form of heme \( b_3 \) is the first time such a species has been identified. It can be supposed that this can be derived from the reduction of a \( \mu \)-oxo bridged FeB(II)-FeC(III) dinuclear center, which would correlate to the 595-nm form of oxidized NOR. It then seems plausible that on reduction of the FeB of the dinuclear center with one electron a proton will also enter the catalytic site leading to a hydroxide-bound ferric heme \( b_3 \) (Fig. 6). MCD studies of this spectral form of heme \( b_3 \) are consistent with this assertion. There are many examples of high spin hemes that exhibit CT bands at \(-630 \text{ nm} \) and these characteristically arise from ferric hemes with His-H\_2O coordination (e.g. in *E. coli* cytochrome-b\(_{595}\) oxidase (16)). Thus it seems most likely that the low pH 635-nm form of heme \( b_3 \) arises from a simple protonation of the putative hydroxide-bound high pH 605-nm form (Fig. 6). It is also important to note that experiments in our laboratory have shown that NOR activity in BTP buffer at pH 6.0 is \( 8\)-fold higher \((70 \text{ s}^{-1}) \) than at pH 8.5 \((-8 \text{ s}^{-1}) \) (data not shown). The acidic nature of this optimal activity is in agreement with previously published data obtained in various buffers (32, 33). At this pH the His-H\_2O form of the enzyme dominates. The higher midpoint redox potential of this species than that of the His-hydroxide form may allow more rapid electron transfer from physiological electron donors such as cytochrome c and pseudooxazirin.

Having identified spectral signals that might correspond to distinct intermediates in the catalytic cycle of NOR, it is now necessary to try to trap these spectral forms in rapid-reaction experiments. Such experiments may in turn provide further insight into the \( \text{N}_2\text{O} \)-generating half of the catalytic cycle.

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