Structural basis for nitrous oxide generation by bacterial nitric oxide reductases

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The crystal structure of the bacterial nitric oxide reductase (cNOR) from Pseudomonas aeruginosa is reported. Its overall structure is similar to those of the main subunit of aerobic and micro-aerobic cytochrome oxidases (COXs), in agreement with the hypothesis that all these enzymes are members of the haem-copper oxidase superfamily. However, substantial structural differences between cNOR and COX are observed in the catalytic centre and the delivery pathway of the catalytic protons, which should be reflected in functional differences between these respiratory enzymes. On the basis of the cNOR structure, we propose a possible reaction mechanism of nitric oxide reduction to nitrous oxide as a working hypothesis.

Keywords: nitric oxide reductase; denitrification; nitrous oxide generation; haem; non-haem iron; haem-copper oxidase superfamily

1. INTRODUCTION

Nitric oxide reductase (NOR) is an iron-containing enzyme and catalyses the reduction of nitric oxide (NO) to nitrous oxide (N₂O), according to the following equation:

\[ 2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow 2\text{N}_2\text{O} + \text{H}_2\text{O}. \] (1.1)

NOR is often involved in the denitrification pathways of micro-organisms, one type of anaerobic respiration, in which nitrogen oxide compounds such as nitrate (NO₃⁻) and nitrite (NO₂⁻) are converted into dinitrogen (N₂). NO is produced as an intermediate in the denitrification process, but, owing to its high cytotoxicity, it is rapidly decomposed into N₂O by the NOR enzyme immediately on production. However, some pathogenic bacteria have NOR for the detoxification of NO produced by the NO synthase of the host’s defence system [1–4]. Therefore it has been interesting to study the chemistry of biological NO reduction leading to N₂O generation by NOR in micro-organisms.

N₂O (the so-called laughing gas), the product of the NOR enzymatic reaction, is a greenhouse gas that is 310 times more powerful than carbon dioxide which also contributes to ozone depletion in the stratosphere. It is now realized that N₂O generation by micro-organisms accounts for a large proportion of the total N₂O emissions on Earth [5]. From an environmental point of view, the molecular mechanism of biological N₂O generation should be elucidated on the basis of the structure of the NOR enzymes.

NOR enzymes have been identified in two types of micro-organisms: in fungi and in bacteria. Although both enzymes catalyse the same chemical reaction (equation 1.1), the protein architectures are completely different from each other. Fungal NOR is a water-soluble enzyme containing one haem iron at its active site; its overall and active site (haem-Cys) structures are the same as those of the monooxygenase cytochrome P450 [6]. In contrast, bacterial NOR is an integral membrane enzyme that contains a binuclear centre consisting of one haem iron and one non-haem iron as its active site. As a result of these structural differences, we expect different mechanisms of NO reduction (N₂O production) for these two systems. In this study, we focus mainly on the molecular structure and function of the bacterial system.

Since 1971 the NO reduction activities of membrane-bound NOR have been reported for many bacteria, such as Alcaligenes faecalis (IAM 1015), Pseudomonas stutzeri, Rhodobacter sphaeroides (IL 106), Halomonas halodenitrificans and Paracoccus denitrificans and so on. Three types of bacterial NORs have been identified: cNOR, qNOR and qCuA-NOR of which the first to be isolated and the most extensively studied was a cytochrome c-dependent NOR (cNOR) which consists of two subunits, NorB and NorC [7–19]. Since the first cNOR was isolated from P. stutzeri, several cNORs from other bacteria have been purified and characterized using biochemical, molecular biological, spectroscopic and chemical techniques to elucidate their structural
and functional properties [18,20–27]. The NorC subunit of cNOR has a haem c with His and Met as the axial ligands, which act as an electron acceptor from an external electron donor (either cytochrome c551 or azurin). The larger subunit of cNOR, NorB, contains three iron (Fe) centres, i.e. two b-type haems and one non-haem Fe (FeB). The low spin b-type haem (haem b) mediates electron transfer from the haem c in the NorC subunit to the binuclear centre which is the active site of cNOR consisting of another b-type haem (haem b3) and FeB.

In 1994, NOR was identified as a member of the haem-copper oxidase (HCO) superfamily [28] on the basis of the similarity of the primary sequence of the NorB subunit of cNOR to the catalytic subunit of cytochrome oxidases (COXs), aerobic respiratory enzymes catalysing O2 reduction (O2 + 4H+ + 4e− → 2H2O). NOR is now believed to share a common ancestor with COX. Considering the molecular evolution of respiratory enzymes after the emergence of O2 on Earth ca 3 billion years ago, part of the ancient respiratory enzyme could have been converted into the NO-reducing enzyme NOR under anaerobic conditions, while another could have been functionally converted into the O2-reducing enzyme COX through several structural modifications.

In 2010, we succeeded in determining the crystal structure of Pseudomonas aeruginosa cNOR in the resting state [29]. This structure is comparable with other cNORs because the amino acid sequence of its NorB subunit has 75 per cent and 94 per cent homology, respectively, with the cNORs of P. denitrificans and P. stutzeri, both of which have been extensively studied. This has provided us with a structural basis for understanding the molecular mechanism of catalytic N2O generation, and the molecular evolution of the respiratory enzyme.

2. STRUCTURAL DESCRIPTION OF PSEUDOMONAS AERUGINOSA CRYSTAL NITRIC OXIDE REDUCTASE

(a) Overall structure

The crystal structure of P. aeruginosa cNOR revealed that the NorB subunit contains 12 transmembrane (TM) α-helices, and has three iron: one in each of the haem b and haem b3, and one as a non-haem iron, FeB, in the TM region (figure 1a). The NorC subunit

Figure 1. Crystal structure of Pseudomonas aeruginosa cytochrome c-dependent nitric oxide reductase (cNOR). (a) Overall structure, (b) binding site of Fab to cNOR, (c) possible electron transfer pathway, and (d) possible proton transfer pathway.
has one $\alpha$-helix in the TM region and one hydrophilic domain typical of a cytochrome $c$-fold in the periplasmic side. The TM helix of NorC interacts with some helices of the NorB subunit. The topology of the helices of the TM region and the arrangement of the metal centres in cNOR are very similar to those of COX, consistent with the notion that NOR and COX have a common evolutionary progenitor. A comparison of the overall structures and reaction centres of NOR and COX provides a key for considering the evolution of domain architecture and the functional conversion of respiratory enzymes between the NO reduction in anaerobic respiration and the $O_2$ reduction in aerobic respiration.

The arrangement of TM helices shows strong similarity between NOR and COXs. For example, the interaction of TM helices between NorB and NorC in cNOR is very similar to the interaction between N and O subunits observed in the $cbb_3$ type of COX from $P.\ stutzeri$ [30]. The $cbb_3$ COX also contains a hydrophilic region with a haem $c$ in the O subunit, although another large hydrophilic subunit (P subunit) containing multiple haem $c$ moieties is associated with the other subunits. Because $cbb_3$ COX has low NO to $N_2O$ reducing activity, it is thought to be evolutionarily the closest to NOR. The $cbb_3$ COX is found in many pathogenic proteobacteria that colonize microaerobic host tissues and in diazotrophs that can simultaneously perform aerobic respiration and nitrogen fixation.

The protein folds and metal species of the hydrophilic region in the periplasmic side differ significantly among members of the HCO superfamily. In contrast to the cytochrome $c$-fold in cNOR and $cbb_3$ COX, the cupredoxin fold is found in the hydrophilic region in $ba_3$ and $aa_3$ COXs, and shows that the two copper ions (CuA site) in this region play an important role in electron transfer [31–35]. The $bo_3$ COX also shows a cupredoxin fold but lacks a metal centre in the hydrophilic region, because the physiological electron donor is not a protein but a small compound (ubiquinol) located inside the membrane [36].

### (b) Active site structure

Two His residues (His60 and His349) serve as ligands of one of the $b$-type haems (figure 1c). Haem $b$ is thought to receive electrons from haem $c$ and transfer them to the binuclear catalytic centre of cNOR, which comprises non-haem iron FeB and haem $b_3$ (figure 2). While His347 is the ligand for haem $b_3$, the three His residues His207, His258 and His259 together with Glu211 are ligands of the non-haem iron FeB. Significant electron density is observed between haem $b_3$ iron and FeB, and as assigned as one oxygen atom ($\mu$-oxo-bridge), because cNOR was crystallized in the ferric form in the resting state [26]. The FeB site has a slightly distorted trigonal–bipyramidal coordination geometry that differs from the geometry observed for the CuB site in COX. In the CuB of COX, the position occupied by Glu211 in cNOR is occupied by a His (His233) that is covalently attached to a highly conserved Tyr residue (Tyr237) of $T.\ thermophilus$ $ba_3$ COX. Glu215 and Glu280, both of which are also conserved Glu residues in NORs, are present near the binuclear centre, indicating a highly electronegative environment at the active site, which is consistent with the prediction based on a resonance Raman study [25].

The carboxylate of Glu280 also interacts with the FeB-coordinated Glu211 through hydrogen bonding.

The electronegative environment of the binuclear pocket of NOR is responsible for the redox potential of the haem $b_3$ iron (60 mV), which is low in comparison with those of the irons of haems $b$ (345 mV) and $c$ (310 mV), and this environment then contributes to activation of the NO molecule bound to the haem $b_3$ and FeB binuclear centre [18]. In addition, the hydrogen-bonding network, including the three Glu residues, can serve as a terminal proton-donating system to facilitate N–O bond cleavage in $N_2O$ and $H_2O$ production.

The open space near the catalytic binuclear centre for the NO binding in cNOR is slightly larger than that observed in COXs. However, when the distance between the two irons of cNOR (3.8 Å) is compared with that observed between haem $a_3$ iron and CuB in COX (4.4 Å), it is not enough to accommodate two NO molecules in cNOR. In oxidases, one $O_2$ molecule binds to the haem iron. The ligation of Glu211 to FeB might be one of the structural reasons for such a crowded environment. Therefore, in the catalytic
cycle of cNOR, some conformational changes at the binuclear centre and its protein side chain ligands must take place in order to position two NO molecules suitably for N–N bond formation. Experimental and theoretical analyses of the cNOR structure in several oxidation and ligand-coordination states are necessary to understand fully the reaction mechanism of NOR at the atomic level.

(c) Electron transfer pathway (including calcium binding)

The electrons used in NO reduction are donated from electron donors (cytochrome \(c_{551}\) or azurin) to haem \(c\) in the hydrophilic (globular) domain of the NorC subunit. The haem \(c\) has His65 and Met112 as axial iron ligands (figure 1c). The electrons are then transferred to the catalytic binuclear centre (haem \(b_1\) and FeB) via haem \(b\). The iron-to-iron distance between haems \(c\) and \(b\) in cNOR is 20.3 Å and the distance between haems \(b\) and \(b_1\) in cNOR is 14.1 Å. These distances are comparable with that between the CuA site and \(b\)-propionates of the two haems of cNOR. This density was also reported in \(cbb_3\) COX [30], but the equivalent position in other COXs is occupied by the positively charged side chains of two conserved Arg residues (Arg449 and Arg450 in \(ba_3\) COX) [31–36]. The distance between two propionate ligands of the calcium in cNOR is as long as that in \(cbb_3\) COX. Possible functions of the \(Ca^{2+}\) ion in cNOR might be stabilization of the interaction between NorB and NorC and/or control of electron transfer between the haems.

The Fab dramatically inhibited the NO-reducing activity of cNOR when cytochrome \(c_{551}\) or azurin was used as an electron donor, whereas the reducing compound phenazine methosulphate (PMS) was not affected. Because PMS can directly donate an electron to either haem \(c\) or the catalytic binuclear centre of cNOR, these data indicate that the Fab-binding site on the surface of NorC hydrophilic domain overlaps with the interaction site of the binuclear centre to the surface in the membrane-spanning region was identified (fig. 3a,b of Hino et al. [29]). Because the location of the channel appears to be similar to that of the O\(_2\) channel of COX, in cNOR this channel could provide a means of NO entry and \(N_2O\) exit. However, it was notable that neither an obvious channel nor a hydrogen-bonding network from the active site of cNOR to the cytoplasmic region was identified, in sharp contrast to the K- and the D-channels of COX, which are considered to be possible proton transfer channels.

(d) Proton transfer pathway

Protons must be transferred from bulk water to the buried active site to facilitate N–O bond cleavage of the transient species to produce \(N_2\) and \(H_2O\). Probably the proton transfer would be through a water channel and a hydrogen-bonding network. In the \(P\ aeruginosa\) cNOR structure, we identified two channels extending from the NorB–NorC interface to the periplasmic side of the enzyme (figure 1d).

In addition, a hydrophobic channel extending from the binuclear centre to the surface in the membrane-spanning region was identified (fig. 3a,b of Hino et al. [29]). Because the location of the channel appears to be similar to that of the \(O_2\) channel of COX, in cNOR this channel could provide a means of NO entry and \(N_2O\) exit. However, it was notable that neither an obvious channel nor a hydrogen-bonding network from the active site of cNOR to the cytoplasmic region was identified, in sharp contrast to the K- and the D-channels of COX, which are considered to be possible proton transfer channels.

Extensive evidence has been obtained from biochemical, biochemical and flow-flash kinetic data, all of which unambiguously indicate that electrons and protons are supplied from the periplasmic space and that NOR reaction is non-electrogenic [37–39]. Therefore, we propose that the channels and hydrogen-bonding network identified in the \(P\ aeruginosa\) cNOR structure serve as the pathway for proton transfer in the catalytic reduction of NO at the binuclear centre. Asp198 (NorB) and Glu57 (NorC) on the protein surface are probably the proton entry site, because both acidic residues are highly conserved in NORs. The proton pathway is expected to be end at the FeB ligand Glu211 that is involved in the hydrogen bond network adjacent to the binuclear centre as described in §2c. However, we consider that the observed distance between the propionate of haem \(b_3\) and Thr330 is too far (8.0 Å) for direct connection. Thus, hydrogen-bonding networks do not completely connect the bulk water region and the catalytic centre of the enzyme. Note that one or two water molecules in the obvious space between Thr330 and the haem propionate would facilitate the formation of the channel and network extending from the periplasmic side to the binuclear centre via Glu211 and Glu280 [24,40–43].

In contrast, there is no connection via a hydrogen bond between the cytoplasm and binuclear centre, and no proton can permeate from the cytoplasm to the periplasm. The observation that the pathway corresponding to the K-pathway in COX is blocked by many hydrophobic residues is consistent with the absence of proton pumping ability of cNOR. However,
it should also be noted that near the terminal proton donor Glu211, cNOR contains a small charged region generated by Glu215, Glu280 and water. This region partly overlaps with the K-pathway of COX. It appears that this charged region of cNOR might reflect an evolutionary step to acquiring the proton delivery pathway from the cytoplasm to the binuclear centre as in COX.

3. MOLECULAR MECHANISM OF NO REDUCTION BY NOR

(a) Reaction mechanism of fungal nitric oxide reductase

It is chemically interesting to elucidate how the N–N bond is formed and how the N–O bond is cleaved in NO reduction (\( \text{N}_2\text{O} \) generation) catalysed by NORs. Prior to discussion of the bacterial system, the fungal system is briefly discussed [44,45]. In contrast to the bacterial NOR, only one haem iron is contained at the active site of the fungal NOR. The haem has the Cys thiolate as the fifth axial ligand of the iron [6], similar to that in monoxygenase cytochrome P450, so that the fungal NOR is sometimes designated as P450nor. In the first step of the catalytic reaction of the fungal system, one NO molecule binds to the ferric haem iron as the sixth ligand to form the stable and first intermediate. Then, the ferric NO complex of P450nor is reduced with two electrons (2e\(^{-}\)) and one proton (H\(^+\)) by direct hydride (H\(^-\)) transfer from the nicotinamide group of NAD(P)H bound to the haem pocket of the enzyme [46]. Eventually, the second and short-lived intermediate is produced, which is in the two-electron-reduced state of the ferric NO complex ([Fe\(^{3+}\)–NO\(^2-\)–H\(^+\)]). The most important finding was that this characteristic intermediate is never generated by the reduction of dithionite (one-electron reducing agent), and dithionite cannot work as the electron donor of fungal NO reduction, suggesting that the H\(^+\) transfer is essential in fungal NO reduction. Upon reaction of the intermediate with a second NO molecule, the N–N bond formed and the N–O bond cleaved with H\(^+\) transferred from bulk water. In other words, in fungal NO reduction, one NO molecule is activated on the haem iron site with two electrons, and reacts with a second NO to give N\(_2\)O, i.e. the so-called donor–acceptor type reaction occurs: (NO)\(^2-\) + NO → hyponitrite + 2H\(^+\) → N\(_2\)O + H\(_2\)O.  

(b) Reaction intermediate in bacterial nitric oxide reduction

In contrast to the fungal system, the active site of bacterial NOR with a binuclear centre comprises two iron forms (haem \( b_3 \) and non-haem Fe\(_B\)), in which the fifth axial ligand of the haem \( b_3 \) iron is the His imidazole, as in myoglobin. Electrons are supplied to the binuclear centre from an exogenous electron donor (azurin or cytchrome c\(_{551}\)) through haem c in the NorC subunit and then the haem \( b \) iron (figure 1). In electron transfer, it is noted that each iron in the binuclear centre could be reduced in a step-by-step manner with one electron, so the two-electron reduced state of the ferric NO haem \( b_3 \) complex would never be generated, unlike the situation in fungal NOR (P450nor). Therefore, a different mechanism might be expected for NO reduction by bacterial NOR.

Thus far, three possible mechanisms of NO reduction by bacterial NOR have been proposed with respect to the coordination and electronic structures of the reaction intermediate, as illustrated in figure 3. In the \( \text{trans} \)-mechanism, the haem \( b_3 \) iron and Fe\(_B\) each bind one NO molecule to form an Fe\(^{2+}\)–NO dimer intermediate. Rapid-freezing electron paramagnetic resonance (EPR) spectroscopic studies on \( P. \text{aeruginosa} \) cNOR detected the haem–Fe\(^{2+}\)–NO and FeB–NO species, which supports the \( \text{trans} \)-mechanism (see §3c). The vibrational spectroscopic characterization of CO-bound qCu\(_m\)NOR is also consistent with the \( \text{trans} \)-mechanism [47,48]. A NOR model complex prepared by Collman et al. [49] that can bind NO to both haem Fe\(^{2+}\) and non-haem Fe\(_B\)^\(3+\) reacts with two equivalents of NO, leading to the formation of one equivalent of N\(_2\)O. These results are consistent with the \( \text{trans} \)-mechanism. In contrast, however, another haem Fe\(^{2+}\)–NO/Fe\(_B\)^\(3+\)–NO model complex is thermally stable and does not seem to react further to give N\(_2\)O upon addition of a proton source [50].

Two other mechanisms, the so-called \( \text{cis} \)-mechanisms, are proposed in which two NO molecules bind to either haem \( b_3 \) iron or Fe\(_B\). Thomson and co-workers [51–53] favour the \( \text{cis} \)-Fe\(_B\)-mechanism because this model has a vacant haem \( b_3 \) site. A rather stable \{Fe\(_{\text{NO}}\)\]^\(7\) species, a potential ‘dead-end’ product, is therefore not formed during the turnover. In the \( \text{cis} \)-haem \( b_3 \) mechanism, the first NO molecule binds to haem \( b_3 \) to form a \{Fe\(_{\text{NO}}\)\]^\(7\) species which is then electrophilically attacked by the second NO molecule [48]. The mechanism looks analogous to, but is not the same as that proposed for fungal NOR (P450nor). In the case of P450nor, the key step is a formation of the short-lived intermediate \{Fe\(_{\text{NO}}\)\]^\(8\) by two-electron (H\(^+\)) reduction of the ferric haem–NO complex, while the one-electron reduced form \{Fe\(_{\text{NO}}\)\]^\(7\) of P450nor never reacted with another NO.

(c) Rapid-freezing EPR studies on the reaction of bacterial nitric oxide reductase with nitric oxide

To examine the molecular mechanism of catalytic N\(_2\)O formation at the binuclear centre of cNOR, we
followed the reaction of fully reduced P. aeruginosa enzyme with NO using rapid mixing-and-quenching techniques [16]. The sample obtained 1 ms after mixing the enzyme and NO gave an ESR spectrum in which a $g = 2$ signal with a three line hyperfine splitting from $^{14}$N and a $g = 4.3$ signal were observed. The former signal originates from a five-coordinated haem $b_3$ Fe$^{2+}$–NO complex ($S = 1/2$), and the latter from the FeB$^{2+}$–NO complex ($S = 3/2$). On the basis of this observation, we proposed that two NO molecules could be shared with two irons in the binuclear centre, and that this species might be the reaction intermediate, supporting the trans-mechanism. However, it is important to bear in mind that the presence of two paramagnetic Fe$^{2+}$–NO species in the single binuclear centre might lead to spin-coupling and an ESR-silent species [48,54].

(d) Molecular mechanism proposed on structural basis

The molecular mechanism of bacterial NOR has not yet been established. However, it is noted that in any proposal for NO reduction by bacterial NOR (figure 3), two NO molecules are accommodated by the haem $b_3$ and FeB binuclear centre, in sharp contrast to COX in which one O$_2$ binds to the haem iron. Taking look at the active site structure of P. aeruginosa cNOR (figure 2), it can be seen that the coordination sphere is so crowded and tightly packed that there is no space to accommodate two NO molecules even after dissociation of the bridging O ligand, $\mu$-oxo-bridge (see §2b). The finding unambiguously suggests that some conformational changes are needed for formation of the reaction intermediate by binding of two NO molecules to the binuclear centre.

Moenne-Loccoz [48] suggested that the Glu carbonylate of the FeB ligand, i.e. Glu211 in P. aeruginosa cNOR, might shift to control the coordination of NO at the binuclear centre. The author's suggestion was based on the crystallographic studies of the di-iron-containing enzymes, such as the R2 subunit of Escherichia coli ribonucleotide reductase, methane monoxygenase and so on, leading to the concept of carboxylate shifts controlling the number of open coordination sites and the range of the Fe–Fe distances during catalytic turnover. Another possible structural
change in the binuclear centre of NOR in the catalytic turnover would be dissociation of one of the ligands (His258, His259 or Glu211) from FeB, thus opening space for accommodation of a second NO molecule in the binuclear centre. Non-haem irons having two His and one Glu/Asp ligands are generally observed in nature, in which one of the coordination sites is occupied by a water molecule [55–57]. In addition, in the crystal structure of quercetin 2,3-dioxygenase, which contains copper Cu having 3 His and 1 Glu, both the Glu-bound and unbound forms are concomitantly observed in the Cu$^{2+}$ state [58,59]. This tends to suggest that either the His or Glu residue could be dissociated from the FeB of cNOR during its catalytic turnover. In either case, the FeB coordination sphere in NOR could possibly be opened to accommodate two NO ligands.

Finally, we propose a possible molecular mechanism for bacterial NO reduction as a working hypothesis for future study, which is based on the trans-mechanism (figures 3 and 4).

— In the resting state (I), the haem iron and FeB are bridged by an O ligand, as observed in the crystal structure (figure 2). The $\mu$-oxo-bridged coordination structure is fully consistent with the proposed structure on the basis of the resonance Raman study.

— However, in the single turnover study of cNOR, we observed the strong ESR signal at $g = 6$, suggesting that the haem iron is in a ferric and five-coordinated state. It is likely that the non-bridged structure of the binuclear centre, rather than the $\mu$-oxo-bridged structure, is involved in the catalytic turnover of cNOR. This state can be designated as the fully oxidized state (2).

— The order of the reduction of the two irons and the NO binding, i.e. formation of 3, 4 and 5, is still complicated. However, it has been noted that the Fe$^{3+}$/Fe$^{2+}$ redox potential of FeB ($E_{m} \approx 300$ mV) is much higher than that of haem $b_3$ (60 mV). Consequently, the two NO molecules bound to the binuclear centre, e.g. in the trans-mechanism, are shared by ferrous haem $b_3$ and FeB (5). In this form, the binding of NO to the ferrous FeB might accompany the dissociation of one of the ligands, either His or Glu.

— After electron transfer from the ferrous irons to the bound NOs, the close proximity of two NO molecules on haem $b_3$ and FeB could promote N–N bond formation, producing hyponitrite as a transient species (6).

— As a transient complex, Richardson and co-workers [60] proposed that the hyponitrite can bridge the two irons of haem $b_3$ and FeB. In this hypothetical structure, the Fe–Fe distance must be at least 4.4 Å, that is, the iron must shift by 0.4 Å from their original (fully oxidized state) positions. However, it might be possible that the hyponitrite could reside on the haem $b_3$ coordination site (7), as proposed for the cis-haem $b_3$ mechanism.

— Protons which are transferred from the periplasmic bulk water through the delivery pathway described above could facilitate N–O bond cleavage of the hyponitrite to produce $N_2O$ and $H_2O$. If the Glu211 carboxylate were to dissociate from FeB in the N–N bond formation step, then it might work as a shuttle for catalytic protons from Glu280 to the hyponitrite.

Although the transient species of bacterial NOR that we have proposed above are analogous to those found in the fungal system, their formation must be different. In contrast to the donor–acceptor reaction in fungal NOR (see §3a), NO reduction by bacterial NOR is a so-called ‘disproportionation’ reaction:

$$2(No^-) \rightarrow$ hyponitrite $+ 2H^+ \rightarrow N_2O + H_2O.$$

In summary, we here propose a possible molecular mechanism of $N_2O$ generation catalysed by bacterial NOR, on the basis of its molecular structure. However, there is no direct evidence to verify the proposal. To test some other possibilities, we shall need to determine the crystal structures of the various ligand-bound forms of cNOR in the ferric and ferrous states at higher resolution, and time-resolved spectroscopic study to directly characterize the electronic and coordination structure of the short-lived species.

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