Electron Transfer Complex between Nitrous Oxide Reductase and Cytochrome c_{552} from *Pseudomonas nautica*: Kinetic, Nuclear Magnetic Resonance, and Docking Studies†

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Received February 28, 2008; Revised Manuscript Received August 20, 2008

ABSTRACT: The multicopper enzyme nitrous oxide reductase (N_2OR) catalyzes the final step of denitrification, the two-electron reduction of N_2O to N_2. This enzyme is a functional homodimer containing two different multicopper sites: CuA and CuZ. CuA is a binuclear copper site that transfers electrons to the tetranuclear copper sulfide CuZ, the catalytic site. In this study, *Pseudomonas nautica* cytochrome c_{552} was identified as the physiological electron donor. The kinetic data show differences when physiological and artificial electron donors are compared [cytochrome vs methylviologen (MV)]. In the presence of cytochrome c_{552}, the reaction rate is dependent on the ET reaction and independent of the N_2O concentration. With MV, electron donation is faster than substrate reduction. From the study of cytochrome c_{552} concentration dependence, we estimate the following kinetic parameters: $K_{mc_{552}} = 50.2 \pm 9.0$ μM and $V_{max c_{552}} = 1.8 \pm 0.6$ units/mg. The N_2O concentration dependence indicates a K_{mN_2O} of 14.0 ± 2.9 μM using MV as the electron donor. The pH effect on the kinetic parameters is different when MV or cytochrome c_{552} is used as the electron donor ($pK_a = 6.6$ or 8.3, respectively). The kinetic study also revealed the hydrophobic nature of the interaction, and direct electron transfer studies showed that CuA is the center that receives electrons from the physiological electron donor. The formation of the electron transfer complex was observed by ^{1}H NMR protein−protein titrations and was modeled with a molecular docking program (BiGGER). The proposed docked complexes corroborated the ET studies giving a large number of solutions in which cytochrome c_{552} is placed near a hydrophobic patch located around the CuA center.

Nitrous oxide reductase (N_2OR)[1] is a copper-containing enzyme that catalyzes the two-electron reduction of N_2O to N_2 ($N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$) (*1, 2*), the last step of the denitrification process ($2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2$), which is coupled to ATP generation.

This enzyme has been isolated from several denitrifying bacteria, but only recently has its crystal structure been reported for three different bacterial species, *Pseudomonas nautica* (*Pn*) (*3*), *Paracoccus denitrificans* (*Pd*) (*4*), and *Achromobacter cycloclastes* (*Ac*) (*5*). N_2OR is a functional homodimeric enzyme containing two different multicopper sites: CuA and CuZ. In the functional dimer, the CuA−CuZ distance is approximately 10 Å, while within the monomer, the distance is ~40 Å (*3, 4*), a distance considered to be inappropriate for an efficient electron transfer (*6*).

The electronic structure of the CuA has been extensively studied, while the one of the catalytic site CuZ has only recently been the object of several studies. The CuA site is located in the C-terminal cupredoxin-like domain and is constituted by a binuclear copper site similar to the CuA site from cytochrome oxidases (*1, 7*). In both enzymes, this center is involved in long-range electron transfer due to the low energy of reorganization between the reduced Cu^+-Cu^+ state and the mixed-valence oxidized $Cu^{1.5+}-Cu^{1.5+}$ state. The peculiar spectroscopic and electronic properties of this center have been exhaustively investigated, especially in cytochrome *c* oxidase (*8, 9*).

The catalytic CuZ site is located in the N-terminal domain that is constituted by a seven-blade β-propeller fold. CuZ is a novel mixed-valence copper center (Cu_4S) with a sulfide ion bridging a distorted tetrahedron of copper atoms, in a unique structure in biology. The Cu_4S core is bound to the protein via the N atoms of seven histidine residues. Five histidine residues bind to the copper ions through their Nε2 atoms (His 80, His 128, His 270, His 325, and His 376), whereas two use their Nδ1 atoms (His 79 and His 437) (*3*).

† This research was supported by FCT (Fundação para a Ciência e Tecnologia) Grants PTDC/QUI/64638/2006 (to I.M.), POCI/QUI/57741/2004 (to J.J.G.M.), and SFRH/BD/30414/2006 (to S.D.) and by the University of Pavia through FAR support (to E.M. and L.C.).

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[1] Abbreviations: *Pn*, *Pseudomonas nautica*; *Pd*, *Paracoccus denitrificans*; *Pp*, *Paracoccus pantotrophus*; *Ac*, *Achromobacter cycloclastes*; MV, methylviologen; DT, sodium dithionite; ET, electron transfer; N_2OR, nitrous oxide reductase; HH, horse heart.

10.1021/bi801375q CCC: $40.75
Published on Web 09/20/2008

A water-derived ligand is proposed to bridge the two copper atoms (Cu_I and Cu_{IV}), where the N_2O is proposed to bind.

N_2OR can be isolated in different forms depending on the procedure followed during the purification (*8, 10, 11*). *Pn* N_2OR can be isolated in two forms (*Blue* and *Purple*) (*10*) that differ in the redox state of the CuA center. Both forms are intermediate oxidation states between an oxidized ($Cu^{1.5+}-Cu^{1.5+}$) state and one-electron-reduced ($Cu^{+}-Cu^{+}$) state, but the *Purple* form is more oxidized than the *Blue* form, in which CuA is mainly in the $Cu^{+}-Cu^{+}$ state.

It has been spectroscopically demonstrated that the aerobically isolated *Pn* N_2OR used for the crystallographic study, described by Brown et al. (*3*), contains a $1Cu^{II}/3Cu^{I}$ redox state with a total spin of $^1/_2$ where the unpaired electron appears to be delocalized between two or more Cu atoms via sulfide ion bridging (*12, 13*), while the CuA is in the reduced $Cu^{+}-Cu^{+}$ state.

The CuZ cluster can be reduced only to a fully reduced state ($4Cu^{I}$) after a prolonged incubation with reduced methylviologen (MV), a powerful reductant [$E'^{\circ} = -0.44$ V vs NHE (*14*)]. Recently, this form has been shown to be catalytically active (*15, 16*), with a k_{cat} of 162.9 s^{-1}, per monomer, when MV is used as the electron donor (*17*).

This slow activation process does not appear to be catalytically relevant, which makes more imperative the need to study the interaction of the enzyme with its putative physiological electron donor to clarify both the activation process and the enzyme catalytic cycle.

Although it has been demonstrated that a cytochrome *c* could transfer electrons to nitrous oxide reductase (*18, 19*), the only detailed kinetic characterization was reported for the N_2OR isolated from *Wolinella succinogenes*, where cytochrome *c* was used as an electron donor. However, this enzyme has the unique feature of presenting an additional domain containing a *c*-type heme, which is not found in any other isolated bacterial N_2OR (*20*).

Recently, Rasmussen and co-workers (*21*) reported a pre-steady-state kinetic study of *Paracoccus pantotrophus* (*Pp*) N_2OR, using horse heart cytochrome *c* as a nonphysiologic electron donor. Moreover, Dooley and co-workers (*17*) were able to activate the *Ac* N_2OR using reduced bovine heart cytochrome *c*.

In *Pd*, under denitrifying conditions, two small redox proteins are expressed, a cytochrome *c*, cytochrome c_{550}, and a type I copper protein, pseudoazurin (*22*). Although none of these proteins were tested as electron donors for this enzyme, a structural model for the electron transfer complex has been proposed on the basis of a theoretical docking study using the structure of *Pd* N_2OR (*23*). The model structure shows that both proteins interact with a well-defined and conserved region of the enzyme surface, in the C-terminal domain near the CuA center.

Pn contains at least five monohemic cytochrome *c* proteins in the periplasm: cytochrome c_{552}, cytochrome c_{553}, cytochrome c_{549}, cytochrome $c_{553(548)}$, and cytochrome *c*′, which are putative candidates for the role of physiological redox partners of N_2OR. In this work, we have identified cytochrome c_{552} as the physiologic electron donor of nitrous oxide reductase and carried out a kinetic study. A model of the electron transfer complex was also obtained using protein docking simulation, and the formation of this complex was observed by NMR spectroscopy.

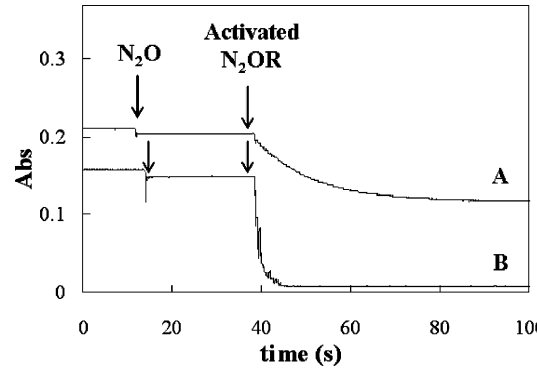


FIGURE 1: Kinetic trace of the modified enzymatic assay using (A) *Pn* cytochrome c_{552} or (B) methylviologen as the electron donor. The assays were initiated by the addition of activated *Pn* N_2OR (70 nM) to a solution containing 1 mM N_2O-saturated water, 0.1 M Tris-HCl (pH 7.6), and 10 μM reduced *Pn* cytochrome c_{552} or 12 μM reduced MV. The absorbance change was followed at 552 nm for cytochrome c_{552} (A) and at 600 nm for MV (B).

MATERIALS AND METHODS

Purification of the Proteins. Pn nitrous oxide reductase was purified as previously described (*10*), with some minor modifications. Enzyme concentrations were determined using the Lowry method (*24*). *Pn* cytochromes were purified from the soluble extract as previously described, with some minor modifications: cytochrome c_{552} (*25*), cytochrome c_{549} (*25*), cytochrome c_{553} (*26*), and cytochrome *c*′ (*26*). Horse heart (HH) cytochrome *c* was purchased from Sigma and used without further purification. The concentration of the cytochromes was determined spectrophotometrically using the extinction coefficients at 552 nm [$\varepsilon = 19.3$ mM^{-1} cm^{-1} (*27*)], 549 nm [$\varepsilon = 47.3$ mM^{-1} cm^{-1} (*28*)], 553 nm [$\varepsilon = 20.8$ mM^{-1} cm^{-1} (*26*)], 399 nm ($\varepsilon = 70$ mM^{-1} cm^{-1}), and 550 nm [$\varepsilon = 29.5$ mM^{-1} cm^{-1} (*29*)] for the reduced forms of cytochrome c_{552}, cytochrome c_{549}, cytochrome c_{553}, cytochrome *c*′,and HH cytochrome *c*, respectively.

Enzyme Activation. Nitrous oxide reductase was activated anaerobically, in a glovebox, using reduced methylviologen, as previously described (*30*). The enzyme (1.4 μM) was incubated at room temperature in a degassed solution containing 3.0 mM methylviologen (MV) and 1.5 mM sodium dithionite (DT) in 100 mM Tris-HCl (pH 7.6) for 180 min. The excess of reductants was removed using a PD10 Sephadex G25 column (Amersham Biosciences), equilibrated with 100 mM Tris-HCl at pH 7.6. The eluted solution (1 mL) was collected and stored at -30 °C until further use. After this step, the enzyme concentration (0.7 μM) was determined separately by the Lowry method.

Activity Assay. The activity assays were performed using different reduced cytochromes or MV as electron donors to the enzyme. Concentrated solutions of each cytochrome *c* (typically 100−150 μM) were reduced by addition of 2.5 mM sodium ascorbate, incubated for 5 min, and then desalted into 100 mM Tris-HCl at pH 7.6, using a PD10 Sephadex G25 column (Amersham Biosciences).

Activity assays were started by adding activated N_2OR (70 nM dimer) to a cuvette containing 100 mM Tris-HCl (pH 7.6), 1.25 mM nitrous oxide (N_2O), and each cytochrome (cytochrome c_{552}, cytochrome c_{553}, cytochrome c_{549}, cytochrome *c*′, or HH cytochrome *c*) or MV at 20−25 μM (Figure 1). The enzymatic activity was determined by

monitoring the decrease in the magnitude of the α-band and the shift of the Soret band of each cytochrome *c*, using a TIDAS diode array spectrophotometer. In the case of MV, the assays were monitored at 600 nm. The concentration of the N_2O-saturated solution was assumed to be 25 mM (*30*). The enzyme specific activity is expressed in units per milligram, corresponding to micromoles of N_2O reduced per minute per milligram of N_2OR dimer.

For the determination of the cytochrome c_{552} concentration dependence of the reaction rates, the assay included 100 mM Tris-HCl (pH 7.6), 1.25 mM nitrous oxide (N_2O), and 3.6, 6, 12, 18, or 36 μM cytochrome c_{552}. Each kinetic assay was initiated by the addition of activated N_2OR (70 nM dimer) to the cuvette. The ionic strength dependence was studied by varying the concentration of NaCl in the cuvette containing 100 mM Tris-HCl (pH 7.6), 1.25 mM nitrous oxide (N_2O), 9 μM cytochrome c_{552}, and 0, 225, 450, or 650 mM NaCl and initiated with 35 nM activated N_2OR dimer.

For the determination of the MV concentration dependence of the reaction rates, the kinetic assays were started by adding 35 nM activated N_2OR dimer to a cuvette containing 100 mM Tris-HCl (pH 7.6), 1.25 mM nitrous oxide (N_2O), and 6.2, 9.3, 28.0, 56.0, 93.4, or 155.6 μM reduced MV. Dithionite was present at stoichiometric amounts relative to MV.

For the determination of the N_2O concentration dependence of the reaction rates, the kinetic assays were started by adding 35 nM activated N_2OR dimer to a cuvette containing 100 mM Tris-HCl (pH 7.6), 91 μM MV or 7.5 μM *Pn* cytochrome c_{552}, and 5, 10, 12.5, 25, 37.5, 62.5, 125, and 500 μM N_2O, added as N_2O-saturated water.

To determine the pH dependence of the reaction rates with both MV and cytochrome c_{552} as electron donors, the kinetic assays were started by adding 35 nM activated N_2OR dimer to a cuvette containing 91 μM MV or 9 μM *Pn* cytochrome c_{552} and 1.25 mM nitrous oxide (N_2O) in a 0.1 M buffer system at the desired pH. The following buffers were used: 0.1 M potassium phosphate buffer at pH 6.2, 6.7, and 7.0 or 0.1 M Tris-HCl at pH 7.6, 8.2, and 8.7.

All assays were repeated at least three times. Initial rate constants were determined by linear fitting of the initial region of each progress curve. The values of K_m and V_{max} were obtained from direct fits of the experimental data with the Michaelis−Menten equation. The pH profiles were fitted with the equations $Act = Act_{max}/(1 + 10^{(pK_a - pH)})$ and $Act = Act_{max}/(1 + 10^{(pH - pK_a)})$ when MV and cytochrome c_{552} were used as the electron donors, respectively.

Direct Electron Transfer Studies. Pn N_2OR was fully oxidized by addition of 10 mM ferricyanide, incubated for 5 min, and then desalted into 100 mM Tris-HCl (pH 7.6) with a PD10 Sephadex G25 column (Amersham Biosciences). *Pn* cytochrome c_{552} was fully reduced by addition of 2.5 mM sodium ascorbate over 5 min; the excess reductant was removed using a PD10 Sephadex G25 column (Amersham Biosciences) in 100 mM Tris-HCl (pH 7.6). The MV was reduced by addition of sodium dithionite under anaerobic conditions. The direct electron transfer was studied by adding a stoichiometric amount of reduced electron donor (cytochrome c_{552} or MV) to a cuvette containing fully oxidized enzyme. The UV−visible spectrum of the sample was monitored before and after the addition of the reduced electron donor.

^{1}H NMR Titration. Protein samples were exchanged with 20 mM sodium phosphate buffer (pH 7.6) using a Sephadex G25 column and concentrated above a Vivaspin membrane with a 30 kDa M_r cutoff for N_2OR or a 5 kDa M_r cutoff for cytochrome c_{552}. The titration was carried out using a solution of 0.475 mM oxidized cytochrome c_{552} in 20 mM sodium phosphate buffer (pH 7.6), 50 mM NaCl, and 10% D_2O. Increasing amounts of oxidized N_2OR were added, until a 3.5 monomer:monomer molar ratio was reached. ^{1}H NMR spectra were recorded on a Bruker Avance 600 spectrometer at 288 K with a spectral width of 90 ppm for 32K data points, and 512 scans were accumulated. Spectra were obtained by presaturating the water signal and processed with TOPSPIN 2.0b provided by Bruker. The chemical shifts were referenced to the H_2O resonance (4.86 ppm at 288 K).

Molecular Docking Simulation. Docking was performed as described in ref *31* using the algorithm BiGGER developed by Palma et al. (*32*). The target protein was the functional dimer of *Pn* nitrous oxide reductase. The coordinates for the enzyme (PDB entry 1QNI), *Pn* cytochrome c_{552} (PDB entry 1CNO), and HH cytochrome *c* (PDB entry 1HRC) were obtained from the RCSB Protein Data Bank (PDB). The BiGGER algorithm provides a complete and systematic search of the rotational space of one protein relative to the other, generating a large number of candidate docking geometries based on the complementarity of the molecular surfaces. The 5000 best solutions thus generated were evaluated and ranked according to a combination of additional interaction criteria that include the electrostatic energy of interaction, the relative solvation energy, and the relative propensity of side chains to interact. For each solution, this combination process produces a "global score". In this particular case, the best solutions were ranked according to global score and hydrophobic score. We then applied a filter to the results on the basis of the distance between the Cu_A center in N_2OR and the iron ions in cytochrome c_{552} heme groups. This procedure is described in Results. The top solutions were evaluated individually using the http://www.biochem.ucl.ac.uk/bsm/PP/server/index.html site to determine the size of the interface of the complex, the gap volume index [as defined by Jones and Thornton (*33*)], and the hydrophobicity.

RESULTS

Activation and Kinetic Assay of N_2OR. The as-purified *Pn* N_2OR [mixture of the *Blue* and *Purple* forms described by Prudêncio et al. (*10*)] exhibited very low activity, 0.090 ± 0.005 unit/mg, when used in a kinetic assay without preactivation with a reducing agent and using MV as the electron donor (without the usual prolonged incubation). In addition, the incubation of this enzyme form with reduced cytochrome c_{552} (the putative physiological redox partner; see below) was not able to activate the enzyme since no activity was detected. Indeed, enzyme activation is achieved only through prolonged incubation (∼1 h) with sodium dithionite and methylviologen, which fully reduces both CuA and CuZ centers, as previously reported (*15*). This activated N_2OR remains active for several hours under anaerobic conditions, even after the complete removal of the reductants (the profile of the activity vs time is not shown).

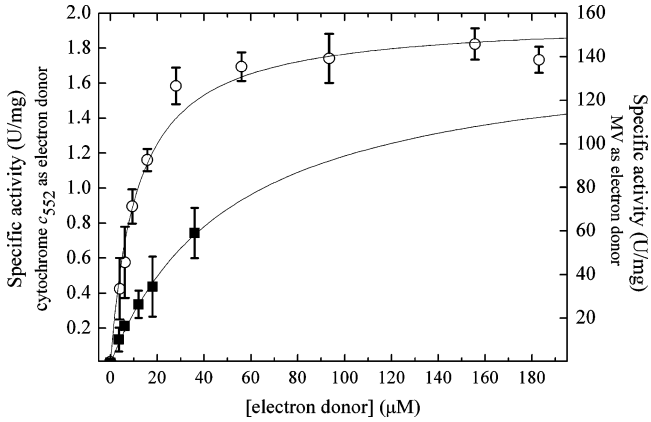


FIGURE 2: Kinetic activity of *Pn* N_2OR as a function of electron donor concentration: reduced methylviologen (○) or *Pn* cytochrome c_{552} (■). The assays were performed in 0.1 M Tris-HCl (pH 7.6), 70 nM activated N_2OR, 1.25 mM N_2O-saturated water, and 6.2−155.6 μM MV or 3.6−36 μM *Pn* cytochrome c_{552}. The experimental data were fitted with the Michaelis−Menten equation, using a K_m of 11.5 ± 3.6 μM and a V_{max} of 157 ± 13 units/mg or a K_m of 50.2 ± 9.0 μM and a V_{max} of 1.8 ± 0.6 units/mg for methylviologen or *Pn* cytochrome c_{552}, respectively.

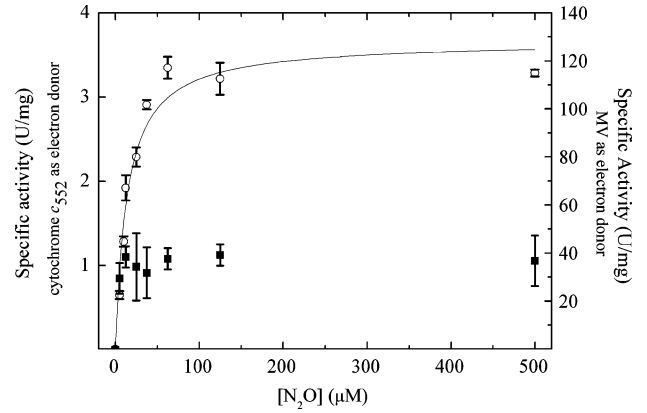


FIGURE 3: Kinetic activity of *Pn* N_2OR as a function of N_2O concentration with methylviologen (○) and cytochrome c_{552} (■) as the electron donors. The assays were performed in 0.1 M Tris-HCl (pH 7.6), 35 nM activated N_2OR, 5, 10, 12.5, 25, 37.5, 62.5, 125, and 500 μM N_2O-saturated water, 91 μM MV, and 7.5 μM *Pn* cytochrome c_{552}. The experimental data were fitted with the Michaelis−Menten equation, using a K_m of 14.0 ± 2.9 μM and a V_{max} of 128 ± 17 units/mg, for methylviologen.

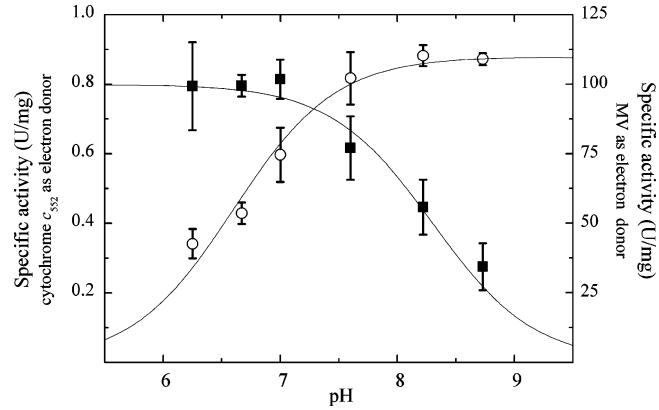


FIGURE 4: pH dependence of the kinetic activity of *Pn* N_2OR using *Pn* cytochrome c_{552} (■) or MV (○) as the electron donor. The assays were performed by adding 35 nM activated N_2OR to a solution containing 9 μM *Pn* cytochrome c_{552} or 91 μM MV and 1.25 mM N_2O-saturated water, in different buffer systems between pH 6.2 and 8.7. The data were nonlinearly fitted using the equation described in Materials and Methods, and pK_a values of 8.3 and 6.6 were calculated for cytochrome c_{552} and MV, respectively.

The activity assay has been slightly changed from the one previously reported (*30*), which was initiated by the addition of N_2O. Actually, contrary to the previous assay, the one described here is designed to separate the effective activity of the enzyme from its activation process (Figure 1). In this new assay, the activated enzyme (fully reduced enzyme from which reductants were removed) is added after the substrate to the reduced electron donor. This procedure allows comparison between the behavior of artificial electron donors, such as methylviologen, with that of one of the putative physiologic redox partners, such as *Pn* cytochromes.

MV as an Electron Donor to N_2OR. The catalytic activity of N_2OR was studied using methylviologen as the electron donor. The profile of the activity versus concentration of methylviologen can be fitted to the Michaelis−Menten equation (Figure 2), with a K_{mMV} of 11.5 ± 3.6 μM and a V_{maxMV} of 157 ± 13 units/mg. The k_{cat} value calculated for *Pn* N_2OR (321 ± 27 s^{-1} per monomer of enzyme) is larger than the one calculated for *Ac* N_2OR [k_{cat} = 162.9 s^{-1} per monomer (*17*)].

The rate dependence of the activity of *Pn* N_2OR (35 nM) with MV on N_2O concentration (5−500 μM) was evaluated while the MV concentration was kept constant at the saturating value (91 μM). The rates follow a hyperbolic behavior, which can be fitted through the Michaelis−Menten equation. The kinetic parameters for the reaction with different N_2O concentrations are 14.0 ± 2.9 μM and 128 ± 17 units/mg for K_{mN_2O} and V_{maxN_2O}, respectively (Figure 3). The k_{cat} in this case is 275 ± 33 s^{-1} per monomer of enzyme.

The ionic strength dependence of the activity was studied at pH 7.6, showing that there is a 20% decrease in activity when the ionic strength is increased from 0 to 650 mM (data not shown). The catalytic activity has a maximum between pH 7.5 and 9 and a pK_a of 6.6, when the ionic strength is 100 mM (Figure 4).

Cytochrome c_{552} as an Electron Donor to N_2OR. To identify the putative physiologic electron donor of N_2OR, different *Pn* cytochrome *c* proteins (cytochrome c_{552}, cytochrome c_{553}, cytochrome c_{549}, and cytochrome *c′*) were used as electron donors in the activity assay described before. The only cytochrome that was able to transfer electrons to the activated enzyme was *Pn* cytochrome c_{552} (Figure 1). In addition, HH cytochrome *c* was also tested as a nonphysiological electron donor, but it was also unable to donate electrons to the activated enzyme.

The kinetic parameters K_m and V_{max} for cytochrome c_{552} could not be determined with accuracy due to the difficulty in collecting data at high cytochrome concentrations. Nevertheless, $K_{mc_{552}}$ and $V_{maxc_{552}}$ were estimated to be 50.2 ± 9.0 μM and 1.8 ± 0.6 units/mg, respectively (Figure 2). Although it is an estimate, the K_m value is on the same order of magnitude as the ones obtained for other physiological electron transfer complexes, such as the one determined for *Pp* pseudoazurin as an electron donor to cytochrome *c* peroxidase (50 μM) (*34*).

The ionic strength and the effect of pH on catalytic activity were studied. The N_2O reduction, catalyzed by N_2OR with cytochrome c_{552} as the electron donor, is independent of ionic strength up to 0.65 M (data not shown). In addition, the catalytic activity is constant at acidic pH, until pH 7, and

decreases at basic pH, with an apparent pK_a estimated to be 8.3 (Figure 4). The rate dependence of the activity of *Pn* N_2OR (35 nM) with cytochrome c_{552} on N_2O concentration (5−500 μM) was evaluated while the cytochrome c_{552} concentration was kept constant (7.5 μM). The rates are constant within experimental error, indicating that the variation of N_2O concentration does not influence the reaction rate (Figure 3). Considering the fact that the k_{cat} for N_2O is an intrinsic characteristic of the enzyme, and that it was possible to determine its value using MV as the electron donor (k_{cat} = 275 s^{-1}), it is reasonable that the rates do not change with N_2O concentration when cytochrome c_{552} is used as the electron donor, since k_{cat} for this electron donor is much lower (k_{cat} = 3.8 ± 1.3 s^{-1} per monomer of enzyme) (see Discussion and Supporting Information).

Direct Electron Transfer Studies. The direct electron transfer between fully oxidized *Pn* N_2OR and stoichiometric amounts of reduced electron donors (MV and *Pn* cytochrome c_{552}) was studied in the absence of substrate, N_2O. The UV−visible spectrum shows that the CuA center is rapidly reduced by both the artificial (MV) and physiological electron donor (cytochrome c_{552}). In the case of MV, the bands at 480, 540, and 800 nm disappear. Due to the absorption overlap with cytochrome c_{552}, just the variation at 800 nm could be analyzed, while it is possible to follow reoxidation of the cytochrome by the decrease in the magnitude of the band at 552 nm. In both experiments, the CuZ center remains oxidized since there is no change in the absorbance band at 640 nm (Figure 5).

^{1}H NMR Titration. As previously reported, the oxidized form of cytochrome c_{552} has a ^{1}H NMR spectrum typical of a low-spin cytochrome *c* (*27*). The four intense downfield resonances, from 13 to 36 ppm, were assigned to the four methyl groups of the porphyrin ring (Figure 6A). Upon addition of N_2OR, cytochrome c_{552} heme methyl resonances M3 and M4 undergo a small downfield shift (−0.010 and −0.016 ppm, respectively) with multiphase behavior, while resonances M1 and M2 do not change (Figure 6B). This chemical shift variation shows that the complex between the two proteins is in a fast exchange regime on the NMR time scale.

The chemical shift variation can be simulated with a K_d of 5 μM (Figure 6C), up to the addition of 1 equiv of monomer N_2OR to cytochrome c_{552}. However, at a 1:1 ratio, there is a clear inflection in the curve, and instead of a plateau, there is an increase in both chemical shift variation and line width (data not shown), which does not reach any plateau even at a ratio of 3:1 (Figure 6B). The line width of methyl M1 increases 234 Hz (0.39 ppm) from a 1:1 ratio to a 3:1 ratio of N_2OR to cytochrome c_{552}. At this ratio, all the heme methyl resonances have an increased chemical shift variation: 0.054, 0.032, 0.100, and 0.113 ppm for M1, M2, M3, and M4, respectively.

Docking of Cytochrome c_{552} to Pn N_2OR. In the first stage of the docking algorithm BiGGER, a set of 5000 models were selected from all possible configurations generated by rotating cytochrome c_{552} (probe) around the surface of nitrous oxide reductase (target protein) in steps of 1 Å and a translation at each 15° step rotation. The second stage of the docking is called the "scoring and ranking", in which these 5000 models were evaluated and ranked according to a global score (see Materials and Methods), or according to each criterion individually. In the top 500 solutions, ranked by either global or hydrophobic score, the cytochrome c_{552} binds at a particular location of the enzyme surface, near the CuA center (Figure 7A,B). The intersection of these two groups of solutions gives eight solutions (Table 1 and Figure 8) that have a distance between one of the heme irons of cytochrome c_{552} and the CuA center of N_2OR of <20 Å, appropriate for the electron transfer (*6*).

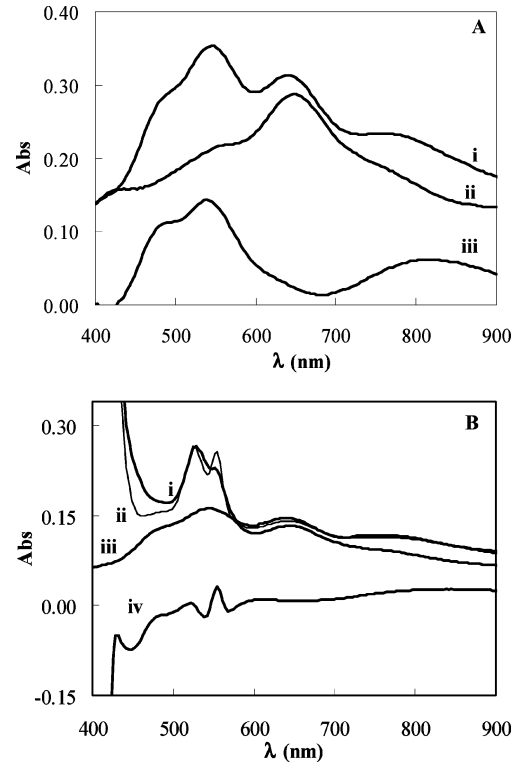


FIGURE 5: Direct electron transfer between fully oxidized nitrous oxide reductase and reduced electron donors (A) MV and (B) *Pn* cytochrome c_{552}, monitored by visible spectra. (A) The reduction of N_2OR by MV was observed at 480, 540, and 800 nm: (i) fully oxidized *Pn* N_2OR, (ii) N_2OR after addition of reduced MV, and (iii) difference spectrum between spectrum i and spectrum ii. (B) The reduction of *Pn* N_2OR by *Pn* cytochrome c_{552} was observed at 800 nm, while the oxidation of the cytochrome was observed at 552 nm: (i) N_2OR spectrum immediately after addition of reduced cytochrome c_{552} (t = 0), (ii) N_2OR spectrum after addition of reduced cytochrome c_{552} (after 30 s), (iii) fully oxidized *Pn* N_2OR, and (iv) difference spectrum between spectrum ii and spectrum i.

These top solutions have an interface accessible area between 868 and 1064 Å^2 (Table 1), which is typical for small complexes following the criteria of Lo Conte et al. (*35*). The gap volume index was also calculated, as defined by Jones and Thornton (*33*), in estimating the complementarity in the complex interface. An average gap index of 3.4 ± 0.5 Å was obtained for the eight chosen solutions (Table 1). In these docking models, 64−86% apolar residues compose the interface area, which is consistent with a hydrophobic interaction (Table 1). Different residues on the N_2OR surface can be identified as the putative entry point for the electron, according to different possible orientations of cytochrome c_{552} in these electron transfer complexes (Table 1 and Figure 8). Horse cytochrome *c* was also used as a probe in a docking study with *Pn* nitrous oxide reductase as the target, and the results show that this small cytochrome binds at the groove formed between the two monomers of

the enzyme, far from the proposed electron entry site, CuA (data not shown).

DISCUSSION

Establishment of the Physiological Electron Donor of N_2OR. Cytochrome c_{552}, cytochrome c_{553}, cytochrome c_{549}, and cytochrome c' isolated from the soluble extract of *Pn* were tested as putative electron donors to *Pn* N_2OR in an assay performed using activated enzyme, as described in Materials and Methods. Cytochrome $c_{553(548)}$ is present in much smaller amounts in the periplasm of this bacterium, in comparison with the other cytochromes, and at this point was excluded as a physiological redox partner. Cytochrome c_{553}, cytochrome c_{549}, or cytochrome c' did not exhibit any catalytic oxidation, within the experimental conditions, upon addition of activated N_2OR, and only when reduced cytochrome c_{552} was used as an electron donor was a detectable catalytic oxidation of the electron donor observed.

The data suggest that cytochrome c_{552} is the physiological electron donor to nitrous oxide reductase. Actually, this cytochrome had already been shown to be the physiological redox partner of other enzymes isolated from the periplasm of *Pn* and involved either in denitrification, as the electron donor to cytochrome cd_1 nitrite reductase (*36*) and nitric oxide reductase (*37*), or in detoxification of hydrogen peroxide, as the electron donor to cytochrome *c* peroxidase (*38*).

Moreover, HH cytochrome *c*, which was previously used as the electron donor to *Pp* N_2OR (*21*), was unable to donate electrons to *Pn* N_2OR, which was similar to what had already been observed for another enzyme isolated form this organism, cytochrome *c* peroxidase (*38*).

It should be noted that *Pn* is a marine organism, living in a high-ionic strength environment, and therefore, we expect that its periplasmic enzymes have evolved to provide high turnover rates under these conditions. The surface of the enzymes so far studied (or from which three-dimensional structure has been determined) is mostly hydrophobic in nature in contrast to that of horse heart cytochrome *c*, which has a relatively high dipole moment, with a ring of positive charges around the exposed heme edge. This difference in charge surface complementarity might be the reason for the result observed in the kinetic study. This also provides an explanation for the fact that no putative electron transfer orientations were obtained in the docking study using HH cytochrome *c* as a probe.

Direct electron transfer studies, performed in the absence of substrate, also provide strong evidence of electron donation from *Pn* cytochrome c_{552} to *Pn* N_2OR (Figure 5). These studies show that both cytochrome c_{552} and MV, an artificial electron donor, are able to donate electrons directly to the CuA center of nitrous oxide reductase, making the CuA center a point of entrance of the electrons needed for the catalysis that occurs at the CuZ center, as previously proposed (*23*).

Kinetic Characterization: Cytochrome c_{552} as the Electron Donor to Pn N_2OR. The detailed kinetic characterization of the N_2OR activity must consider that the reaction depends on both N_2O and reducing agent concentrations. This multisubstrate enzymatic reaction could proceed in two general cases that are described in detail in the Supporting Information. In particular, the reaction could require the binding of two substrates to give a ternary adduct, or the reaction could occur in a sequence of two separate steps involving each substrate. Our experimental data obtained when cytochrome c_{552} is used as the electron donor perfectly

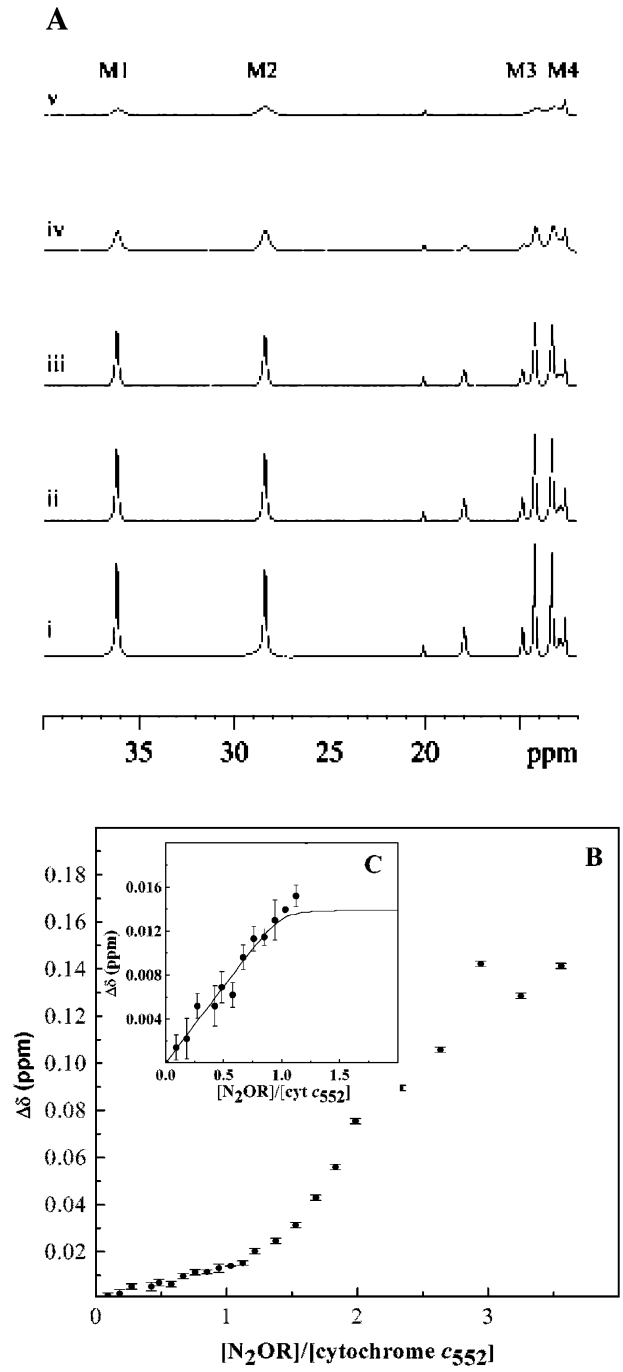


FIGURE 6: ^{1}H NMR titration of *Pn* cytochrome c_{552} with nitrous oxide reductase. In panel A, the low-field spectral region (40−12 ppm) containing the cytochrome M1, M2, M3, and M4 heme methyl resonances is shown. The experiment was performed as described in Materials and Methods, and the protein samples were as follows: (i) $R = 0$, 472 μM cytochrome c_{552}; (ii) $R = 0.5$, 440 μM cytochrome c_{552} and 220 μM N_2OR; (iii) $R = 1.0$, 410 μM cytochrome c_{552} and 410 μM N_2OR; (iv) $R = 2.0$, 365 μM cytochrome c_{552} and 730 μM N_2OR; (v) $R = 3.6$, 308 μM cytochrome c_{552} and 1109 μM N_2OR. Panel B shows the chemical shift variation of cytochrome c_{552} heme methyl M4 with increasing molar ratios of nitrous oxide reductase. Panel C shows the fitting curve simulated for a single binding site with a K_d of 5.0 μM and a δ_{max} of 0.014 ppm, until a ratio of 1:1 is reached.

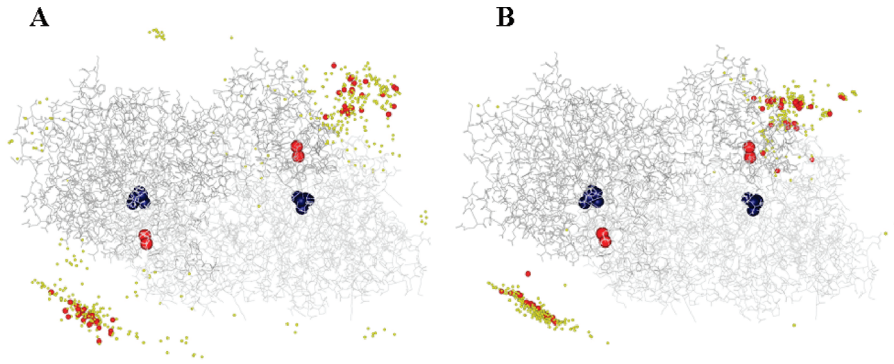


FIGURE 7: (A) Five hundred top model complexes ranked by global score of the docking of the N_2OR dimer with the cytochrome c_{552} dimer. (B) Five hundred top model complexes ranked by the hydrophobic score of the docking of the N_2OR dimer with the cytochrome c_{552} dimer. In panels A and B, the geometric center of the cytochrome c_{552} of the 500 putative model complexes is represented as a yellow sphere, while the top 50 are represented as larger red-colored spheres. The two copper atoms of the CuA center are colored red; the catalytic center is colored blue, and the polypeptide of each nitrous oxide reductase monomer is colored light or dark gray.

Table 1: Characteristics of the Eight Top Model Complexes Obtained by Molecular Docking Simulation of *Pn* Cytochrome c_{552} to *Pn* N_2OR

probe	global score	hydrophobicity (kcal/mol)	interface area (Å^2)	gap volume index (Å)	apolar residues[a] (%)	CBC−Cu distance (Å)	CBC−AA distance (Å)	residue closest to CBC
1	9.2	−7.0	938	3.1	75	10.7	1.5	Gln497
2	10.4	−6.1	1064	2.6	80	10.3	2.4	Asp519
3	3.3	−5.7	868	3.9	57	11.4	3.0	Ala495
4	3.0	−5.3	995	3.5	64	14.1	1.6	Gly499
5	4.3	−5.0	961	4.0	86	13.8	3.8	His566
6	10.4	−4.8	871	4.0	84	14.0	4.7	Pro496
7	5.9	−4.6	1002	3.4	74	13.9	2.8	Ile340
8	3.1	−4.4	980	2.9	82	11.8	2.2	Val421

[a] The percentage of the interface area of N_2OR composed of apolar atoms.

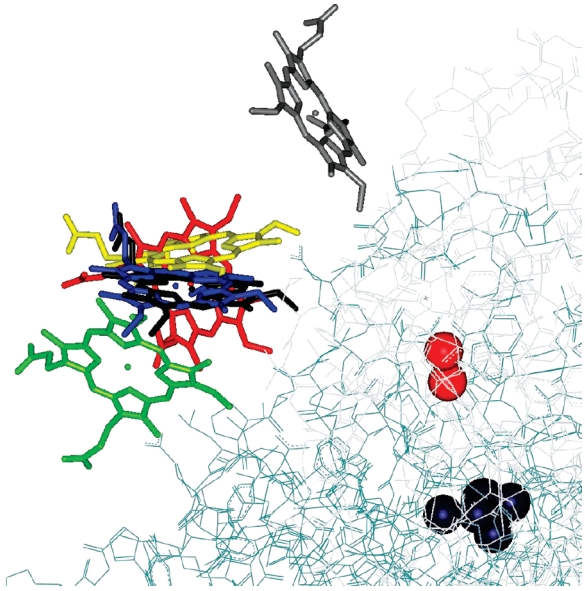


FIGURE 8: Top putative docking positions obtained after analysis of the docking between the *Pn* cytochrome c_{552} dimer and the *Pn* N_2OR dimer. Only the heme groups of six of the top eight models are displayed: model 1 in gray, model 2 in black, model 5 in green, model 6 in yellow, model 7 in red, and model 8 in blue. These numbers correspond to the numbering in Table 1. Models 3 and 4 are not displayed, because they are located near the CuA center of the other N_2OR monomer. The two copper atoms of the CuA center are colored red; the catalytic center is colored dark blue, and the polypeptide chain of each nitrous oxide reductase monomer is colored light or dark gray.

agree with the latter case, as shown by the simulation provided in the Supporting Information (Figures S1 and S2). Therefore, we conclude that the rates do not depend on N_2O concentration (Figure 3), indicating that this substrate is involved in a fast step of the catalytic cycle. However, the rates depend on cytochrome c_{552} concentration, indicating that its reaction (including the formation of the complex between the two proteins, the electron transfer reaction, and the dissociation of the complex) is slower than the reduction of N_2O and is the rate-limiting step of the enzyme turnover. Data analysis allowed determination of the kinetic parameters: $K_{mc_{552}} = 50.2 \pm 9.0\ \mu M$, and $V_{maxc_{552}} = 1.8 \pm 0.6$ units/mg, at pH 7.6 (Figure 2).

Compared to the nonphysiologic HH cytochrome *c* tested with N_2OR from *Pp* [$K_m = 6 \pm 3\ \mu M$ and $V_{max} = 0.034 \pm 0.002$ units/mg (*18*)], *Pn* cytochrome c_{552} is shown to be a more efficient electron donor, although in those studies the *Pp* enzyme was not activated, as in this work.

The reactivity pattern observed with MV as the electron donor is different. The reaction rates depend on both N_2O and MV concentrations, showing apparent saturation behavior (Figures 2 and 3). It should be noted that the global rates are much higher with MV than those observed when cytochrome c_{552} is used as the electron donor (Figure 2). This is in agreement with a much faster electron transfer with the nonphysiologic reducing agent. The enzyme reduction becomes a fast pre-equilibrium with the N_2O reduction as the slow step of the turnover cycle.

The K_m value for cytochrome c_{552} is higher than the one calculated for MV, indicating a lower affinity of N_2OR for the cytochrome, and the k_{cat} for cytochrome is much lower. In fact, MV is the reductive agent necessary to turn the inactive as-isolated N_2OR into the active fully reduced form, and the kinetic data show a stronger affinity for the enzyme. However, there is no evidence for the site of interaction of MV with the enzyme surface, while for the cytochrome, the

contact takes place in the region surrounding the CuA center, as described below.

Overall, as already suggested by Wunsch et al. (*39*), the activity assay with MV may not probe the internal electron flow between the CuA and CuZ centers of N_2OR. Nevertheless, both the artificial and physiologic electron donors were able to reduce the CuA, as shown in the direct electron transfer experiment (Figure 5). This evidence suggests that MV might donate electrons to both CuA and CuZ sites, justifying the high turnover number obtained and the different behavior in the pH dependence and the low but not negligible dependence on ionic strength for MV. Because MV is a small molecule, its binding to the enzyme is just diffusion-controlled, and the k_{off} rate affects the overall reaction rate to a lesser extent.

On the other hand, the negligible influence of the activity over a wide range of ionic strength suggests an interaction between cytochrome c_{552} and N_2OR mainly driven by hydrophobic forces. Moreover, this cytochrome has been previously shown to be the redox partner of *Pn* cytochrome *c* peroxidase, and a similar effect of ionic strength on activity was observed (*38*).

The pH effect also elucidates a different behavior of the two molecules as electron donors. MV-mediated catalysis shows a maximum activity at pH >8, with a pK_a of ~6.6, as already observed in past studies of N_2OR from different sources (*18, 40*). Instead, the activity promoted by cytochrome c_{552} as the electron donor decreases at pH >7, presenting a pK_a of 8.3. A similar behavior has already been observed for *Pp* N_2OR (*18*), where the reaction rate with HH cytochrome *c* is faster at lower pH. This different pH dependence and the slower reaction rate for the cytochrome-mediated reaction (2 orders of magnitude lower than that for the MV donor system) might suggest that the electron transfer between cytochrome and N_2OR is the rate-limiting step and the pH dependence of the reaction can be attributed to the protein−protein interaction or to the reduction process of the CuZ site (vide infra), as proposed previously for the N_2O concentration dependence kinetic studies.

In contrast, Rasmussen et al. (*21*) found that when nonphysiologic HH cytochrome *c* is used as the electron donor, not electron donation but N_2O reduction is the rate-limiting step in the catalysis even if the internal ET (in particular intra-ET between CuA and CuZ centers) is slow. These authors also suggested that the rapid turnover promoted by the MV donor system could bypass the electron transfer path used by the cytochrome, which seems to be evident in the results presented here.

Characterization of the Electron Transfer Complex between Cytochrome c_{552} and N_2OR. (i) ^1H NMR Titration. The chemical shift variation observed for methyl M4 and M3 [M1 and M2 have almost negligible variations (data not shown)] upon addition of increasing amounts of N_2OR up to 1:1 ratio (N_2OR monomer:cytochrome c_{552} monomer) is small (Figure 6A). The possibility that the small shift variation observed could be due to formation of a nonspecific complex can be ruled out since just two of the heme methyl resonances are affected, until a ratio of 1:1 is reached. Moreover, as cytochrome c_{552} is the electron donor to N_2OR, the complex between these two proteins is expected to be specific.

Until this 1:1 ratio was reached, the chemical shift variation could be simulated with a K_d of 5 μM, an estimate that is not very reliable due to the high concentrations of protein used in the NMR experiments.

The small chemical shift variation is then attributed to a weak perturbation of the heme vicinity surrounding upon binding of cytochrome c_{552}. Cytochrome c_{552} structure shows that methyl M4, in the porphyrin ring, is the most solvent exposed heme methyl in a favorable position for electron transfer. Moreover, heme methyl M4 is close to the CBC methyl group, the most probable point for electron transfer in our docking simulation (see below), while the other heme methyls are not exposed and may not take an active part in the electron transfer process.

It should be noted that the chemical shift variation and the observed line broadening are not due to a paramagnetic effect related to the increasing amount of N_2OR which contains several paramagnetic centers (in this experiment, N_2OR presents a CuA center in the $Cu^{1.5+}-Cu^{1.5+}$ mixed-valance state while CuZ is in the $1Cu^{2+}-3Cu^{1+}$ oxidized state). In fact, the paramagnetic effect on resonance shifts and broadening could be due to two contributions: contact mechanism and dipolar contribution (or Curie). The contact mechanism is excluded since there are no covalent bonds between the porphyrin in cytochrome c_{552} and the paramagnetic centers of N_2OR. The dipolar contribution is very sensitive to the distance between the protons and the paramagnetic center (with an r^{-6} dependence) and to the correlation time for the spin inversion in the metal center (τ_s) (*41*). Both the large distance obtained in the docking simulation, where the CuA is at least 10.4 Å from methyl M4 (see below), and the very low τ_S observed in coupled copper clusters (*41*) suggest that the dipolar contribution is negligible.

The larger chemical shift variations observed at protein ratios higher than 1:1 can be attributed to the formation of larger complexes, in which there might be nonspecific binding of N_2OR to the complex. The high molecular mass (>150 kDa, one dimer of cytochrome c_{552} and two dimers of N_2OR) of these complexes implies a dramatic broadening of the resonances of cytochrome and a concomitant shift related to the slow tumbling of the large aggregate. The line width is a function of the correlation time (molecular tumbling time), which depends on the molecular size. The presence of aggregates would explain the broadening of the heme methyl resonances observed at higher ratios but may not explain the further chemical shift observed unless it is an induced effect due to the formation of the high-molecular mass complex, but not directly due to the binding of the protein at the contact surface. This hypothesis is further supported by the equivalent chemical shift variation and broadening observed for all the methyl groups that is independent of their spatial arrangement in the adduct between the two proteins.

(ii) Docking of Pn Cytochrome c_{552}. In agreement with the kinetic data, the interface analysis of the modeled complexes shows that the interaction between N_2OR and cytochrome c_{552} is mainly hydrophobic. In fact, in our docking models, the composition of the N_2OR surface involved in the ET complex contains 64−86% apolar residues (Table 1). This has already been observed for other electron transfer complexes, but these values are higher than

the average reported for *c*-type cytochromes (*42*). Moreover, the hydrophobic interface has a low dielectric constant, which is favorable for electron transfer (*43*).

The small interface area of the complexes indicated in Table 1 (868−1064 Å^2) is also characteristic of short-lived complexes (<1200 Å^2) (*35*), and the average gap index of 3.4 ± 0.5 Å is close to the one found for redox protein complexes (4.7 ± 1.5 Å) (*42*), which is consistent with poor geometric fitting and poorly packed interfaces in this type of complex.

The hydrophobic and poorly packed interfaces enable the interaction with different redox partners. This does not seem to be the case for N_2OR that is proposed to have only one redox partner but, on the other hand, can explain the ability of cytochrome c_{552} to donate electrons to at least two other *Pn* periplasmic enzymes, cytochrome *c* peroxidase (*38*) and nitrite reductase cd_1 (*36*). As described by Williams et al. (*43*), who introduced the concept of "pseudospecific" docking, hydrophobic interactions are less specific and less directional than electrostatic ones.

With regard to the HH cytochrome *c*−N_2OR docking study, the absence of interaction may provide an explanation for the fact that no electron transfer was observed between reduced horse cytochrome *c* and activated N_2OR, in the kinetic assay. Although this is a single example, it shows that this docking program can also be used to discriminate between putative electron donors, prior to acquiring experimental data, as long as the three-dimensional structure of the intervenient proteins is known.

Although our eight top model complexes were not energy-minimized, it is possible to compare them with the ones obtained for *Pd* N_2OR and cytochrome c_{550}: the distance between the heme ring and one of the coppers of CuA in the models of the this study is in the same range (10.3−14.1 Å) (Table 1) as the one found for *Pd* N_2OR complexes (12.8−14.3 Å) (*23*); in both cases, the interface has a hydrophobic patch in the central area; and the same residues are found in close contact with the redox partners (A495, P496, H566, and D519), which comprise a conserved surface region near the CuA center.

Suggested Electron Transfer Pathway and Reduction Process of CuZ. The interface of the modeled complexes can be analyzed in identifying putative residues involved in the electron transfer pathway. Cytochrome *c* oxidase (COX) presents the same binuclear copper center, CuA, and the amino acid chain in the proximity of the CuA site is similar for both COX and N_2OR. Recent structural studies assert that in cytochrome *c* oxidase a tryptophan exposed on the surface is essential for the electron transfer from a cytochrome to the CuA center (*44*). In N_2OR, isolated from either *Pd* or *Pn*, there is no Trp residue in the expected position that could be implied in the electron transfer. Mattila et al. (*23*), who performed a docking study on N_2OR from *Pd* and two putative electron donors, pseudoazurin and cytochrome c_{550}, proposed that residues Pro565 and His635 for *Pd*, occupying a position analogous to that of Trp121 in cytochrome *c* oxidase, are involved in the electron transfer pathway. In fact, Pro565 occupies the same position as Trp121 in the polypeptide chain, while the imidazolic ring of His635 replaces the indolic ring of the tryptophan. In other small copper proteins, like pseudoazurins, a histidine (that coordinates the copper ion) and a proline residue that is located adjacent to this histidine are proposed to be the point of entry or leaving of the electron (*34, 45*).

As this region is well-conserved, these amino acids correspond to Pro496 and His566 in *Pn* and were found in the interface of the top eight solutions. Moreover, residue Asp519 (corresponding to Asp558 in *Pd*) appears to be exposed on the surface and available for the electron transfer. The homologue of this amino acid in cytochrome *c* oxidase from *Pd*, Asp178, has been shown to be important for the binding of cytochrome *c* (*44*). This is the only residue that is common to all known N_2OR and COX structures, and in all these, the Asp carboxylic group is hydrogen bonded to His526, the terminal ligand of the CuA center.

At the moment, it is not possible to define a unique ET pathway and binding interface. The eight best solutions chosen have different heme orientations and involve slightly different interfaces, but in all of them, those three conserved residues (Pro496, Asp519, and His566) are present in the complex interface (data not shown). These docking models also support the view that electrons from cytochrome c_{552} must be delivered to CuA and then to CuZ.

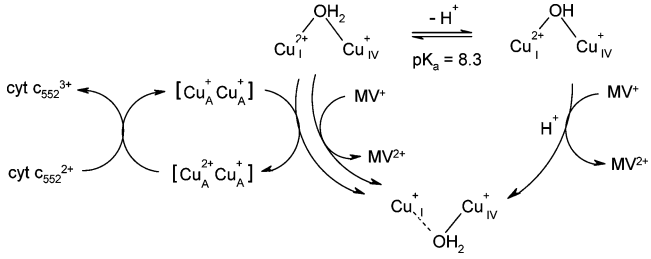
The electron transfer from cytochrome c_{552} to N_2OR that is necessary to complete the catalytic cycle that occurs in the CuZ center controls the enzymatic activity, and the pH dependence suggested that a group with a pK_a of 8.3 must be in the protonated form to enable the reaction to occur. It is known that cytochrome c_{552} potential is higher at acidic pH and decreases at basic pH (with a pK_{ox} of 7.3 ± 0.1 and a pK_{red} of 9.0 ± 0.2) (*27*), indicating that donation of an electron from the cytochrome becomes less favorable in this range. As the kinetic studies gave an opposite result and this pK_a cannot be correlated to any residue on the protein−protein interface, our attention is then turned to the reduction process of the CuZ center in N_2OR.

As within the CuZ center the pair of Cu^I and Cu^{IV} atoms is the site of N_2O binding and reduction, we propose that the protonation of a water molecule that bridges these two copper atoms (with a pK_a of 8.3) controls the electron transfer process. This Cu pair cycles among three states during turnover, $Cu^{II}Cu^{II}$, $Cu^{II}Cu^{I}$, and $Cu^{I}Cu^{I}$, according to the accepted mechanism of N_2O reduction (*46*), and the fully oxidized form of the enzyme Cu_ICu_{IV} pair is achieved upon N_2 release. From a variety of model studies on binuclear copper complexes containing N-donor ligands, it is known that deprotonation of a bridging water molecule bound to a couple of Cu^{II} ions typically occurs in the range between pH 7 and 8 (*47*). Perhaps more important is the fact that deprotonation of the $Cu^{2+}-H_2O-Cu^{2+}$ species to the $Cu^{2+}-OH-Cu^{2+}$ form systematically leads to a large reduction in the $E°(Cu^{II}/Cu^{I})$ values (*48, 49*).

However, in the stepwise reduction of the CuZ center during catalysis, it is the second reduction step, from $Cu^{2+}-H_2O-Cu^{+}$ to $Cu^{+}-H_2O-Cu^{+}$, which is expected to be more difficult and probably rate-limiting (*48*). For this mixed-valent state, one-hole $Cu^{2+}-H_2O-Cu^{+}$ species, we also expect that the redox potential will be lowered upon deprotonation to the corresponding hydroxo species, $Cu^{2+}-OH-Cu^{+}$.

Considering the second one-electron reduction by cytochrome c_{552} as the rate-limiting step of the catalytic cycle, that hypothesis would also explain the determined pK_a of 8.3 found in the experiment, as it is expected that a bound

Scheme 1



water will be slightly less acidic in the mixed-valent $Cu^{2+}-H_2O-Cu^{+}$ species than in the $Cu^{2+}-H_2O-Cu^{2+}$ species. Thus, the activity of N_2OR at pH >9 is completely inhibited because the bridge between Cu_I and Cu_{IV} within the CuZ cluster is fully deprotonated and CuA (i.e., cytochrome c_{552}) cannot reduce CuZ. This problem does not exist when methylviologen is used as the electron source, because it is a strong reductant and can directly reduce CuZ, without the need to deliver electrons to this site through CuA. The activity of N_2OR mediated by this nonphysiological electron donor is controlled by the protonation state of a group with a pK_a of ~6.6, which could be reasonably associated with His566. A schematic picture of the key reduction pathway of the Cu_ICu_{IV} pair in CuZ is shown in Scheme 1.

According to a recent study (*50*), the water molecule acting as a bridging ligand of the Cu_ICu_{IV} pair remains in the deprotonated state in a pH range from 6 to 10, and reduction of CuZ by methylviologen is controlled by a group with a pK_a of 9.2 (likely to be K397 in *Pn* N_2OR). The latter data refer to the bimolecular reaction between methylviologen and the as-isolated N_2OR; in this work, the pH effect was studied for the turnover of the activated enzyme, and different profiles were obtained whether methylviologen or cytochrome c_{552} was used as the electron donor. Therefore, the interaction of the enzyme with both the substrate and the electron donor is important. As we know from this study, the interaction of cytochrome c_{552} with N_2OR is specific, while in the case of methylviologen, it may occur in more than one site, with the most effective one being different in the absence or presence of bound N_2O. Moreover, it is also possible that during turnover the other Cu atoms of the CuZ cluster, Cu_{II} and Cu_{III}, take a more active part than what is currently hypothesized.

In any case, the reduction process of N_2O, after binding to the fully reduced Cu_ICu_{IV} pair, could be facilitated by the presence of a protonated water ligand within the CuZ site, as this could be the source of the proton assisting the cleavage of the N−O bond and release of N_2 (*46*).

CONCLUSION

In conclusion, we reported here a new activity assay that separates the activation of N_2OR from the catalytic reduction of N_2O and allows the comparison between different electron donors. Cytochrome c_{552} from *Pn* was recognized as the electron donor of N_2OR, and the first kinetic characterization of N_2OR with its physiological partner was carried out. The artificial electron donor, methylviologen, the reductive agent that is required to activate the enzyme, was shown to be a more efficient electron donor. Nevertheless, differences in the mechanism and interaction with the enzyme are pointed out. In particular, the electron transfer reaction is the rate-limiting step when cytochrome c_{552} is the electron donor, while the reduction of N_2O is the slower reaction when reduced MV is used as the electron donor. The determined pK_a values and the different ionic strength dependence suggest that MV might interact not only with the CuA center (which was observed in the direct electron transfer experiments) but also directly with the CuZ center through a different reduction pathway for the physiological electron donor. The pH dependence of the enzymatic activity when using cytochrome c_{552} as the electron donor also suggests the presence of a water bridging ligand at the CuZ center. For cytochrome c_{552}, both the kinetic and docking studies indicate the presence of a hydrophobic patch near the CuA center, which is the electron entry site.

ACKNOWLEDGMENT

We thank Ludwig Krippahl for all the help with BiGGER calculations and Melanie Hassler for some experimental assistance.

SUPPORTING INFORMATION AVAILABLE

Detailed kinetic treatment and simulation (using the kinetic parameters determined here) showing the reaction of nitrous oxide reductase as a specific case in a more general analysis of multisubstrate enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801375Q