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Structure and properties of the catalytic site of nitric oxide reductase at ambient temperature

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ABSTRACT

Nitric oxide reductase (Nor) is the third of the four enzymes of bacterial denitrification responsible for the catalytic formation of laughing gas (N_2O). Here we report the detection of the hyponitrite ($HO–N=N–O^-$) species ($\nu_{N-N} = 1332\ cm^{-1}$) in the heme b_3 Fe–Fe_B dinuclear center of Nor from *Paracoccus denitrificans*. We have also applied density functional theory (DFT) to characterize the bimetallic-bridging hyponitrite species in the reduction of NO to N_2O by Nor and compare the present results with those recently reported for the N–N bond formation in the ba_3 and caa_3 oxidoreductases from *Thermus thermophilus*.

1. Introduction

Denitrification is the anaerobic use of bacteria of nitrogen oxide species as terminal electron acceptors in place of O_2. It is the only process that returns large amounts of fixed nitrogen to the atmosphere [1]. The reduction of nitric oxide (NO) to laughing gas (N_2O) which is the committed step in denitrification, is also an "escaping" process, resulting in the release of large amounts of N_2O, a greenhouse gas that is implicated in atmospheric ozone depletion into the atmosphere [1–3]. Several themes have emerged in the studies of biological control of nitric-oxide chemistry [1–5]. Apparently, evolution has clearly selected means by which to take advantage of the unique NO chemistry, and a number of enzymes have evolved that are able to activate NO kinetically, and control their redox chemistry. In bacterial respiration the reduction of NO to N_2O by terminal oxidoreductases supports the hypothesis of a common evolutionary origin of bacterial denitrification and aerobic respiration. Comparison of the enzymes responsible for the activation of NO in denitrification and bacterial respiration may provide the means to identify conserved structural features, which can be assumed to be involved in basic functions common to both classes of enzymes [4–12].

Nitric oxide reductase (Nor) from *Paracoccus denitrificans* contains four redox centers: three heme groups and one non-heme Fe_B atom [4,5]. Heme *c* is bound to NorC subunit and functions as the electron entry site of the enzyme. NorB contains a low-spin heme *b* and a five-coordinate, high-spin heme b_3, which, together with a non-heme iron atom form the dinuclear, NO reduction site. The molecular mechanism of the reduction of NO to N_2O by Nor and the structures of the key intermediates have not yet been determined.

The most commonly encountered metals in the biological reduction of NO to N_2O are iron and copper in mononuclear, binuclear, and dinuclear centers. In P450Nor the electrons needed for the reduction of NO are directly transferred from NADH ($2NO + NADH + H^+ \rightarrow N_2O + H_2O + NAD^+ + H_2O$) [10]. This way, a single molecule of NO binds at the heme Fe, and addition of two electrons to heme Fe^{3+}–NO yields the two electron reduced species $Fe^{2+}–N=O^-$. A second NO molecule attacks the N atom of the ferrous–NO species to transiently yield hyponitrite ($HONNO^-$), and thus the N–N bond formation. Cleavage of the N–O bond produces the ferric enzyme, N_2O and H_2O. Proposed mechanisms for the NO reduction by Nor involve two NO molecules in the dinuclear center. Based on the Soret and UV–Raman experiments of the reaction of ba_3 oxidase with NO, a reaction scheme for the initial binding of two NO molecules to the oxidized heme a_3–Cu_B binuclear center in which the formation of the N–N bond required the addition of an electron was demonstrated [7,8]. The hyponitrite species is formed subsequent to the formation of a transient heme a_3 Fe^{3+}–NO species and the addition of the second NO molecule to Cu_B. Given that, upon addition of NO to the oxidized enzyme, heme a_3 is in the ferric form, then Cu_B must be reduced upon addition of NO, producing Cu_B^{1+} and NO_2^-. Addition of a second NO molecule to Cu_B^{1+} yields the Cu_B^{1+}–NO complex.

The Soret excitation resonance Raman spectra of the reactions of oxidized and reduced Nor with NO have been reported [11,12]. In this report we have extended our original work on the NO binding to Nor and have applied UV resonance Raman spectroscopy to investigate the

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reaction of NO with the dinuclear center of Nor [11,12]. Our results indicate the formation of the hyponitrite (HO–N = N–O$^-$) species (ν_{N-N} = 1332 cm^{-1}) in the heme b_3 Fe–Fe$_B$ dinuclear center of Nor from *P. denitrificans*. We have also applied density functional theory (DFT) to characterize the heme Fe/Fe$_B$ hyponitrite species. With the identification of the hyponitrite species the mechanism for the 2e$^-$/2H$^+$ reduction of NO to N_2O and the role of the non-heme Fe_B^{3+} in Nor versus Cu_B^{2+} in heme-copper oxidases in the reaction mechanism can be described with more certainty.

2. Materials and methods

2.1. Experimental section

Nitric oxide reductase was isolated from *P. denitrificans* cells according to previously published procedures [5,11]. The activity of the enzyme was measured according to [5] and was 40 μmol/mg/min (46e-/s). The samples were concentrated to 150 μM in 20 mM Tris pH 7.4 containing 0.05% dodecyl β-D-maltoside and stored in liquid nitrogen until use. The concentration of Nor was determined using an $\varepsilon_{411} = 3.11 \times 10^5$ M$^{-1}\cdot$cm^{-1}. Oxidized samples were exposed to 1 atm NO in an anaerobic rotating quartz cell for the Raman measurements. The hyponitrite species in Nor was formed upon addition of reductant (final concentration 130 μM NOR/500 μM dithionite) to the NO-exposed oxidized enzyme. The UV Raman spectra were acquired as described previously [8].

2.2. Computational

Hyponitrite complex was studied in cases of singlet through septet and doublet through sextet states with +3 and +2 overall charges respectively at the riblyp/tzvp level of theory with ecp-10-mdf effective core potential on iron atoms as implemented in Turbomole 5.8.0 software package (Development of the University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 1989–2007, TURBOMOLE GmbH, since 2007; available from http://www.turbomole.com). The quartet species of total charge +2 converged to a structure consistent with our previous DFT study [13] while all other species have either a distorted porphyrin (singlet, doublet), almost one spin (~0.80) localized on porphyrin (quintet, septet), or dissociated hyponitrite substrate (octet), and thus, they were rejected. For the sextet species, the vibrational frequency calculation gave inconsistent results to our previous DFT study on Cu$_B$–Fe binuclear center [13].

3. Results

The Fe^{3+}–NO stretching and the Fe^{3+}–N–O bending modes of ferric nitrosyl-heme proteins/enzymes have been detected under Soret excitation, [11] however, the N–O stretching mode in the 1900–1925 cm^{-1} region has been observed only under UV-excitation [14]. It was suggested that there is little orbital conjugation between the NO ligand and the heme and because such conjugation is not necessary to occur for UV–RR detection, it was proposed that the UV–RR enhancement of the N–O stretching mode is the result of a localized π–π* transition [14]. RR excitation in the UV region enhances strong bands derived from aromatic residues such as tyrosine (Y) and tryptophan (W) [14].

Fig. 1 (trace A) shows the UV–RR spectrum of oxidized Nor (trace A) and the NO bound forms at pH 7.5 in the absence (traces B and C) and presence (traces D and E) of reductant. The strong bands in the spectra arise from 1618 (Y8a, W1), 1553 (W3), 1458 (W5), 1359 (W7) and 1339 (W7). Traces B ($^{14}N^{16}O$) and C ($^{15}N^{16}O$) are of the oxidized NO-bound form (heme Fe^{3+}–NO). The difference spectrum B–C shows that the formation of the heme Fe^{3+}–NO complex is not followed by protein changes that could be detected in the spectrum. Trace D was obtained after addition of four equivalents of reductant to

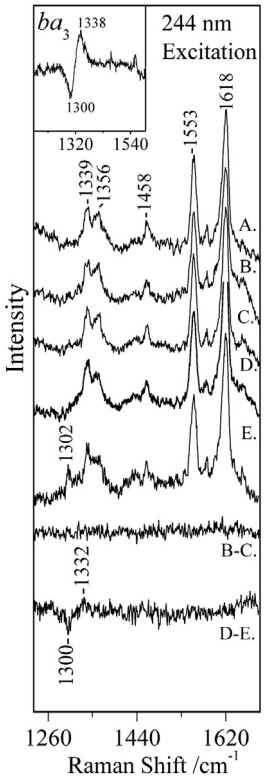

Fig. 1. UV-resonance Raman spectra of oxidized Nor (trace A), upon addition of $^{14}N^{16}O$ (trace B) and $^{15}N^{16}O$ (trace C) at pH 7.5. Traces D and E are the spectra of the oxidized Nor–$^{14}N^{16}O$ and Nor–$^{15}N^{16}O$ complexes, respectively, upon addition of reductant (final concentration 130 μM NOR/500 μM dithionite). Trace B–Trace C is the difference spectrum of the $^{14}N^{16}O$-minus $^{15}N^{16}O$–Nor adduct. Trace D–Trace E is the difference spectrum of $^{14}N^{16}O$-$^{15}N^{16}O$. For comparison, the inset shows the ba_3-$^{14}N^{16}O$ minus ba_3-$^{15}N^{18}O$ difference spectrum [8]. The excitation laser wavelength was 244 nm and the incident laser power 150 μW. Different samples of Nor were accumulated for 4 min each, resulting in a total accumulation time of 100–120 min for each spectrum.

the ^{14}NO bound form of the oxidized enzyme (final concentration 130 μM Nor/500 μM dithionite). A small but noticeable increase in the intensity of the 1339 cm^{-1} mode is detected. Trace E was obtained after the addition of reductant to the ^{15}NO-bound form of the oxidized enzyme. A relative decrease in intensity and bandwidth of the 1339 cm^{-1} mode and the appearance of a new mode at 1302 cm^{-1} is observed. Similar observations were detected in the reaction of ba_3 with NO, ^{15}NO and $^{15}N^{18}O$. The presence of the 1302 cm^{-1} mode in both the ba_3 and Nor samples upon explosion to ^{15}NO strongly indicates the formation of the same species in both enzymes. The difference spectrum D–E ($^{14}N^{16}O$–$^{15}N^{16}O$) show the presence of a weak peak/trough pattern at 1332/1300 cm^{-1}, as compared to that observed in ba_3 (Fig. 1, inset) [8]. We suggest that the weak intensity of the peak/trough pattern at 1332/1300 cm^{-1} is due to the low concentration and/or short life time of the observed Nor/NO species. In the sodium, silver and mercury salts of the hyponitrous acid the fundamental frequency of the N–N bond of the hyponitrite ion (HO–N = N–O$^-$) is at 1392 cm^{-1} [15]. We assign the mode at 1332 cm^{-1} in Nor as the N–N stretching vibration of the hyponitrite species because of the $^{15}N^{16}O$ isotope shift and the similarity in the frequency with that observed in ba_3, and the similarity of its frequency to that reported for the hyponitrite ion (HO–N = N–O$^-$) [8,15]. The 6 cm^{-1} difference between the hyponitrite-bound to the binuclear center of ba_3 and that bound in the dinuclear center of Nor indicates that the N–N bond has less double bond character when bound to Nor. The small intensity of the 1332/1300 cm^{-1} pair, as compared with that observed at 1338/1300 cm^{-1} in ba_3, indicates the small population of the hyponitrite species detected under our