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Roles of Four Iron Centers in *Paracoccus halodenitrificans* Nitric Oxide Reductase

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Reactions of *Paracoccus halodenitrificans* nitric oxide reductase (NOR) containing four iron centers, a low spin heme*c*, a low spin heme *b*, a high spin heme *b* and a non-heme iron, have been studied to show the roles of each iron center. Soon after reacting the resting (oxidized) NOR with L-ascorbate, the low spin heme *c* and low spin heme *b* were reduced to a considerable extent but the high spin heme *b* was still in the oxidized form and was reduced slowly. When CO acted on the reduced NOR, the high spin heme *b* center changed to a low spin state. On the other hand, when NO acted on the resting NOR, no apparent spectral change was observed. However, when NO acted on the reduced NOR (a steady state condition, excess dithionite is present), both of the low spin centers changed to be partly in the oxidized form. A small but clear new EPR signal with g = 4.1 appeared together with some new signals at the g = 2 region soon after the action of NO on the reduced NOR. During incubation at room temperature the nitrosyl-heme signal typical of 5-coordination developed. These results suggested that both the high spin-heme *b* center and the non-heme iron are the reaction centers and their reductions are indispensable for the enzyme process in contrast to the reaction mechanism proposed for the P-450 type NOR(P-450nor).

The conversion of NO to N_2O in the denitrification process has been a black box because the relevant enzyme has not been isolated until recently because of its instability and difficulty of the enzyme assay (1-3). Two types of nitric oxidase reductase, cytochrome *bc* complex (NOR) and P-450 (P-450 nor) have been successfully purified from denitrifying bacteria (4-10) and from a fungus (11), respectively. Recently, the crystal structure of the latter has been reported and its reaction mechanism has became more clear (12). However, the structural and mechanistic studies of the former have not progressed much in spite of its importance, the wide distribution on the earth and the relevance to the global nitrogen cycle.

The cofactors contained in NOR have been revealed to be a low spin heme *c*, a low spin heme *b*, a high spin heme *b* and possibly a non-heme iron from spectroscopic and gene analyses (4-10, 13-15). The absorption spectrum of the reduced NOR is indicative of the feature from the low spin heme *c* and low spin heme *b*. The feature from the high spin heme *b* is not clear because of the masking effect by the low spin heme centers, although the band at about 595 nm in the resting enzyme was assigned to coming from it (8, 9). The presence of the three heme centers was clear from magnetic circular dichroism, cryogenic EPR and resonance Raman spectroscopies (8,9,14,15).

The studies on the structure genes of NOR have been very interesting (13,16-19). NOR is a heterodimer of a smaller subunit (NorC) and a larger subunit (NorB), the former has a binding site for a heme *c* and the latter binding sites for two hemes *b* and a non-heme iron. NorB has a striking sequence homology with that of the subunit I of bacterial cytochrome oxidases (COX). In line with this, we have recently sequenced the structure gene of *Paracoccus halodenitrificans* NOR and it has been found that NorB has a 19% sequence homology with the subunit I of the *Paracoccus denitrificans* COX (13). The twelve transmembrane segments have been similarly arranged in NorB and the subunit I of COX, giving the homologous architecture of the molecules. In addition, the ligand histidines for the low spin heme *b*, high spin heme *b*, and non-heme iron in NorB were found to be similarly positioned with those for heme *a*, heme a_3 and CuB in COX, respectively, except one of three histidines and the presence of the additional glutamate(s) for the non-heme iron. Saraste et al. (20, 21) and Zumft et al. (1, 22) have anticipated that COX has evolved from NOR. In addition, we recently found the presence of a putative proton transfer pathway in NorB (23). Two protons

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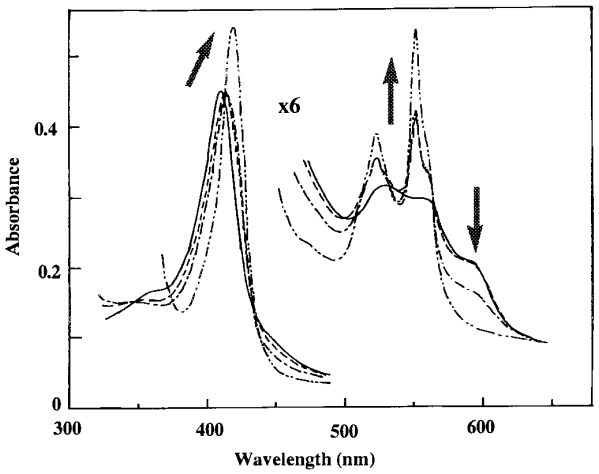

FIG. 1. Absorption spectra of NOR reacted with L-ascorbate (—— as isolated NOR, - - - obtained soon after reaction with L-ascorbate, — - — obtained after 1 min. incubation, and — - - — obtained after 30 min. incubation). 1.5 ml of NOR (0.16 mg of protein/mL) dissolved in 20 mM HEPES-NaOH buffer (pH 7.0) containing 0.05 % n-dodecyl-β-D-maltoside, 0.01% 2-phenylethanol, and 0.25 mM benzamidine was reacted with 1 mg of sodium L-ascorbate.

are required to convert 2NO to N_2O and H_2O. It is possible that the pathway to transfer the protons to be reacted have transformed to the pathway to transport the vectorial proton when COX evolved from NOR.

In spite of the importance of NOR, the roles of the four iron centers have not been well understood. Although it has been supposed that the low spin heme centers function as electron mediator from a soluble

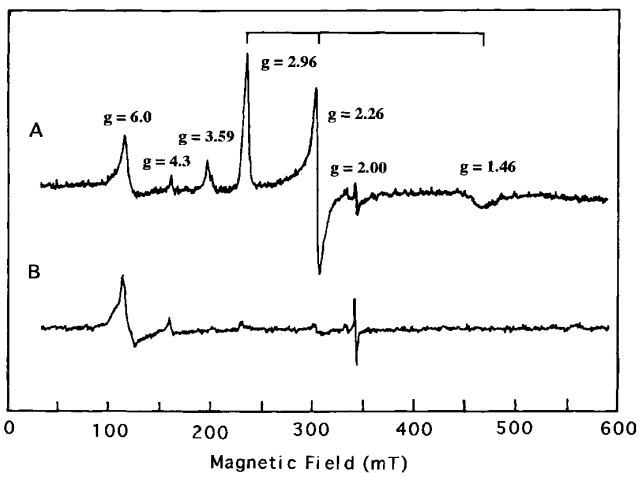

FIG. 2. EPR spectra of NOR in the oxidized form (A) and L-ascorbate-reacted NOR (B) at 3 K. The enzyme concentration was 1.5 mg/mL in 20 mM HEPES-NaOH buffer (pH 7.0) containing 0.05 % n-dodecyl-β-D-maltoside, 0.01% 2-phenylethanol, and 0.25 mM benzamidine. Sample for B was frozen soon after reacting L-ascorbate on NOR. Instrument conditions: frequency 9.483 GHz, microwave power 5.1 mW, modulation frequency 100 kHz, modulation amplitude 0.95 mT, time constant 0.08 s, and scan rate 6.5 min.

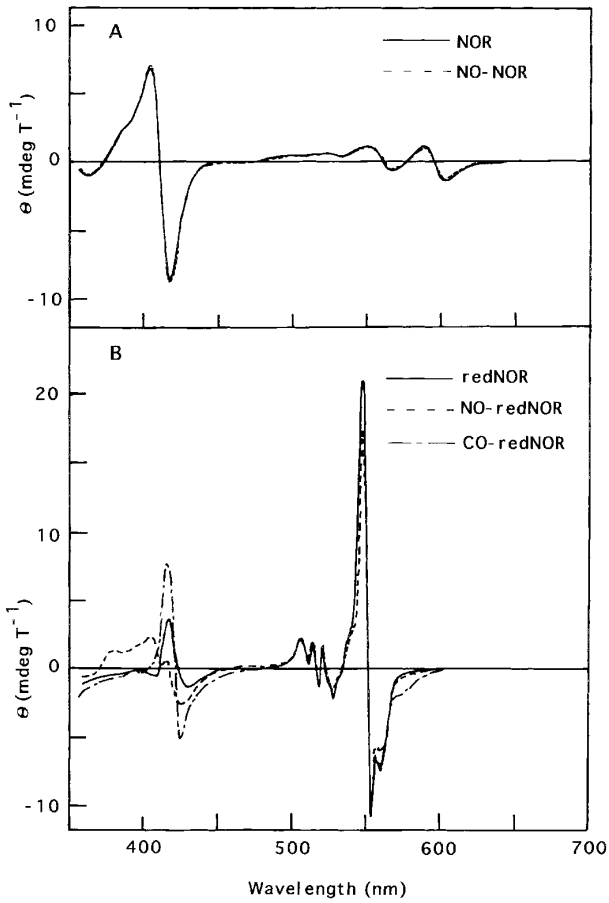

FIG. 3. Magnetic circular dichroism spectra of the resting (oxidized) NOR (——) and the NO-reacted NOR (- - -) (A) and the reduced NOR (——), the NO-reacted reduced NOR (- - -) and the CO-reacted reduced NOR (— - —) (B). NOR (1.1 mg/mL concentration) was dissolved in 20 mM HEPES-NaOH buffer (pH 7.0) containing 0.05 % n-dodecyl-β-D-maltoside, 0.01% 2-phenylethanol, and 0.25 mM benzamidine. The magnetic field applied was 1.4 T.

cytochrome *c* to the high spin heme *b* center and also possibly a non-heme center, there has been very few data to discuss the reaction mechanism. In the present communication we show both the high spin heme *b* and non-heme iron are involved in the reaction process. Cytochrome *bc* type NOR is supposed to follow a quite different reaction mechanism from that of P-450nor.

MATERIALS AND METHODS

NOR was purified from *Paracoccus halodenitrificans* as reported (9). All procedures for spectral measurements were performed under an anaerobic condition using quartz cells attached to a three-way stop cock on their heads. To react NO and CO on the reduced NOR, 5% NO gas (Ar 95%) and 100% of CO gas were used, respectively.

The absorption spectra were measured on a JASCO Ubest 50 spectrometer and the MCD spectra on a JASCO J-720W spectropolarimeter equipped with an electromagnet at 1.4T. The X-band EPR spectra were obtained with a Bruker ESP-300E spectrometer attached to an Oxford liquid helium cryostat at 3 -12 K and with a JEOL FE-2X spectrometer at 77 K.

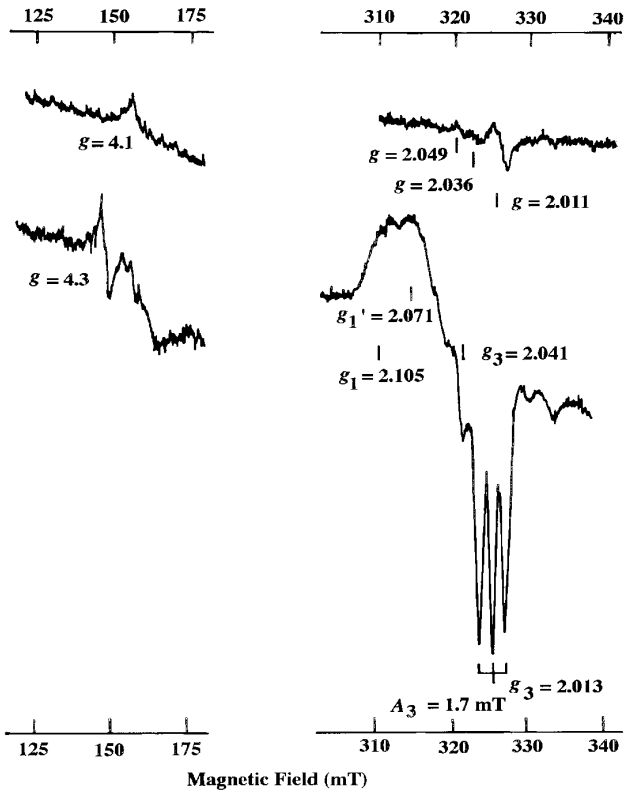

FIG. 4. EPR spectra of the NO-reacted reduced NOR at 77 K. The top spectrum was obtained soon after the action of NO and the bottom spectrum after incubation for 15 min at room temperature. Instrument conditions: frequency 9.18 GHz, microwave power 6.3 mW, modulation frequency 100 kHz, modulation amplitude 1 mT, time constant 0.03 s, and scan rate 8 min.

RESULTS AND DISCUSSION

The reaction with L-ascorbate. Soon after addition of a large excess of L-ascorbate on the resting (oxidized) NOR, in which all irons are in the ferric form, the bands due to the reduced low spin heme *c* and low spin heme *b* appeared at 551 and 556 nm, respectively (Fig. 1). The band due to the oxidized high spin heme *b* at ca. 595 nm did not decrease. However, it gradually decreased concomitantly with the further slight increase in the bands at 551 and 556 nm, and the spectrum for the completely reduced form was obtained finally.

The EPR spectrum of the resting enzyme showed the signals due to the low spin heme *c* at g = 2.96, 2.26, 1.46, the low pin heme *b* at g = 3.59 and the high spin heme *b* at g = 6.0 (Fig. 2) (Cheesman et al. (14) assigned the signals for heme *c* and low spin heme *b* of *Pseudomonas stuzeli* NOR in the reversed way). While the highly sensitive signal at g = 4.3 was strong in the former preparation (9), it was considerably small and it could be assigned to be an adventitious Fe^{3+}. The sharp signal at g = 2.00 has been assigned to be due to the non-heme iron by Girsch and de Vries (8). Although the signal is very small in our NOR differing from their result, it was not saturated easily differing from an organic radical (data not shown) and accordingly, we also assigned it to coming from the non-heme iron. When L-ascorbate was acted on NOR, the intensities of the low spin signals decreased dramatically but the signal of the high spin signal was intact in harmony with the result for absorption spectra (Fig. 2). As for the g = 2.00 signal, it was not clear because semidehydroascorbate masked the region g ~ 2. Nevertheless, all these spectral features seem to indicate that the low spin centers are electron mediators and the high spin center is the reaction site.

The reactions with NO and CO. MCD spectra of the resting and NO-acted resting NOR (Fig. 3) are practically same, showing that NO does not bind to the resting NOR. However, the MCD spectra of the NO- and CO-acted reduced NOR's changed from that of the fully reduced NOR (Fig. 3). When the reduced NOR was treated with CO, the features characteristic to the reduced low spin heme *c* and low spin heme *b* (545 to 560 nm) were intact, but another smaller low spin-like band (negative band) appeared at ca. 575 nm and the Soret band region showed only the low spin character differing from that of the resting enzyme (Fig. 3). On the reaction of NO the peculiar features between 545 and 560 nm characteristic to the low spin hemes *c* and *b* and the feature for the Soret band region characteristic to the high spin heme *b* changed unequivocally. The intensities of the sharp bands at 547, 555 and 560 nm decreased and the features at the Soret band region were that of a mixture of high and low spin characters, indicating that low spin centers are partly in the oxidized form under the steady state condition. These behaviors exerted by NO and CO on the reduced NOR strongly suggest that NO and CO are bound to the high spin heme *b* center to be in the low spin form.

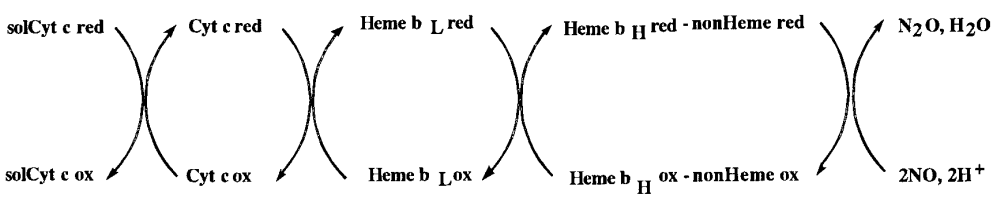

FIG. 5. The reaction scheme of NOR starting from the soluble cytochrome *c* to the high spin hem *b*-non heme iron binuclear center.

The corresponding EPR spectra showed a small but clear signal at g = 4.1 soon after reacting the reduced NOR with NO (Fig. 4). In addition, the small signals were also observed at g = 2.040, g = 2.036 and g = 2.011. The similar signals originating from Fe^{3+}-NO^{-} have been reported for the NO-acted non-heme iron proteins (24, 25). When the NO-acted reduced NOR was incubated at room temperature, the strong signal typical of the 5-coordinate nitrosyl-heme developed (g_1 = 2.105, g_2 = 2.041, g_3 = 2.013, A_3 = 1.7 mT) (26). This species is considered to be formed by the strong binding of NO to Fe^{2+} so as to eliminate the axial ligand at the opposite site. Concomitantly, the signal due to the adventitious Fe^{3+} (g = 4.3) appeared even at 77 K, although its intensity was still considerably weak. Together with the results from MCD spectra, behaviors shown by EPR spectroscopy strongly suggest that both the high spin heme*b* and the non-heme iron are directly concerned in the reduction of NO. Nevertheless, the signal intensities due to the intermediates (g = 4.1) seem to be very weak compared to the 5-coordinate-nitrosyl heme signal due to a denatured form of the enzyme. This is probably because the main intermediate is EPR-undetectable or its population is considerably low due to a short life time. Very recently, Moënne-Loccoz and de Vries (15) proposed that the NO-bound heme *b* is 5-coordinate during reaction based on the resonance Raman spectra of the CO-NOR. However, the signal typical of the 5-coordinate nitrosyl heme increased during incubation, suggesting it may come from a denatured form. The 6-coordinate low spin state seems to be more probable than of the 5-coordinate state as for the intermediate form of the high spin heme *b* center from the MCD spectra of the NO and CO reactions (Fig. 3).

Fig. 5 shows the roles of four iron centers. Electrons furnished from the soluble cytochrome *c* are gated by the heme *c* on the periplasmic side of NorC and transferred to the binuclear center composed of the high spin heme *b* and non-heme iron via the low spin heme *b* in NorB. Two NO's are supposed to interact with the reduced binuclear centers differing from the case of P-450nor, in which NADH furnishes two electron to the NO bound to the central Fe^{3+}. The binuclear structure might be favorable for the reductive bimolecular process.

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