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Structural characterization of a binuclear center of a Cu-containing NO reductase homologue from *Roseobacter denitrificans*: EPR and resonance Raman studies

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Abstract

Aerobic phototrophic bacterium *Roseobacter denitrificans* has a nitric oxide reductase (NOR) homologue with cytochrome *c* oxidase (CcO) activity. It is composed of two subunits that are homologous with NorC and NorB, and contains heme *c*, heme *b*, and copper in a 1:2:1 stoichiometry. This enzyme has virtually no NOR activity. Electron paramagnetic resonance (EPR) spectra of the air-oxidized enzyme showed signals of two low-spin hemes at 15 K. The high-spin heme species having relatively low signal intensity indicated that major part of heme b_3 is EPR-silent due to an antiferromagnetic coupling to an adjacent Cu_B forming a Fe–Cu binuclear center. Resonance Raman (RR) spectrum of the oxidized enzyme suggested that heme b_3 is six-coordinate high-spin species and the other hemes are six-coordinate low-spin species. The RR spectrum of the reduced enzyme showed that all the ferrous hemes are six-coordinate low-spin species. ν(Fe–CO) and ν(C–O) stretching modes were observed at 523 and 1969 cm^{-1}, respectively, for CO-bound enzyme. In spite of the similarity to NOR in the primary structure, the frequency of ν(Fe–CO) mode is close to those of aa_3- and bo_3-type oxidases rather than that of NOR.

Keywords: Cytochrome *c* oxidase; Nitric oxide reductase; Respiratory; *Roseobacter denitrificans*; EPR spectroscopy; Resonance Raman spectroscopy

1. Introduction

Many prokaryotic and mitochondrial terminal oxidases in aerobic respiration belong to a large superfamily called heme–copper oxidase family. Enzymes of the superfamily have common structural feature, binuclear center, formed by heme–iron and adjacent copper called Cu_B [1–3]. At the binuclear center, oxygen is reduced to water and the redox reaction is coupled to the translocation of protons across the bacterial cell membrane or the mitochondrial inner membrane [2]. Cytochrome *c* oxidases (CcO) of aa_3-type have been the most intensively studied member of the family that contains low-spin heme *a*, high-spin heme a_3, and Cu_B in the catalytic subunit [4,5]. Amino acid residues that ligate those redox metal centers and distances between the metals are well known because the crystal structures were reported for the complexes from *Paracoccus denitrificans* [6] and bovine [7,8]. The heme–copper oxidase family includes bo_3-type quinol oxidase and cbb_3-type CcO [4,5]. These oxidases possesses *o*- or *b*-type high-spin heme, respectively, in the binuclear center. The catalytic sites have been investigated spectroscopically and magnetically, e.g. resonance Raman (RR), Fourier transform infrared (FTIR), and electron paramagnetic resonance (EPR) studies on bo_3-type quinol oxidase suggested that it has similar properties in the structure of binuclear center with aa_3-type CcO [9–12]. A cbb_3-type oxidase, on the other hand, is reported to be different from other heme–copper oxidases in that it may have a relatively open structure in the binuclear center [13]. Nitric oxide reductase (NOR), one of the terminal enzymes in denitrifying respiration, is the most distant member of the superfamily [14,15]. It contains low-spin heme *c* in the NorC subunit and low-spin heme *b*, high-spin heme b_3, and

Abbreviations: CcO, cytochrome *c* oxidase; EPR, electron paramagnetic resonance; NOR, nitric oxide reductase; RR, resonance Raman

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non-heme iron in the NorB subunit. The binuclear center is formed by heme b_3 and non-heme iron [16], and it has been characterized by EPR and RR studies [16–22]. High-spin heme b_3 of this enzyme was suggested to be five-coordinate high-spin species both in the oxidized and reduced forms [20], and the frequency of ν(Fe–CO) stretching mode is reported to be substantially low (476 cm^{-1}) compared to those of heme–copper oxidases [20].

Roseobacter denitrificans is a marine aerobic phototrophic bacterium that contains bacteriochlorophyll *a*. It synthesizes a complete photosynthetic apparatus when grown under dark aerobic condition, and the synthesis is suppressed when grown under light condition [23]. *R. denitrificans* does not grow anaerobically unless the culture medium contains alternative electron acceptor, such as trimethylamine *N*-oxide or nitrate [24,25]. *Roseobacter* belongs to α-3 subclass of proteobacteria together with facultative phototrophs *Rhodobacter* and aerobic heterotrophs *Paracoccus* [26,27].

We have isolated a cytochrome *cb*-type complex with CcO activity from *R. denitrificans* [28]. The enzyme is composed of two subunits homologous with NorC and NorB subunits of NOR. Primary structures of the two subunits are 68.7% and 78.6% identical to those of NorC and NorB, respectively from *P. denitrificans* [28]. Heme and metal compositions of this enzyme were analyzed by forming pyridine hemochrome and by using inductively coupled plasma mass spectrometry, respectively, and were indicated to be in a stoichiometry of heme *c*: heme *b*: Cu = 1:2:1 [28]. Two subunits of the enzyme are detected at the position of 18 and 37 kDa in the SDS-PAGE analysis. The former is positive to heme staining, indicating that this polypeptide contains a heme *c* [28]. The latter has six histidine residues that are conserved and ligate two hemes and a non-heme metal in the catalytic subunit of the heme–copper oxidases [28]. This suggests that the larger subunit binds two hemes *b* and a copper atom and one of the hemes *b* forms a binuclear center with a copper atom as in the cases of the heme–copper oxidases. We temporarily call this enzyme "NOR-homologue", because the enzyme differs from NOR in that it contains a copper atom as the non-heme metal and has virtually no NOR activity [28]. Since the features that the enzyme possesses CcO activity and associates a copper atom as the non-heme metal are common with heme–copper oxidases, it is of great interest to compare the structure of the catalytic site of this unique enzyme to those of heme–copper oxidases and NOR.

In this paper, we report EPR and RR studies on the NOR homologue. The binuclear center that has been suggested to be formed by heme b_3 and Cu_B [28] is confirmed from EPR spectrum of the oxidized form. RR spectra suggest that the heme b_3 is in a six-coordinate high-spin state in the oxidized form and in a six-coordinate low-spin state in the reduced form. The latter property is peculiar to this enzyme in the members of heme–copper oxidase superfamily. The frequency of ν(Fe–CO) stretching mode of the CO-bound form is similar to those of aa_3- and bo_3-type oxidases rather than that of NOR. This suggests that aa_3- and bo_3-type oxidases and the NOR homologue share some common structural properties in the catalytic sites, while NOR does not. We discuss the results from this study with respect to comparison with the enzymes of heme–copper oxidase superfamily.

2. Experimental procedures

2.1. Purification of the NOR homologue from *R. denitrificans*

The NOR homologue was purified from aerobically grown *R. denitrificans* according to the procedure previously reported [28] with some modifications. Membrane fractions were prepared as previously reported [28] and the NOR homologue was solubilized with 1% (w/v) sucrose monocaprate. After stirring for 2 h at 4 °C, the solubilized preparation was centrifuged at 370,000 × *g* for 3 h. The supernatant was applied to anion-exchange chromatography on a DEAE-Sepharose CL-6B column (3.5 × 5.0 cm) that had been equilibrated with 50 mM Tris–HCl buffer (pH 7.2) containing 0.3% (w/v) sucrose monocaprate. The column was washed with the same buffer containing 0.13 M NaCl, and proteins were eluted by 200 ml of liner salt gradient from 0.13 to 0.5 M NaCl. The fractions with CcO activity were pooled and concentrated by VIVASPIN 6 (VIVASCIENCE) to about 1 ml. The preparation was applied to gel-filtration on a Sephacryl S-200 column (2.5 × 95 cm) which had been equilibrated with 50 mM Tris–HCl buffer (pH 7.2) containing 0.3% (w/v) sucrose monocaprate and 100 mM of NaCl. Proteins were eluted from the column and the eluates with CcO activity were applied to a Mono Q HR5/5 column (1 ml, Pharmacia Biotech). The column, which is adapted to a FPLC system (Pharmacia Biotech, model GP-250 PLUS), had been equilibrated with 20 mM Tris–HCl (pH 7.2) containing 0.3% (w/v) TritonX-100. TritonX-100 yielded better results than sucrose monocaprate for the separation of the proteins at this step. After the column was washed with the same buffer containing 0.15 M NaCl, proteins were eluted by a liner salt gradient from 0.15 to 0.7 M NaCl. The NOR homologue was eluted at around 0.3 M NaCl and the preparation was diluted 2-fold with the same buffer and reloaded on the same column. Purified preparation was obtained by the rechromatography and stored at − 80 °C after freezing by liquid nitrogen. Enzyme 1.2–1.4 mg was obtained using 45–50 g wet weight of *R. denitrificans* cells. To concentrate the enzyme preparation using membrane filters, detergent was exchanged from TritonX-100 to sucrose monocaprate. The purified preparations were loaded on the MonoQ or the DEAE column, which had been equilibrated with 50 mM Tris–HCl (pH 7.2) containing 0.5% (w/v) sucrose monocaprate. The column was washed with the same buffer and the enzyme was eluted with the buffer containing NaCl. The

eluates were concentrated using VIVASPIN 6 and diluted with 50 mM Tris–HCl (pH 7.2) containing 0.3% sucrose monocaprate. This was repeated twice to remove NaCl. The concentrated enzyme was used for the measurement of EPR and RR spectra.

2.2. Activity assay

CcO activity was measured with reduced horse heart cytochrome *c* as an electron donor, by monitoring the absorption change at 550 nm using an UV-2400 spectrophotometer (Shimadzu, Kyoto, Japan). The reaction was started by addition of the sample to reaction mixture containing 50 mM Tris–HCl (pH 7.2), 0.3% (w/v) sucrose monocaprate, 100 mM NaCl, and horse heart cytochrome *c*. The reaction was measured at room temperature.

2.3. EPR spectra

EPR measurements were carried out at 5 or 15 K at X-band (9.218 GHz) microwave frequency with a Varian E-12 EPR spectrometer equipped with an Oxford flow cryostat (ESR-900).

2.4. Optical spectra

Absorption spectra were measured with an UV-2400 spectrophotometer (Shimadzu). Enzyme preparation was diluted with 50 mM Tris–HCl buffer (pH 7.2) containing 0.3% (w/v) sucrose monocaprate for the measurement. Reduced form of the enzyme was prepared by adding dithionite solution (final 10 mM) into degassed enzyme solution under Ar atmosphere. Reduced enzyme was exposed to CO for 60 min in an anaerobic cell to prepare CO-bound form.

2.5. RR spectra

RR spectra were obtained with a single polychromator (Ritsu Oyo Kogaku, DG-1000) equipped with a liquid N_2-cooled charge coupled device (CCD) detector (Prinetone Instruments, Model LN/CCD 1100-PB). The excitation wavelengths used were 413.1 nm from a Kr^+ ion laser (Spectra Physics, Beam Lock 2060) for the oxidized, reduced, and CO-bound form of the enzyme, and 422.6 nm from a diode laser (Hitachi Metals Model ICD-430) and 406.7 nm from a Kr^+ laser for CO-bound form of the enzyme. The laser power at the sample point was adjusted at 0.1–1 mW for the CO-bound form to minimize photodissociation of the heme-bound CO, at 0.1 mW for the oxidized form to avoid photo-reduction of the enzyme, and at 7 mW for the reduced form. Raman shifts were calibrated with indene and potassium ferrocyanide to an accuracy of ± 1 cm^{-1} for intense isolated bands. The reduced form was prepared by adding freshly prepared sodium dithionite solution (final 15 mM) into the degassed enzyme solution under N_2 atmosphere. The CO-bound form was prepared by addition of CO gas into an airtight Raman cell containing the reduced form of the enzyme. All measurements were performed with a spinning cell (diameter $\phi = 5$ mm, 2000 rpm) at room temperature. The enzyme concentration for RR experiments was 20 μM in 50 mM Tris–HCl (pH 7.2) containing 0.3% (v/w) sucrose monocaprate.

3. Results

3.1. CcO activity of the NOR homologue from R. denitrificans

The NOR homologue was purified from *R. denitrificans* as described above. With horse cytochrome *c* as an electron donor, 1 mg of the enzyme oxidized 3.1 ± 0.5 (S.D.) μmol of cytochrome *c* per minute.

3.2. EPR spectroscopy

EPR spectra of the air-oxidized form of the NOR homologue were measured at 5 and 15 K (Fig. 1). Each of the spectra are very similar to the spectrum measured with oxidized NOR [18]. Signals in both of the spectra (Fig. 1) suggest that this enzyme contains one high-spin heme and two low-spin hemes. One of the low-spin heme showed signals with rhombic trio at $g_z = 2.96$, $g_y = 2.28$, and $g_x = 1.45$. Another low-spin species exhibited a high g_{max} at $g = 3.57$. High-spin heme signal was observed at $g = 6.30$, but the intensity was relatively weak to that of $g = 2.96$ low-spin heme signal. In addition, the EPR signal corresponding

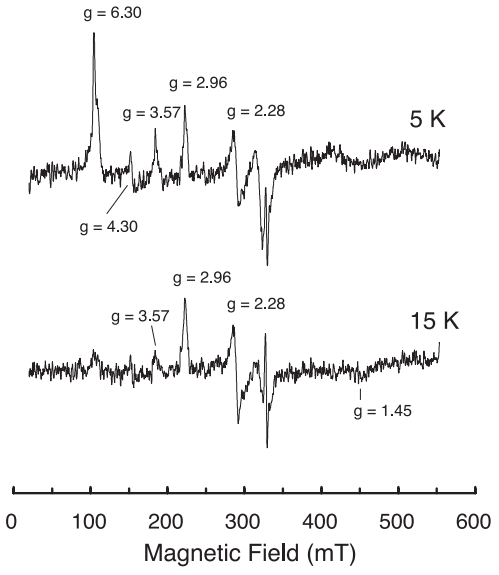

Fig. 1. X-band EPR spectra of the NOR homologue in the oxidized form. The enzyme concentration was 90 μM in 50 mM Tris–HCl (pH 7.2) containing 0.3% sucrose monocaprate. Measurements were performed under the following conditions: microwave power, 10 mW; microwave frequency, 9.218 GHz; amplitude of 100 kHz magnetic field modulation, 1 mT.

to Cu_B^{2+} was not observed both at 5 and 15 K. It indicates that a major part of the high-spin heme was spin–spin exchange-coupled to the Cu_B center and was EPR-invisible as usual for heme–copper oxidases in the oxidized form [10]. The $g=6.30$ signal may be derived from a small amount of uncoupled high-spin heme. The uncoupling between high-spin heme and Cu_B may be caused by denaturation of the binuclear center.

3.3. Optical spectra

The optical absorption spectra of the NOR homologue were measured in the air-oxidized form (Fig. 2 (a)), dithionite-reduced form (b), and CO-bound form (c). The results were basically the same as those previously reported [28]. The enzyme showed absorption maxima at 411 and 530 nm in the oxidized form, and maxima at 421, 522, and 551 nm, and a shoulder at around 560 nm in the reduced form. The spectral features in the α-band region confirmed the presence of heme *b* and heme *c*. The CO-bound form of the enzyme displayed absorption maxima at 420, 521, and 551 nm, and a shoulder at around 560 nm. In the difference spectrum between the traces c and b (Fig. 2, inset), there are maxima at 418, 535, and 570 nm, and troughs at 430, and 551 nm.

3.4. RR spectra

RR spectrum of the high-frequency region includes the so-called marker bands whose frequencies are known to be sensitive to the iron coordination, spin, and oxidation states

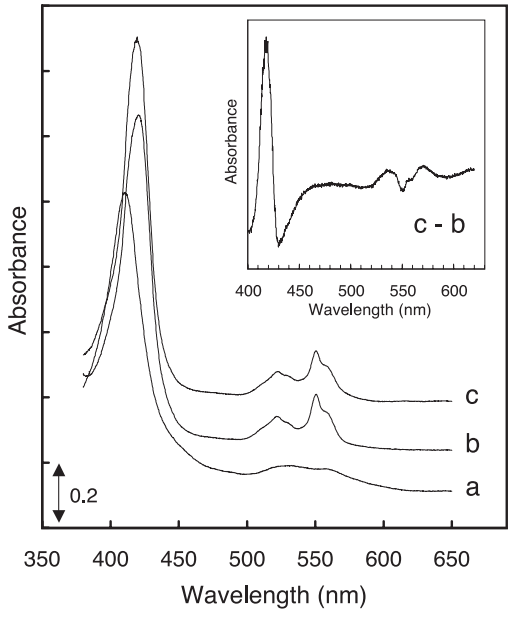

Fig. 2. Optical spectra of air-oxidized (a), dithionite-reduced (b), and CO-bound form (c) of the NOR homologue. Reduced and CO-bound forms of the enzyme were prepared under anaerobic conditions. Inset is a difference spectrum obtained by subtracting trace b from trace c. Enzyme concentration was 3.6 μM.

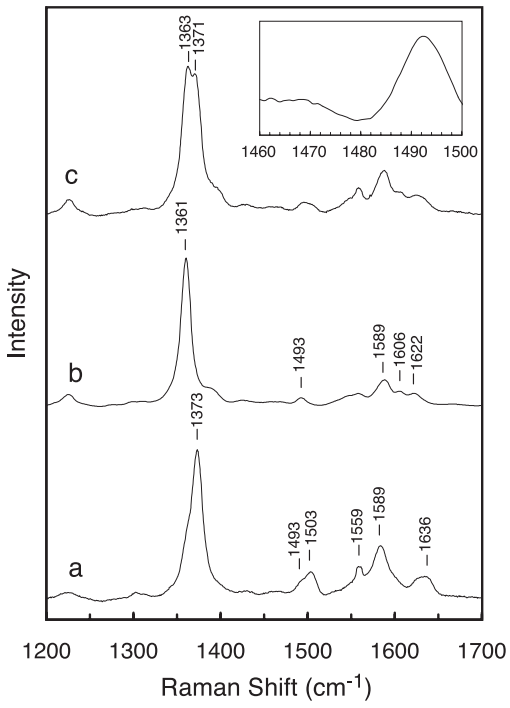

Fig. 3. High-frequency RR spectra of oxidized (a), reduced (b), and CO-bound form (c) of the NOR homologue. Inset shows an amplified RR spectrum of the reduced enzyme (trace b) in the 1460–1500 cm^{-1} region. Experimental conditions were: excitation wavelength, 413.1 nm; laser power at the sample point, 0.1 mW for spectra a and c, 7 mW for spectrum b; accumulation time, 30 min for spectra a and c, 5 min for spectrum b; at room temperature; enzyme concentration, 20 μM.

of the heme [29]. Fig. 3 shows the RR spectra of air-oxidized (a), reduced (b), and CO-bound form (c) of the enzyme in the high-frequency region excited at 413.1 nm. In the oxidized form, the ν_4 band was observed at 1373 cm^{-1}, a frequency characteristic of ferric heme. The ν_3, ν_2, and ν_{10} bands were observed at 1503, 1589, and 1636 cm^{-1}, respectively, indicating the presence of six-coordinate low-spin heme. These bands may derive from a heme *c* and one of two hemes *b*. Also present in this spectrum are modes at 1493 (ν_3) and 1559 (ν_2) cm^{-1}. The 1559 cm^{-1} (ν_2) band indicates that heme b_3 is in a six-coordinate high-spin state. Considering the previous studies using model compounds [30], the frequency of the ν_3 band, 1493 cm^{-1}, seems to be high as it derived from six-coordinate high-spin heme, but a similar case was observed in a RR spectrum of cbb_3-type CcO from *Rhodobacter sphaeroides* [31].

In the RR spectrum of reduced enzyme (Fig. 3, trace b), ν_4 band was observed at 1361 cm^{-1} indicating that all three hemes are in the reduced state. The ν_3 band at 1493 cm^{-1} and ν_2 band at 1589 cm^{-1} establish the presence of six-coordinate low-spin heme species. On the other hand, no obvious features for the five-coordinate heme species are observed in the spectrum, e.g. ν_3 band of the five-coordinate high-spin heme b_3 of reduced NOR from *P. denitrificans* was observed at 1470 cm^{-1} in the RR spectrum [20] but the spectrum of the NOR homologue does not show the

corresponding band clearly (Fig. 3, inset). This suggests that ferrous *heme-b_3* iron of the NOR homologue is in a six-coordinate low-spin state. This is also suggested from RR spectrum of the reduced enzyme in the 200–250 cm^{-1} range. Vibration of the Fe-ligand along the axis normal to the heme b_3 was observed at 208 cm^{-1} in the RR spectra excited at 413.1, 422, or 442 nm, but its intensity was very weak (data not shown). The frequency of 208 cm^{-1} is appropriate for ν(Fe-His) stretching mode, which has been observed in a range from 210 to 215 cm^{-1} in the RR spectrum of heme a_3 in aa_3-type CcO [32,33], and this vibration mode is not resonance enhanced in the six-coordinate state [34]. The weak intensity of the ν(Fe-His) mode indicates that the bulk of the ferrous heme b_3-iron of the NOR homologue are in the six-coordinate low-spin state. The presence of the six-coordinate catalytic heme in the reduced form discriminates this enzyme from other members of heme–copper oxidase superfamily. Upon the addition of CO to the reduced form of the enzyme, another band appeared at 1371 cm^{-1} (Fig. 3, trace c) and its intensity was diminished at a higher laser power (data not shown). These indicate the coordination of CO to the ferrous heme b_3 and the photo-dissociation of CO from the heme, respectively. A similar feature has been reported in the RR spectrum of CO-bound NOR: the ν_4 mode is observed at 1360 and 1371 cm^{-1}, and the latter is assigned to CO-ligated heme b_3 component [20].

The RR spectrum of ^{12}CO-bound form of the NOR homologue reveals bands at 523 and 567 cm^{-1} (Fig. 4, trace a), both of which are sensitive to $^{12}C/^{13}C$ isotopic substitution and shift to 520 and 561 cm^{-1}, respectively, in the ^{13}CO derivative (Fig. 4, trace b). The shifts are obvious for two bands at 523 and 567 cm^{-1} in the difference spectrum between ^{12}CO- and ^{13}CO-bound forms (Fig. 4, bottom trace). The bands at 523 and 567 cm^{-1} are assigned to the Fe–CO stretching [ν(Fe–CO)] and Fe–C–O bending [δ(Fe–C–O)]. RR spectra in the 1700–2100 cm^{-1} range are shown in Fig. 5. The ν(C–O) stretching mode of the ^{12}CO-bound form (a) is observed at 1969 cm^{-1}, which shifts to 1927 cm^{-1} in the ^{13}CO isotopic substitution (b). The assignment is evident in the difference spectrum between the ^{12}CO and ^{13}CO adducts.

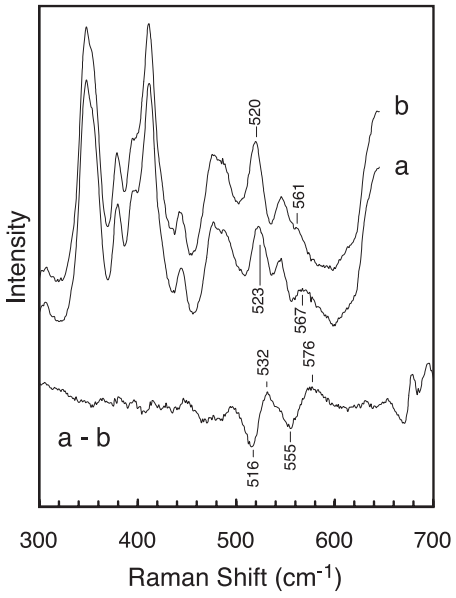

Fig. 4. RR spectra of ^{12}CO- (a) and ^{13}CO-bound form (b) of the NOR homologue in the 300–700 cm^{-1} region. Bottom trace is a difference spectrum obtained by subtracting trace b from trace a. Experimental conditions were: excitation wavelength, 406.7 nm; laser power at the sample point, 0.85 mW; accumulation time, 30 min; at room temperature; enzyme concentration, 20 μM.

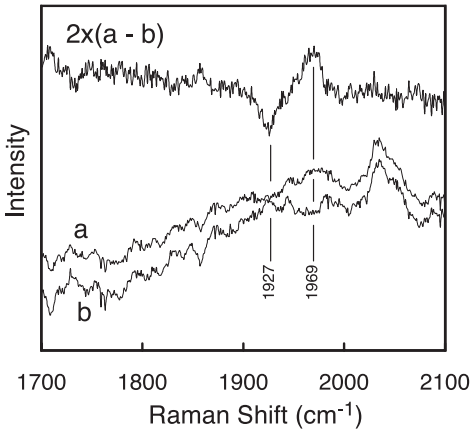

Fig. 5. RR spectra of ^{12}CO- (a) and ^{13}CO-bound form (b) of the NOR homologue in the 1700–2100 cm^{-1} region. Top trace is a difference spectrum obtained by subtracting trace b from trace a. It is amplified 2-fold. Experimental conditions were: excitation wavelength, 422.6 nm; laser power at the sample point, 0.43 mW; accumulation time, 1 h; at room temperature; enzyme concentration, 20 μM.

4. Discussion

4.1. Coordination structure of the binuclear center

EPR spectra of the NOR homologue in the oxidized form revealed features peculiar to two low-spin hemes and one high-spin heme (Fig. 1). A high g_{max} value at $g=3.57$ can be assigned to a low-spin heme *c* and a rhombic trio at $g=2.96$, $g=2.28$, and $g=1.45$ to a low-spin heme *b*. Another *b*-type heme of this enzyme in the high-spin state is probably coupled antiferromagnetically to an adjacent Cu_B as usual for the resting heme–copper oxidases, resulting in an EPR-silent binuclear center.

4.2. Binuclear center in the reduced NOR homologue

As described above, RR spectroscopy suggested that majority of the ferrous heme b_3-iron is in the six-coordinate low-spin state. It is an anomaly for the enzymes of heme–copper oxidase superfamily. aa_3-, bo_3-, and cbb_3-type oxidases and NOR have a five-coordinate high-spin heme in

the reduced state [9,20,31,35]. Yoshikawa et al. [36] proposed, in the resting state of aa_3-type CcO, peroxy species is a bridging ligand between Fe^{3+} and Cu_B^{2+}, while Ostermeier et al. [37] proposed a water molecule bound to heme a_3-iron and a hydroxy ion bound to Cu_B. In the oxidized NOR, an oxo-bridged heme/non-heme diiron center (Fe^{3+}–O–Fe^{3+}) is proposed from the RR studies using model compound [21]. The NOR homologue may also have a bridging ligand in the oxidized binuclear center because ferric heme b_3 is suggested to be in the six-coordinate state and EPR silence of heme b_3 and Cu_B implies antiferromagnetic coupling through a ligated species. As ferric heme b_3 is reduced to ferrous state, the bridging ligand would be ejected from the site, and then, another ligand would bind to the ferrous heme b_3 immediately. The sixth ligand of the ferrous heme b_3 is unknown at present. Candidates would be amino acid residues near Fe_{b3}^{2+} that have side-chain capable of generating ferrous low-spin heme species [38]. H194, one of the putative Cu_B ligands [28] corresponding to H240 in bovine cytochrome aa_3 [7], is a potential sixth ligand of ferrous heme b_3. Imidazole group of H194 might bind both Cu_B and heme–Fe or bind only to heme–Fe in the reduced form. This speculation would be plausible to explain the six-coordinate low-spin state of ferrous heme b_3, but it is somewhat incompatible with the following features of the optical spectra. In the reduced spectrum (Fig. 2, trace b), the intensity of the shoulder at around 560 nm is small coming from two equivalents of bis-histidine ligated low-spin heme *b*, compared to the intensity of the peak at 551 nm coming from one heme *c* (trace b). In the CO-bound difference spectrum (Fig. 2, inset), the trough at 430 nm is a feature of the low-spin ferrous *b*-type heme [39] but features in the α-band region are typical of high-spin ferrous *b*-type heme [39,40].

The frequencies of ν(Fe–CO) and ν(C–O) modes are rich in information to infer the environments of catalytic site. Fig. 6 shows the well-known ν(Fe–CO) versus ν(C–O) correlation plot characteristic of hemoproteins with a proximal nitrogenous ligand and with a proximal thiolate ligand. The frequencies of ν(Fe–CO) and ν(C–O) of hemoproteins and many model compounds are known to correlate linear negatively [42,46,50]. This negative correlation is attributed to the Fe $d_\pi \rightarrow$ CO π^* back-donation: as it increases, the Fe–CO bond is strengthened and the C–O bond is weakened [50]. The data points for aa_3- (◆, ○) and bo_3-type (×) oxidases, however, are far off from the curve of proteins with proximal histidine. The reason had been well discussed and the studies combining spectroscopic analysis and mutational technique showed that it is attributed to steric effects caused by close proximity of Cu_B to the distal side of heme a_3/o_3 [51,52]. The replacement of a histidine residue, which ligates Cu_B, brings perturbation in the Cu_B site and the frequency of ν(Fe–CO) mode decreased to the degree such that the data points fall on the correlation curve [51,52]. The data point for cbb_3-type CcO from *R. capsulatus* (+) lies slightly below the ν(Fe–CO) versus ν(C–O) correlation curve (Fig. 6). It is

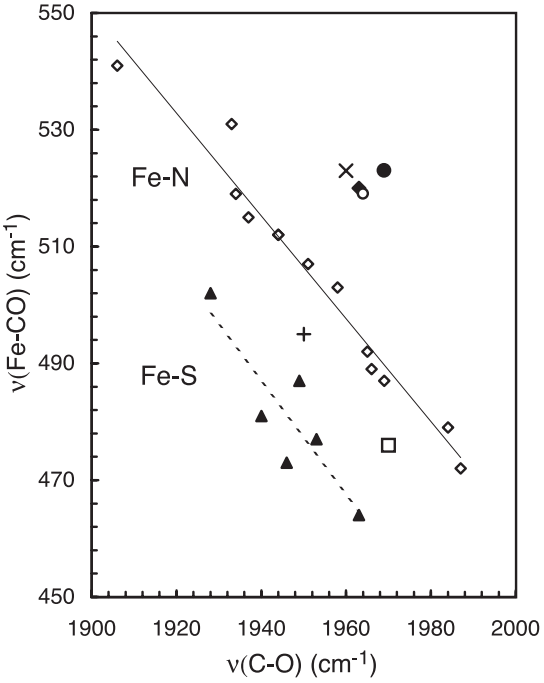

Fig. 6. Correlation between frequencies of the ν(Fe–CO) versus ν(C–O) of hemoproteins. The solid curve is for proteins with a proximal nitrogenous ligand (◇) such as hemoglobin [41], myoglobin [41], horseradish peroxidase [42], heme–heme oxygenase [43], GC [44], and CooA [45], and others [46]. The dashed curve is for proteins with a thiolate proximal ligand (▲) such as cytochrome P-450 [42,46] and nitric oxide synthase [47]. The NOR homologue from *R. denitrificans* is designated by ●, aa_3-type CcO from bovine by ◆ [48], aa_3-type CcO from *R. sphaeroides* by ○ [48], bo_3-type quinol oxidase from *Escherichia coli* by × [48], cbb_3-type CcO from *Rhodobacter capsulatus* by + [13,49], and NOR from *P. denitrificans* by □ [20]. Histidine residue is the heme proximal ligand in these six enzymes.

translated that this enzyme has a relatively open structure in the heme b_3–Cu_B pocket, compared to the α-form of aa_3- and bo_3-type oxidases, and lacks the distal effects exerted by Cu_B [13]. On the other hand, the data point for NOR from *P. denitrificans* (□) lies far below from those of the other enzymes described above. The frequency of ν(Fe–CO) mode is 43–47 cm^{-1} lower than the mode observed in aa_3- and bo_3-type oxidases. Moënne-Loccoz and de Vries [20] suggested that the weak bonding between Fe and C atoms may be attributed to an unusual environment in the heme b_3–Fe pocket: it is negatively charged and the steric effects that distort Fe–C–O linkage are minimized.

The CO-bound NOR homologue from *R. denitrificans* reveals ν(Fe–CO) and ν(C–O) modes at 523 and 1969 cm^{-1}, respectively, and the data point (●) is far off from the correlation curve and is close to the points of aa_3- and bo_3-type oxidases (Fig. 6). It suggests that the Fe–C–O unit is tilted and/or distorted by the steric effects, as in the cases of those oxidases [33,42,46,50–52]. This result was unexpected because the binuclear center of this enzyme is composed of *b*-type heme and the farnesyl side chain, which might provide the stabilization to the binuclear center [13], is

absent as in the cases of cbb_3-type oxidase and NOR. Furthermore, a tyrosine residue, Y244 in bovine cytochrome aa_3 [7], which is conserved among aa_3- and bo_3-type oxidases, is replaced to glutamic acid in this enzyme [28] and NOR [15], and to glycine in cbb_3-type oxidase [15]. The tyrosine residue is close to the binuclear center [6–8] and may play a role of hydrogen donor to dioxygen bound to heme–Fe [53] and may stabilize the catalytic site [54–56] by forming the covalent link with one of the histidine ligands of Cu_B [55,56]. In spite of these differences, the results from RR spectra of the CO-bound form (Fig. 6) suggested that some steric environments in the heme distal side are similar to aa_3- and bo_3-type oxidases.

Enzymatic properties of the NOR homologue that it catalyzes O_2 reduction but virtually not NO reduction [28] also suggest the common structural feature in the binuclear center with aa_3- and bo_3-type oxidases [57,58]. In these enzymes, structural environment in the catalytic site must be specialized to reduce O_2 and not adopted to reduce NO. cbb_3-type oxidase, on the other hand, was reported to have the highest activity to catalyze NO reduction within the heme–copper oxidases [58,59]. This enzyme must have structural similarities with NOR in the catalytic site to bind two molecules of NO and reduce them to N_2O.

4.3. Physiological properties of the NOR homologue and structural property of the heme b_3

Molecular activity of the NOR homologue as CcO is calculated to be 3.0–4.1 e^-/s. This is considerably low compared to other heme–copper oxidases, e.g. Arslan et al. [60] reported molecular activity of 90–100 e^-/s for cbb_3-type oxidase. They assayed the activity with horse cytochrome *c* as an electron donor and calculated by measuring the change in absorbance at 550 nm in common with our procedure. Although their assay condition was not exactly the same as our assay, it is obvious that the cbb_3-type oxidase has much higher activity compared to this enzyme. Recently, we purified aa_3-type CcO from *R. denitrificans* as a two-subunit enzyme (Matsuda Y., and Arata H., unpublished data). This revealed at least 10-fold higher activity compared to the NOR homologue (data not shown). Although we cannot completely rule out the possibility that the enzyme is partially inactivated during the purification steps, the NOR homologue appears to have intrinsically low CcO activity due to the structural constraint coming from six-coordinate low-spin ferrous heme b_3. The closed structure may be unsuitable for effective turnover of the reaction. An observation consistent with this is the low sensitivity to cyanide. It has been reported that the concentration required for 50% inhibition of CcO activity is 1.3 μM for aa_3-type oxidase of *R. sphaeroides* [61] and is about 1 μM for cbb_3-type oxidase of *Pseudomonas stutzeri* [62]. On the other hand, CcO activity of the NOR homologue is inhibited only about 40% in the presence of 100 μM of KCN [28]. This might be related to the unique coordination structure of the ferrous heme b_3.

Candela et al. [63] reported kinetic and potentiometric studies using membrane fragments of *R. denitrificans*. One of their important conclusions is that this bacterium does not possess quinol oxidase activity. In addition, they suggested that the bacterium has aa_3-type and *cb*-type terminal oxidases. The presence of aa_3-type oxidase is confirmed by purification of the enzyme (Matsuda Y., and Arata H., unpublished data). High-potential heme *b* that they observed in the redox titration of the membrane might come from the NOR homologue, or it might come from cbb_3-type oxidase since we have identified a heme *c*-containing 28-kDa polypeptide which has a N-terminal amino acid sequence similar to that of mono-heme subunit of cbb_3-type oxidase [28]. Probably the aerobic respiration is carried out mainly by aa_3-type and/or cbb_3-type oxidases and the contribution of the NOR homologue appeared to be small [28]. The physiological role of this unique enzyme remains yet to be clarified.

In conclusion, we performed spectroscopic analyses of the NOR homologue from *R. denitrificans*. It was suggested that this enzyme has some intriguing properties in the binuclear site. The frequencies of ν(Fe–CO) and ν(C–O) are close to those of cytochrome aa_3- and bo_3-type oxidases in spite of the similarity with NOR in the primary structure. In the last decade, it has been generally accepted that heme–copper oxidases evolved from NOR [14], and during evolution, non-heme iron would have been replaced by copper. The NOR homologue from *R. denitrificans* may have evolved from NOR by the same replacement of the non-heme metal, which might have brought structural features similar to those of oxygen-reducing enzymes in the catalytic site. Heme *b* at the binuclear site is suggested to be in a six-coordinate low-spin state in the reduced form. This is peculiar to this enzyme in the heme–copper oxidase superfamily, and may cause the low molecular activity of the NOR homologue.

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