Spectral Properties of Cytochrome c_{553} and a Membrane-Bound Cytochrome *b* from *Alcaligenes xylosoxidans* GIFU 1051

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Cytochrome c_{553} and cytochrome *b* were isolated from soluble and membrane fractions of *Alcaligenes xylosoxidans* GIFU 1051, respectively, and their spectroscopic characterizations have been performed. Cytochrome c_{553} has been shown to be in the low spin state both in the oxidized and reduced forms at room temperature by the absorption and magnetic circular dichroism (MCD) spectra, while the high spin heme has been also observed in the cryogenic electron paramagnetic resonance (EPR) spectra. Exogenous small ligands such as NO and CO bind to cytochrome c_{553} by expelling the axial His ligand. The membrane-bound cytochrome *b* has two heme *b* centers in a protein molecule which are the active centers of NO reductase, the cytochrome *bc* complex, although the enzyme activity was rather low. The absorption, MCD, and EPR spectra of the membrane-bound cytochrome *b* showed that two heme *b* centers are in the different electronic states, the high and low spin states. Cytochrome c_{553} is the natural electron donor to the membrane-bound NO reductase, although the interprotein electron-transfer could not been studied because of the low enzyme activity of the cytochrome *b* subunit.

Alcaligenes grows under both aerobic and anaerobic conditions using NO_x such as NO_3^-, NO_2^-, NO, and N_2O as the electron acceptors.[1] Recently, it has been proposed from the comparative amino acid sequence study that terminal oxidases in the respiration processes, cytochrome oxidase and quinol oxidase were evolved from NO reductase and N_2O reductase in the denitrification process.[2] Therefore, urgent and thorough studies on this denitrification process are required from chemical, biochemical evolutional, and biophysical points of view. However, there has been disagreement about whether the enzyme which converts NO to N_2O is present or not, since NO reductase is a very unstable membrane-bound protein and no bioassay to quantitatively analyze NO has been established. However, NO reductase has been recently isolated from several denitrifying bacteria: *Pseudomonas stuzeri*,[4] *Paracoccus denitrificans*,[5—7] and *Achromobacter cycloclastes*[8] and has been revealed to be the complex of a cytochrome *b* subunit and a cytochrome *c* subunit.

Cytochrome reductase (the complex of cytochrome *b*, cytochrome c_1, and other subunits including an iron-sulfur protein) participates in O_2 respiration system, functioning as a vectorial proton pump in the membrane separating periplasm and cytosol.[3] This enzyme is also supposed to participate in the anaerobic NO_3^- or NO_2^- respiration process. A small sized cytochrome *c* present in periplasm (a soluble cytochrome *c*) has been considered to mediate electron between cytochrome reductase and NO reductase.

$$\underset{(\text{cyt } bc_1)}{\text{cytochrome reductase}} \rightarrow \text{cytochrome } c \rightarrow \underset{(\text{cyt } bc)}{\text{NO reductase}}$$

In order to reveal the structure and function of the organization components of the electron transport chain in denitrifiers, we isolated the mediator protein of electron-transfer, cytochrome *c* (since the reduced form of the protein gives a sharp α band at 553 nm, we call it cytochrome c_{553}) from the soluble fraction of *Alcaligenes xylosoxidans*, and characterized it using absorption, MCD, and EPR spectroscopies. Further, we isolated a B type cytochrome, the active center of NO reductase, from the membrane fraction in the presence of dodecyl-β-D-maltoside. Although a membrane-bound C type cytochrome has been eliminated from NO reductase during preparation, the membrane-bound cytochrome *b* sparingly showed the enzyme activity. The spectroscopic characterization of the membrane-bound cytochrome *b* subunit has been performed using absorption, MCD, and EPR spectroscopies.

Experimental

Materials. *Alcaligenes xylosoxidans* GIFU 1051 was a gift from Prof. Shinnichiro Suzuki of Osaka University and was cultivated as reported[9] except that an anaerobic condition was maintained at the final stage of the large scale cultivation. By flushing the inside of the incubator with nitrogen gas we could minimize the progress of the terminal oxidases.

All chemicals used were of the highest grade commercially available.

Purification. The cultured *Alcaligenes xylosoxidans* GIFU 1051 was sonicated and ultracentrifuged to separate the soluble and membrane fractions.

The soluble fraction was dialyzed against 0.01 M (M = mol dm^{-3}) phosphate buffer (pH6) and loaded onto a CM-32 (a cation exchange resin, Whatman) column. By repeating chromatographies using CM-32 at pH 5 and 6 (0—0.5 M NaCl), we

purified cytochrome c_{553}, cytochrome c' and two azurins.[10]

The membrane fraction was treated with the same amount (w/w) of dodecyl-β-D-maltoside with total protein. The solubilized membrane proteins were chromatographed on a DEAE-Sephadex A-50 (an anion exchange resin, Pharmacia) (Tris-HCl of pH 8.5, 0—0.5 M NaCl), hydroxyapatite (Bio-Rad) (0.1—0.5 M phosphate buffer, pH7), and a Sephacryl S-300 (a gel-chromatography resin, Pharmacia) (Tris-HCl of pH 8.5) to give a single band of cytochrome *b* on a SDS/PAGE (Tris, SDS and PAGE are tris(hydroxymethyl)aminomethane, dodecyl sulfate, and polyacrylamide gel electrophoresis, respectively).

Heme and Protein Determinations. Protein concentrations of cytochrome c_{553} and the membrane-bound cytochrome *b* were determined by the Lowry method[11] and bicinchonic acid method[12] with bovine serum albumin as standard, respectively. Heme composition was determined by the pyridine ferrohemochrome method.[13] The acidic acetone method was used to determine covalent or non-covalent linkage of the prosthetic group to the proteins.[14]

SDS/PAGE electrophoreses gave the single band for cytochrome c_{553} (13 kDa) and the membrane-bound cytochrome *b* (66 kDa).

Enzyme Assay. Cytochrome reductase activity[15] and NO reductase activity[16] were measured according to literature methods.

Spectroscopies. Optical spectroscopy was performed with a JASCO Ubest-50 or with a Shimadzu UV-3100 spectrophotometer. MCD spectra were measured on a JASCO J-720W spectropolarimeter equipped with an electromagnet at the magnetic field of 1.4 T. EPR spectra were measured with a JEOL FE-2X and a Bruker ESP-300E attached to an Oxford liquid helium cryostat. Operating parameters were as listed in figure legends.

Results

Heme Determinations. The acidic acetone method to extract non-covalently bound heme and heme-staining on SDS/PAGE electrophoresis evidenced that cytochrome c_{553} has the covalently bound C type heme and the membrane-bound cytochrome *b* has the non-covalently bound heme. The pyridine ferrohemochrome method gave the stoichiometric data, showing that cytochrome c_{553} and the membrane-bound cytochrome *b* have one C type heme and two B type hemes in a protein molecule, respectively.

Absorption and MCD Spectra of the Ferric and Ferrous Cytochrome c_{553}. Absorption spectra of the oxidized (as isolated) and reduced (sodium hydrogensulfite) cytochrome c_{553} are shown in Fig. 1. The Soret (γ) band appeared at 409 nm in the oxidized form and at 418 nm in the reduced form. The three broad bands were observed at around 493, 523, and 560 nm in the Q (α, β) band region of the oxidized form. On the other hand, the reduced protein gave bands at 553, ca. 530 (shoulder), and 523 nm typical for C type heme.[17]

MCD spectroscopy has been widely used in porphyrin and hemeprotein studies to probe the oxidation state, spin state, and axial ligand.[18,19] Cytochrome c_{553} gave the MCD spectra characteristic of the low spin heme both in the oxidized and reduced forms.

EPR Spectra of Cytochrome c_{553} in the Oxidized Form. The EPR spectrum at 10 K (Fig. 2) showed two species: one had the spin Hamiltonian parameters, $g_{\perp}=5.9$ and $g_{//}=2.0$ for an axial symmetry and the other had $g_z=2.95$, $g_y=2.26$,

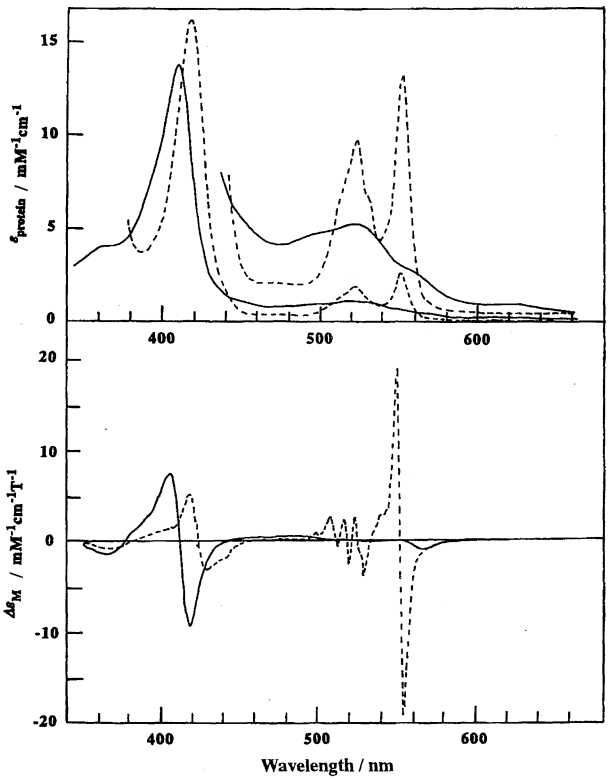

Fig. 1. Absorption (top) and MCD (bottom) spectra of the oxidized (as isolated) (solid line) and reduced (sodium hydrogensulfite) (dashed line) cytochrome c_{553} at pH 5 (0.1×10^{-3} M phosphate buffer) and at room temperature. Protein concentration was 3.0×10^{-6} M.

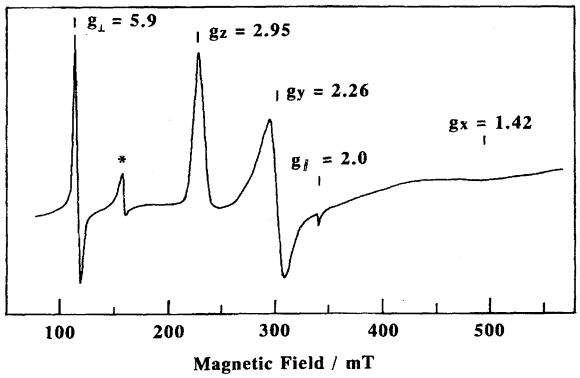

Fig. 2. EPR spectrum of cytochrome c_{553} in the oxidized form. Experimental conditions: frequency, 9.523 GHz; modulation amplitude, 0.96 mT; microwave power, 1 mW; temperature 10 K. Asterisk comes from impurities in the cavity. Protein concentration was 6.0×10^{-6} M in 0.1×10^{-3} M phosphate buffer.

and $g_x=1.42$ for a rhombic symmetry. The former is typical of high spin heme and the latter of low spin heme. The spin Hamiltonian parameters for the low spin component are in the range reported for the 6-coordinate C type heme with the set of axial ligands, His and Met.[18,19] The extra signal having $g=4.2$ will be due to a small amount of an adventitious non-heme ion or from a stain in the cavity which could not be completely eliminated even by compensating the background

spectrum.

The Action of NO and CO onto Cytochrome c_{553}. NO acted on both the oxidized and reduced cytochrome c_{553} and CO on the reduced cytochrome c_{553} as probes to reveal the structure and electronic state of the heme in the active site. The clear difference between 5- and 6-coordinate nitrosylhemes in absorption spectra can be found in the Soret band (391—401 nm for the 5-coordination and 412—419 nm for the 6-coordination) and in the appearance of a weak absorption at around 480 nm only in the 5-coordination.[20] The absorption spectrum of the NO-acted reduced cytochrome c_{553} (Fig. 3) showed the feature for the 6-coordinate nitrosylheme. In the MCD spectrum of the NO-acted reduced cytochrome c_{553} the absorption intensity of the Soret band was comparable with the band in the Q band region, indicating that the 6-coordinate heme is predominant.[21] On the other hand, when NO acted upon the oxidized cytochrome c_{553}, several bands appeared at 371, 360, ca. 340, and 338 nm in addition to the Soret band at 410 nm.

In harmonyous with the MCD spectrum, the corresponding EPR spectrum (Fig. 4) gave the signal originating from the 6-coordinate NO-heme with the parameters, g_1=2.073, g_2=2.008, g_3=1.984, A_2=1.53 mT, and an extra signal at g_{II-1}=2.042. No superhyperfine splitting due to the axial imidazole group was observed, indicating that the apical position opposite to NO was not occupied by a His residue but by a Met residue.

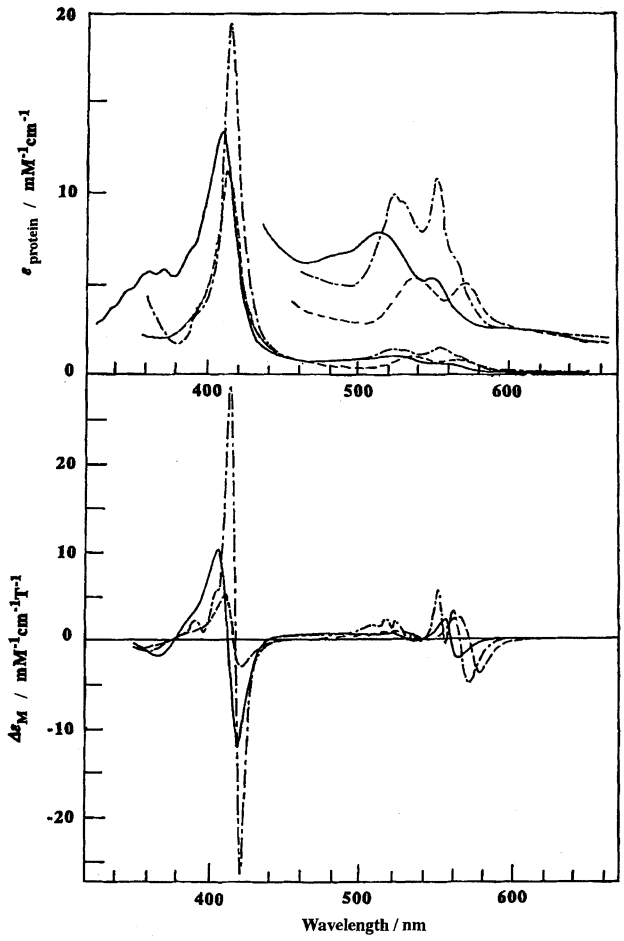

Fig. 3. Absorption (top) and MCD (bottom) spectra of the NO-acted oxidized (solid line), NO-acted reduced (dashed line), and CO-acted reduced (dot-dashed line) cytochrome c_{553}.

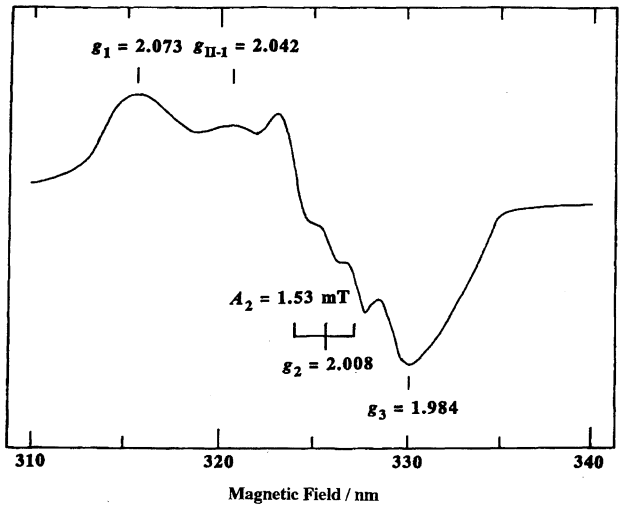

Fig. 4. EPR spectrum of NO-acted reduced cytochrome c_{553}. Experimental conditions: frequency, 9.162 GHz; modulation amplitude, 1 mT; microwave power, 6.125 mW; temperature, 77 K.

When CO acted on the reduced cytochrome c_{553}, the Soret band made blue shift of 2 nm concomitantly with the 60% increase in its intensity. Further, a small shoulder band appeared at ca. 395 nm. On the other hand, the intensities of the α band at 553 nm and the β band at 523 nm were decreased, being accompanied by the new shoulders at ca. 565, 533, and 510 nm.

Absorption Spectra of Membrane-Bound Cytochrome *b*. Absorption spectra of the oxidized (as isolated) and reduced (sodium hydrogensulfite) membrane-bound cytochrome *b* are shown in Fig. 5 together with their MCD spectra. The reduced form gave the sharp α band at 560 nm and a shoulder at 552 nm, indicating that the high and low spin hemes are present in the protein molecule. On the other hand, in the MCD spectrum of the ferric form, the Soret band was apparently stronger than the Q band, and in the ferrous form, in turn, the Q band region is more peculiar than the Soret band region as in the case of cytochrome c_{553}.

EPR Spectra of Cytochrome *b*. Both the low and high spin hemes were observed in the low temperature EPR spectra of the oxidized cytochrome *b* (Fig. 6). The former gave the signal with the parameters g_1=3.25, g_2=2.23, g_3=1.47, and the latter $g_\perp$=5.97 and $g_{//}$=2.0. The signal intensities were highly dependent on temperature. The impurity due to a non-heme component was also observed at g=4.3. Alternatively, the signal came from a stain in the cavity as similarly described above.

The Action of NO on Membrane-Bound Cytochrome *b*. The NO-acted reduced cytochrome *b* gave the EPR signals derived from the 5- and 6-coordinate hemes (Fig. 7). The former signal has the parameters, g_1=2.106, g_2=2.038,

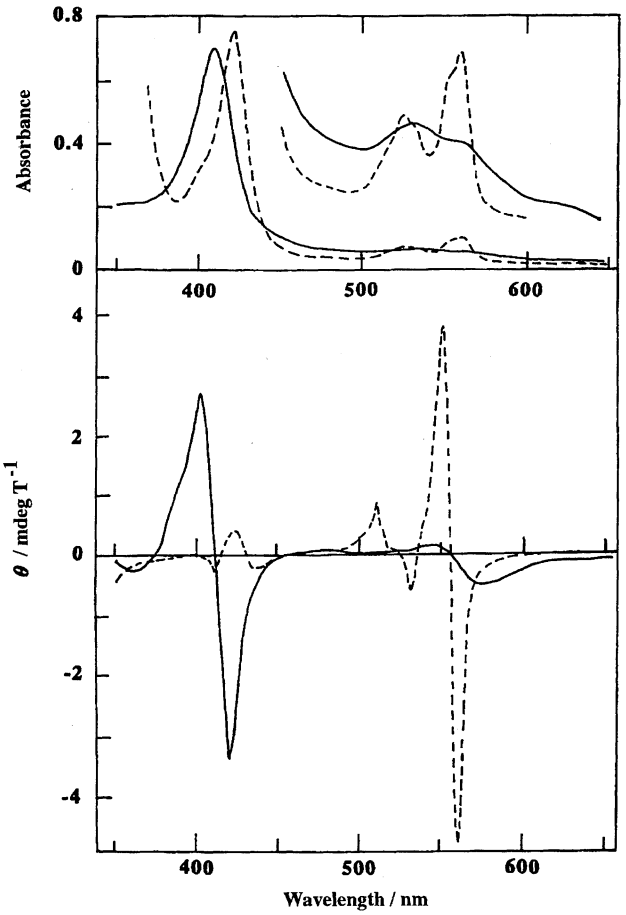

Fig. 5. Absorption (top) and MCD (bottom) spectra of the oxidized (as isolated) (solid line) and reduced (dithionite) (dashed line) cytochrome *b* at pH 8 (20×10^{-3} M phosphate buffer with 1% (w/v) dodecyl-β-D-maltoside) and at room temperature. Protein concentration was 0.19 mg mol^{-1}.

g_3=2.009, A_1=1.25 mT, and A_3=1.67 mT, and the latter has g_1'=2.071, g_3'=1.972, and A_3'=1.25 mT. The component g_2' was not detected because it superimposed on the g_3 component. While the three hyperfine splittings due to the N atom (I=1) in NO were observable clearly, the superhyperfine splittings were not explicitly resolved even in the second order derivative spectrum.

Discussion

Absorption and MCD spectra of cytochrome c_{553} (Fig. 1) indicated that the heme contained is typical of C type[17] and the central iron is in the low spin state both in the oxidized and the reduced forms.[18] On the other hand, the EPR spectra at below 20 K (Fig. 2) showed that the high spin heme is also present. This could originate in a deformation of the protein molecule, which finally leads to a certain temperature dependence of the electronic state of the central heme, although we did not measure the absorption spectrum at a low temperature. Alternatively, an artifact formed by freezing gave the sharp high spin heme signal. Since the sensitivity of this non-heme iron in EPR spectroscopy is considerably high, the amount will not be significant compared to the low spin

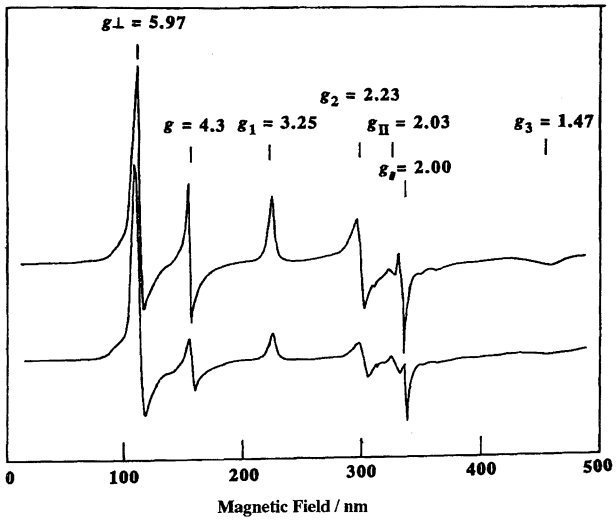

Fig. 6. EPR spectrum of cytochrome *b* in the oxidized form. Experimental conditions: frequency, 9.420 GHz; modulation amplitude, 1 mT; microwave power, 5 mW; temperature, 12 K (top spectrum) and 3 K (bottom spectrum).

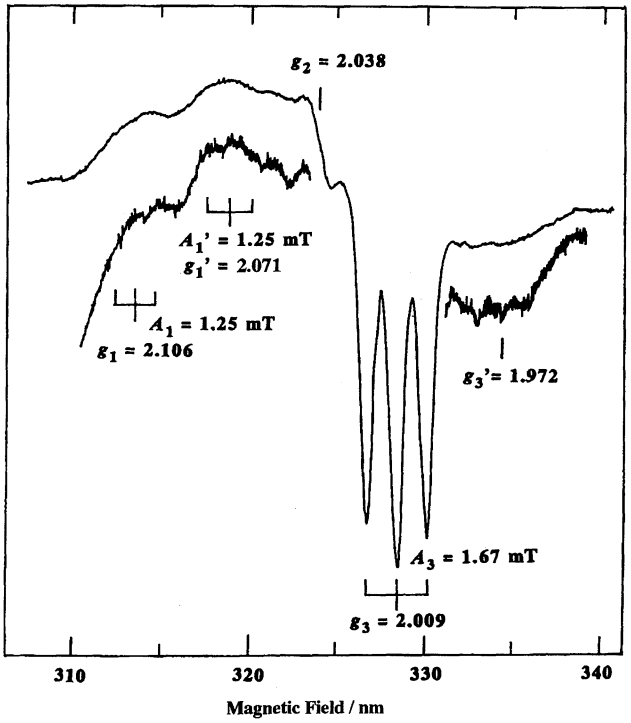

Fig. 7. EPR spectrum of NO-reduced cytochrome *b*. Experimental conditions: frequency, 9.229 GHz; modulation amplitude, 0.1 mT; microwave power, 5 mW; temperature, 77 K.

species. Further, the g=4.2 signal was also observed. If this was derived from a heme, its electronic state is in the condition, $\Delta/\lambda \leq 1$, where Δ represents the energy difference between ^{6}A and ^{4}A state and λ the spin-orbit interaction. However, a stain in the cavity is the most probable origin of the signal, which was not easy to remove by subtracting the background spectrum.

As for the axial ligand the EPR parameters strongly sug-

gest that the set of Met and His residues is most probable among various possible combinations of ligand groups.[18,19] This cytochrome c_{553} is supposed to mediate electron transfer between cytochrome reductase (cytochrome bc_1 complex) and NO reductase (cytochrome *bc* complex)[3] and accordingly, it corresponds to the mitochondrial *c*, whose axial ligands are also Met and His residues.

An NO molecule was bound to an apical position of the heme in cytochrome c_{553}, giving the 6-coordinate nitrosylheme (Figs. 3 and 4). Since in the EPR spectrum the hyperfine structure due to N ($I=1$) did not split further due to the His residue coordinated at another apical position (a triplet of triplets), it is considered that the NO molecule expelled the His ligand from the apical position. This is contradictory to the case of the NO-ferrous cytochrome *c* (horse heart), in which the superhyperfine splittings due to the axial imidazolyl group are apparent.[22] The absorption and MCD spectra also supported the binding of NO to give the 6-coordinate nitrosylheme. With the action of CO on the reduced cytochrome c_{553}, both the absorption and MCD spectra changed appreciably. Although it is apparent from the spectral changes that CO was bound to the ferrous iron, the details of the binding mode and the resulting electronic state of the heme are not known. The ligand field of CO is very strong similarly to NO.

The α band in the absorption spectrum of the reduced membrane-bound cytochrome *b* at 560 nm was very sharp, being accompanied by a shoulder band at ca. 552 nm (Fig. 5). Although C type heme in the low spin state usually gives a sharp band at around 550 nm, the absence of C type heme is evidenced by the fact that no covalent-bound heme was extracted by acetone. Since the pyridine ferrohemochrome method clearly showed that two B type hemes are contained in a protein molecule, the shoulder at ca. 560 nm is considered to originate in the high spin B type heme. The EPR spectrum also showed the presence of two types of heme *b* (Fig. 6): One is apparently the low spin heme and another the high spin heme. In line with this, when this cytochrome *b* was treated with L-ascorbate under Ar (spectra not shown), the rates of development of the two bands at 560 and 552 nm were different (apparently the 552 band developed faster than the 560 nm band) and accordingly, it is also supposed that these two bands come from the different hemes. The Soret band of the high spin Fe^{3+} heme was masked by the strong derivative-shaped Soret band of the low spin Fe^{3+} heme in the MCD spectrum of the oxidized membrane-bound cytochrome *b*. However, the spectral feature of the reduced cytochrome *b* seems to be a mixture of the high and low spin Fe^{2+} hemes in the Soret band region.

NO gave the 5- and 6-coordinate nitrosylhemes shown in Fig. 7. The 5-coordinate nitrosylheme was formed since the bonds between the ferrous ion and two axial ligands were cleaved. This took place because NO is so highly electron-withdrawing as to cleave the bond between the ferrous ion and the opposite axial ligand. The superhyperfine splitting was not ascertained in the 6-coordinate nitrosylheme because of the masking effect of the 5-coordinate nitrosylheme. On the other hand, by the action of CO on the reduced cytochrome *b*, the absorption intensities of the Soret band and the slope of the 560 nm band increased (spectra not shown). This indicates that CO binds to the reduced heme *b* (it is not known whether CO binds to one of the B-type hemes or to both). The absence of the shoulder band at the longer wavelength region of the Soret band also evidenced the coordination of CO.[23]

As the origin of this membrane-bound cytochrome *b*, cytochrome reductase (cytochrome bc_1 complex)[24] or NO reductase (cytochrome *bc* complex) can be considered. A membrane-bound cytochrome *c* (ca. 17 kDa), which had originally complexed with the membrane-bound cytochrome *b*, was eliminated during the final stage of the purification. The cytochrome *b* did not show the cytochrome reductase activity but showed the NO reductase activity although considerably weak (0.04 U/min·mg protein, one unit of the enzyme activity corresponds to 1×10^{-6} mol of NO consumed per min). The stability of the present *Alcaligenes* NO reductase was not improved by using other detergents to solubilize this membrane protein. NO reductases from several other strains of *Alcaligenes* have been also very unstable when solubilized from membrane. This indicates that no artificial detergent is suitable to solubilize and stabilize NO reductases from *Alcaligenes*. Therefore, we could not study the interprotein electron transfer reaction between cytochrome c_{553} and the membrane-bound cytochrome *b* subunit of NO reductase. However, we recently succeeded in isolating highly active NO reductase and a soluble cytochrome *c* from *Paracoccus halodenitrificans*[25] according to the methods similar to those described in the present paper. The spectral features of the protein were analogous to those of the present *Alcaligenes* protein.

The molecular weight of the cytochrome *b* subunit of cytochrome reductase reported hitherto is 40 kDa for *Rhodobacter sphaeroides*,[26] 43 kDa for bovine,[27] 32 kDa for spinach,[28] and 39 kDa for *Paracoccus denitrificans*.[29] On the other hand, the molecular weight of the cytochrome *b* subunit of NO reductase reported hitherto is slightly small: 34 and 37 kDa for *Paracoccus denitrificans*,[5,6] 38 kDa for *Pseudomonas stutzeri*,[4] and *Achromobacter cycloclastes*.[8] While the molecular weight of 66 kDa seems to be too high as coming from NO reductase and cytochrome reductase, it has been reported that the cytochrome *b* subunit of NO reductase easily aggregates by boiling or incubation before electrophoresis to give the apparent molecular weight of 65 kDa.[7] An appreciable discrepancy between the molecular masses of membrane-bound proteins determined by electrophoresis and the sequence analysis easily takes place due to the highly hydrophobic character of the membrane proteins.

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08249214.

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