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Spectral Properties of Nitric Oxide Complexes of Cytochrome *c'* from *Alcaligenes* sp. NCIB 11015†

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ABSTRACT: For the purpose of clarifying the stereochemistry of the heme environment and the electronic structure of the heme iron in cytochrome *c'* from *Alcaligenes* sp. NCIB 11015, the spectral properties of the nitric oxide (^{14}NO and ^{15}NO) derivatives of cytochrome *c'* have been investigated. The electron paramagnetic resonance (EPR) and electronic spectra vary with the change of pH either at room temperature or at 77 K. The results of EPR and electronic spectroscopies on NO–*Alcaligenes* cytochrome *c'* at physiological pH indicate that the heme iron to histidine (Fe–N_ϵ) bond of cytochrome *c'* is very weak and is cleaved upon the coordination of a nitrosyl group. From a comparison of the electronic spectra for NO–cytochrome *c'* from various species, the decreasing order of the Fe–N_ϵ bond strength among species has been considered to be *Rhodopseudomonas palustris* > *Rhodospirillum rubrum* > *Alcaligenes*. The reaction of NO with *Alcaligenes* ferric cytochrome *c'* results in the formation of NO–ferrous cytochrome *c'*, which is interpreted on the basis of a reductive nitrosylation.

Nitric oxide is coordinated as a heme sixth ligand to heme iron in hemoproteins, and the nitrosyl derivatives of hemoproteins have spectral properties that vary with the environmental difference of the heme and the electronic structure of the heme iron. Since nitric oxide has an unpaired electron, the nitrosylhemoproteins have been extensively studied by EPR[1] spectroscopy. Studies on the nitrosylhemoproteins have been carried out also by the use of electronic, IR, resonance Raman, MCD, and NMR spectroscopies [Yoshimura (1983a) and references cited therein]. On the other hand, studies on the model complex nitrosyl(porphyrinato)iron(II) under various conditions have given significant clues to understanding

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[1] Abbreviations: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; CD, circular dichroism; IR, infrared; NMR, nuclear magnetic resonance; *Alcaligenes*, *Alcaligenes* sp. NCIB 11015; *R.*, *Rhodospirillum*; *Rps.*, *Rhodopseudomonas*; *Chromatium*, *Chromatium vinosum*; *A.*, *Azotobacter*; cyt, cytochrome; Hb, hemoglobin; Mb, myoglobin; CCP, cytochrome *c* peroxidase; IDO, indoleamine 2,3-dioxygenase; PPIX, dianion of protoporphyrin IX; PPIXDME, dianion of protoporphyrin IX dimethyl ester; TPP, dianion of *meso*-tetraphenylporphyrin; SDS, sodium dodecyl sulfate; IHP, inositol hexakis(phosphate); NMeIm, 1-methylimidazole; Tris, tris(hydroxymethyl)aminomethane.

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the various properties of these nitrosylhemoproteins [Kon & Kataoka, 1969; Yoshimura and Ozaki (1984) and references cited therein]. Thus nitric oxide has so far been employed as a useful probe for elucidating the heme environment and the heme group of hemoproteins such as hemoglobins, myoglobins, cytochromes, oxygenases, peroxidases, catalases, nitrite reductases, and sulfite reductase [Yoshimura (1983b) and references cited therein].

Cytochromes *c′*, which belong to a unique class of *c*-type cytochromes, are found in photosynthetic, denitrifying, and nitrogen-fixing bacteria [Meyer and Kamen (1982) and references cited therein]. Although the physiological functions of cytochrome *c′* have not yet been elucidated, its unusual spectroscopic and magnetic properties have become of interest in recent years. In particular, the suggestion of Maltempo that the intermediate-spin ($S = {}^3/_2$) state is involved in the ground state of heme iron in ferric cytochromes *c′* has attracted attention to the relation between the unusual properties of these proteins and the stereochemistry of the heme environment (Maltempo et al., 1974; Maltempo, 1974, 1975; Maltempo & Moss, 1976). This has also stimulated study of the synthesis and characterization of the intermediate-spin or mixed ($S = {}^3/_2, {}^5/_2$) spin state of ferric porphyrin complexes (Reed et al., 1979; Masuda et al., 1980, 1982; Scheidt et al., 1983). On the basis of extensive studies for structural and magnetic properties of these complexes, the stereochemistry of heme iron(III) involving the intermediate-spin state has been demonstrated to be as follows: short Fe(heme)–N(pyrrole) bond distance comparable to the low-spin ferric porphyrin complexes and longer Fe–axial ligand bond distance than those of low- and high-spin derivatives (Maltempo, 1974; Weber, 1982). The former heme stereochemistry can destabilize the $d_{x^2-y^2}$ orbital of iron(III), and the latter one can stabilize the d_{z^2} orbital, and consequently the intermediate-spin state may be induced. Thus, the elucidation of the stereochemistry of the heme environment for cytochrome *c′* bears a significant implication in understanding the heme iron electronic configuration involving an intermediate-spin state.

The heme group of a number of hemoproteins such as hemoglobins, myoglobins, cytochrome P-450, and peroxidases is situated in a hydrophobic pocket of the polypeptide chain, and the heme iron can react not only with neutral ligands but also with anionic ligands. On the other hand, although the heme group of cytochromes *c′* is also situated in the hydrophobic pocket (Weber et al., 1980, 1981), the heme iron can only react with nitric oxide and carbon monoxide in the ferrous form and with nitric oxide in the ferric form over the pH range 5.2–12 (Taniguchi & Kamen, 1963). From the comparison of the amino acid sequence of various cytochromes *c′* (Ambler et al., 1981) with the tertiary structure of *R. molischianum* cytochrome *c′* (Weber et al., 1980), the probable heme fifth ligand in native cytochromes *c′* is a histidine residue, and the sixth coordination position of heme iron can be vacant. The distal heme surface, which is called the "cage" by Gibson and Kamen (1969), is surrounded by hydrophobic amino acid residues to restrict access of exogenous ligands except NO and CO (Weber et al., 1981). The findings that the mechanism of CO binding with the cytochromes *c′* differs depending on the origin of cytochrome *c′* (Cusanovich & Gibson, 1973) suggest that the environment of the cage also differs for each cytochrome *c′*.

The reaction of NO with cytochrome *c′* has been so far performed for the proteins from *R. rubrum* (Taniguchi & Kamen, 1963), *Rps. palustris* (De Klerk et al., 1965), *Alcaligenes* (Iwasaki & Shidara, 1969), and *A. vinelandii* (Yamanaka & Imai, 1972), and all have been monitored only by the electronic absorption spectroscopy at room temperature. In these works, NO was examined for reactivity with cytochrome *c′* as a ligand but was not employed as a probe mentioned above.

Alcaligenes cytochrome *c′* has been first purified and characterized by Iwasaki and co-workers (Suzuki & Iwasaki, 1962; Iwasaki & Shidara, 1969). The cytochrome *c′* consists of a dimer with equivalent subunits (subunit M_r 14000) (Cusanovich et al., 1970; Ambler, 1973). We have discussed the spectral properties of *Alcaligenes* cytochrome *c′* under various conditions by the use of electronic, EPR, resonance Raman, CD, and MCD spectroscopies in a separate paper (Yoshimura et al., 1985), which indicated that heme axial ligand(s) can be markedly affected by varying the pH of the medium and by the presence of a detergent. In this study, we have characterized the spectral properties of NO complexes of *Alcaligenes* cytochrome *c′* under various conditions by reference to the accumulated information on models for nitrosylhemoproteins. Further, the electronic spectra of nitrosylcytochromes *c′* from various sources reported so far are compared, and the differences in strength of heme iron to proximal histidine bond for each cytochrome *c′* are discussed.

Materials and Methods

Nitric oxide was purchased from Takachiho Trading Co. or was generated by the reaction of $NaNO_2$ with an aqueous solution of ascorbic acid. ^{15}NO gas was obtained by the latter method by using $Na^{15}NO_2$ (99.2% enrichment, Prochem). NO gas was passed through a cold trap (at about −90 °C) and a KOH column to remove N_2O and higher nitrogen oxides. All other chemicals used were of reagent grade of highest grade available.

Cytochrome *c′* from *Alcaligenes* sp. NCIB 11015 was isolated and purified by the method described previously (Iwasaki & Shidara, 1969). The reduced form of the protein was prepared by the addition of a minimum quantity of solid sodium dithionite to a solution of the ferric form under anaerobic conditions, unless otherwise noted.

The reaction of NO was carried out in a Thunberg-type tube with optical cuvette (path length, 1 cm) or with an EPR tube. Cytochrome *c′* solutions were carefully deoxygenated in the Thunberg tube either (a) by repeated evacuating and flushing with pure argon or nitrogen gas or (b) by repeated freezing and thawing in vacuo. Then the solutions were reduced and equilibrated with NO gas of slightly below 1 atm. After the solution was allowed to stand for 10 min, EPR and electronic spectra were measured at 77 K and 20 °C, respectively. In order to measure reversely the EPR at 20 °C and electronic spectra at 77 K, these solutions were anaerobically transferred into the respective quartz flat cells immediately after the above measurements had been completed. The spectra obtained by method b in the step of deoxygenation were the same as those obtained by method a, suggesting that the protein is fairly stable. All cytochrome *c′* solutions except that at pH 13.4 were used for the NO reaction immediately after pH adjustments. The one at pH 13.4 was used after allowing the solution to stand for 1 week in a frozen state.

EPR measurements were carried out by using a JES-ME-3X spectrometer with 100-kHz field modulation at 20 °C and at 77 K, which was calibrated with a Takeda-Riken frequency counter TR-5211A and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical powder ($g = 2.0036$) and Mn(II) in MgO ($\Delta H_{3-4} = 86.9$ G) as a standard. The second-derivative display was obtained by the use of 80-Hz field modulation. The electronic absorption spectra were recorded on a Shimadzu MPS-5000

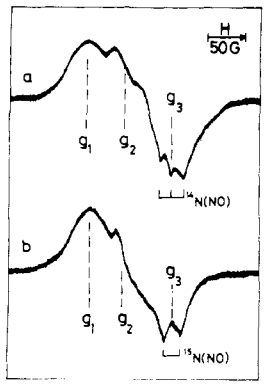

FIGURE 1: EPR spectra of NO–cytochrome *c'* from *Alcaligenes* sp. NCIB 11015 at 20 °C and at pH 7.2 (50 mM phosphate buffer): (a) ^{14}NO derivative (g_1 = 2.088; g_2 = 2.05; g_3 = 2.016, $a_3(^{14}N)$ = 16 G); (b) ^{15}NO derivative (g_1 = 2.085; g_2 = 2.05; g_3 = 2.016, $a_3(^{15}N)$ = 23 G). Instrument settings: modulation frequency and amplitude, 100 kHz and 5 G; microwave frequency and power, 9.447–9.449 GHz and 10 mW.

spectrophotometer at 20 °C (path length, 1 cm) and at 77 K (path length, 2 mm).

Heme concentrations were determined from electronic absorption measurements by using the alkaline pyridine hemochrome method [ϵ_{mM} at 550 nm = 29.1 (Drabkin, 1942)] or the millimolar extinction coefficient of the spectra for *Alcaligenes* ferric cytochrome *c'* under various pHs described previously (Iwasaki & Shidara, 1969; Cusanovich et al., 1970). Heme concentrations of EPR and electronic spectral samples were in the ranges 0.03–1.00 and 0.02–0.03 mM, respectively.

The buffers used here were as follows: pH 1.5, 100 mM Gly·NaCl/HCl; pH 5.3, 50 mM KH_2PO_4/Na_2HPO_4; pH 7.2, 50 mM NaH_2PO_4/Na_2HPO_4; pH 9.1, 60 mM Tris/HCl; pH 11.0, 100 mM K_2HPO_4/KOH; pH 13.4, 100 mM Gly·NaCl/NaOH. It is noted that these buffers have been reported to show a pH change on freezing (Orii & Morita, 1977; Williams-Smith et al., 1977). In this study, the pH values on freezing are used in consideration of those findings as described in a separate paper (Yoshimura et al., 1985).

RESULTS

EPR Spectra for NO Derivatives of Alcaligenes Cytochrome c' at pH 7.2. The EPR spectra for ^{14}NO and ^{15}NO derivatives of *Alcaligenes* cytochrome *c'* at pH 7.2 are shown in Figures 1 (at 20 °C) and 2 (at 77 K). Both the spectra exhibited the line shape characteristic of randomly oriented systems with rhombic symmetry and the existence of three *g* values. The spectra at 77 K apparently exhibited a greater *g* anisotropy as compared with those at 20 °C. As is visualized in the higher magnetic field side of g_3 absorption in Figure 2A-b,B-b, an additional g_3' absorption was also observed at 77 K.

The three *g* values (g_1, g_2, and g_3) of the main absorption were essentially identical with those of five-coordinate model nitrosylhemes[2] such as Fe(PPIXDME)(NO) and Fe(TPP)(NO) in nondonor solvents (Wayland & Olson, 1974; Kon, 1975; Yoshimura, 1978) and Fe(PPIX)(NO) intercalated into SDS micelles (Kon, 1975; Christahl et al., 1982), though the EPR spectrum was well resolved in comparison with those of the model nitrosylheme. The g_1, g_2, and g_3 absorptions split into a triplet or a doublet, which were originated from the

[2] In this paper, the five-coordinate and the six-coordinate nitrosylhemes represent species without and with a ligand at the axial position trans to the nitrosyl group, respectively.

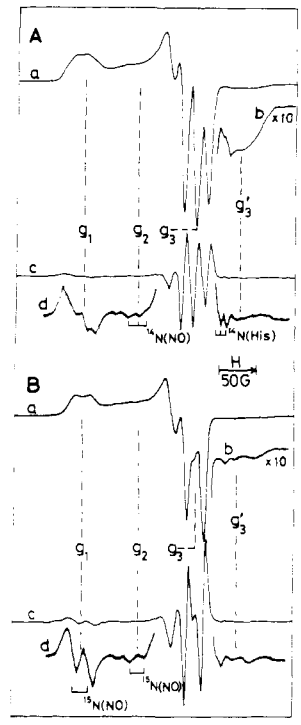

FIGURE 2: EPR spectra of NO–cytochrome *c'* from *Alcaligenes* sp. NCIB 11015 at 77 K and at pH 7.2 (50 mM phosphate buffer): (a) first derivative; (b) expansion of the ordinate of (a); (c) second derivative; (d) expansion of the ordinate of (c). (A) ^{14}NO derivative (g_1 = 2.106, $a_1 \approx$ 14 G; g_2 = 2.058, a_2 = 13 G; g_3 = 2.010$_4$, a_3 = 16.0 G; g_3' = 1.98). (B) ^{15}NO derivative (g_1 = 2.107, a_1 = 20 G; g_2 = 2.058, a_2 = 18 G; g_3 = 2.010$_3$, a_3 = 22.2 G; g_3' = 1.97). Instrument settings: modulation frequency and amplitude, 100 kHz and 2 G; microwave frequency and power, 9.174–9.176 GHz and 10 mW.

hyperfine interaction of the unpaired electron with the ^{14}N (I = 1) or ^{15}N (I = $^1/_2$) nucleus of the NO group, respectively. From the facts that the observed g_3 value is essentially equal to the free spin value and that the hyperfine splittings of NO nitrogen are clearly resolved at the g_3 absorption, the concerned signal can be assigned to the g_z absorption, and the unpaired electron of the NO group is considered as delocalized over the iron d_{z^2} orbital. The sharp three-line hyperfine splittings as observed in the g_3 absorption (Figure 2A) had been also detected in spectra for NO derivatives of hemoglobins in the presence of IHP (Rein et al., 1972) or SDS (Kon, 1968), and those of mutant hemoglobins M (Nagai et al., 1979).

In the additional g_3' absorption, the *g* value is close to g_y value of six-coordinate model nitrosylheme (Yoshimura et al., 1979; Yoshimura, 1980, 1982, 1983b; Christahl et al., 1982). The splitting of 7 G was detected in the region between the g_3 and g_3' absorptions, where a triplet of triplets is usually observed in the EPR spectra of the six-coordinate nitrosylheme with a nitrogenous base as an axial ligand (Yonetani et al., 1972; Yoshimura et al., 1979). In the triplet of triplets, the hyperfine coupling constants due to ^{14}N nuclei of NO and nitrogenous base are reported to be 20–23 and 5–7 G, respectively (Yoshimura, 1980, 1982). Hence, the splitting in Figure 2A-b can be ascribed to a part of such a triplet of triplets.

There are a few NO–hemoproteins whose EPR spectra were measured at room temperature. Those are NO derivatives of hemoglobin, myoglobin, and cytochrome *c* (Trittelvitz et al., 1972; Henry & Banerjee, 1973), which have a six-coordinate nitrosylheme with a histidine at the axial position trans to nitrosyl group. The EPR spectra in Figure 1 clearly differ from those spectra, suggesting that NO–cytochrome *c'* at room

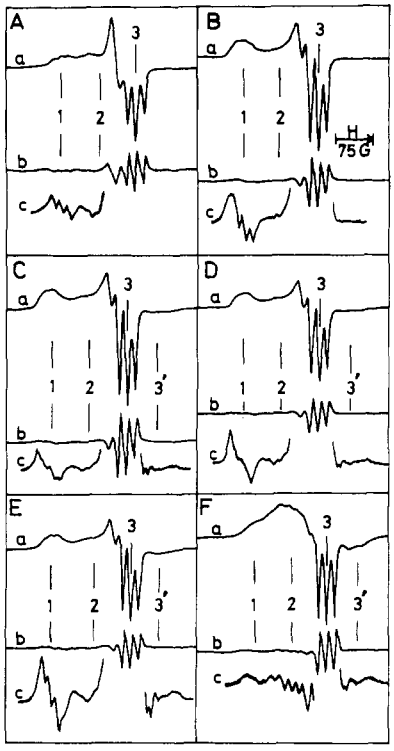

FIGURE 3: EPR spectra of NO–cytochrome *c′* from *Alcaligenes* sp. NCIB 11015 at 77 K under various pH conditions: (a) first derivative; (b) second derivative; (c) expansion of the ordinate of (b). The buffers used are described in the text. The numbers 1, 2, 3, and 3′ represent g_1, g_2, g_3, and g_3', respectively. pH conditions: (A) pH 1.5 (g_1 = 2.105, a_1 = 15 G; g_2 = 2.056; g_3 = 2.010$_4$, a_3 = 16.4 G); (B) pH 5.3 (g_1 = 2.106, a_1 = 15 G; g_2 = 2.059; g_3 = 2.010$_4$, a_3 = 15.8 G); (C) pH 7.2 (see the legend of Figure 2A); (D) pH 9.1 (g_1 = 2.105; g_2 = 2.058, a_2 = 13 G; g_3 = 2.010$_4$, a_3 = 16.1 G; g_3' = 1.97); (E) pH 11.0 (g_1 = 2.114, a_1 = 14 G; g_2 = 2.056; g_3 = 2.010$_8$, a_3 = 15.7 G; g_3' = 1.98); (F) pH 13.4 (g_1 = 2.102; g_2 = 2.054, a_2 = 15 G; g_3 = 2.010$_1$, a_3 16.6 G; g_3' = 1.97). Instrument settings are given in legend of Figure 2.

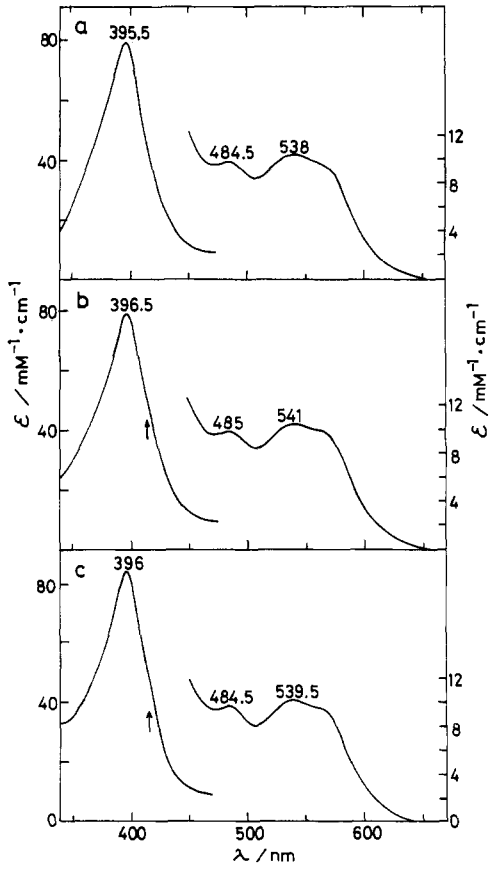

FIGURE 4: Electronic spectra of NO–cytochrome *c′* from *Alcaligenes* sp. NCIB 11015 at 20 °C under various pH conditions: (a) pH 5.3; (b) pH 7.2; (c) pH 11.0. The buffers used are described in the text. The arrow represents a faint shoulder.

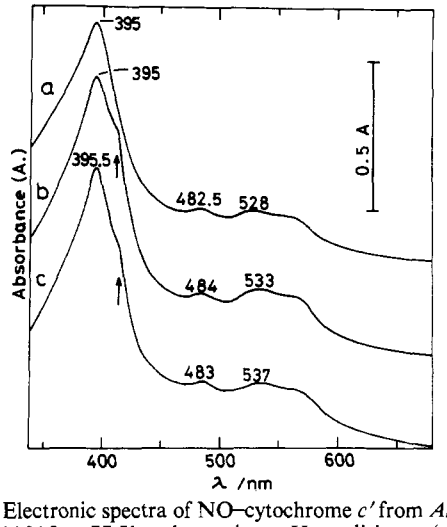

FIGURE 5: Electronic spectra of NO–cytochrome *c′* from *Alcaligenes* sp. NCIB 11015 at 77 K under various pH conditions: (a) pH 5.3; (b) pH 7.2; (c) pH 11.0. The buffers used are described in the text. The arrow represents a faint shoulder at around 415 nm.

temperature has a five-coordinate nitrosylheme. The EPR spectra of NO–cytochrome *c′* reduced by use of ascorbic acid were apparently identical with those in Figures 1 and 2.

pH Dependence of EPR and Electronic Spectra of NO Derivatives of Alcaligenes Cytochrome c′. Both the EPR and electronic spectra for NO derivatives of *Alcaligenes* cytochrome *c′* varied with the change of pH values (Figures 3–5).

In the EPR spectra (Figure 3), the only main absorption with three *g* values (g_1, g_2, and g_3) was observed at pH 1.5 and 5.3, while the main and the additional (g_3') absorptions were observed at pH 7.2. The relative intensity ratio of the g_3 absorption to the central signal of the g_3 absorption slightly increased with increasing pH value (0.04 at pH 7.2, 0.06 at pH 9.1 and 1.10, and 0.16 at pH 13.4). At pH 1.5, the clear ferric cytochrome *c′* solution became slightly turbid with reduction and then suspended upon the subsequent reaction with NO.

The electronic spectra for NO–cytochrome *c′* at pH 5.3, 7.2, and 11.0 are illustrated in Figures 4 (at 20 °C) and 5 (at 77 K). The spectral data at pH 7.2 shows a fairly good coincidence with those at pH 7 reported by Iwasaki and Shidara (1969). The three spectra resembled one another as a whole. However, a faint shoulder is visualized at around 415 nm in spectra b (pH 7.2) and c (pH 11.0), while it is not in spectrum a (pH 5.3).

The general features of the electronic spectra for NO–cytochrome *c′* were similar to those for a five-coordinate model nitrosylheme, Fe(PPIXDME)(NO) (Yoshimura & Ozaki, 1984). The relative intensity ratio of α- to β-band (α/β ratio), however, was below unity in the spectra for NO–cytochrome *c′*, while it was above unity in those for the five-coordinate model. The shoulder at around 415 nm was observed even more clearly at 77 K than at 20 °C. The Soret band of six-corrodinate model nitrosylheme and hemoproteins was observed in the range 410–420 nm (Table I). Thus, the shoulder at around 415 nm may be indicative of the slight existence of the six-coordinate form in NO–cytochrome *c′*.

Reaction of NO with Alcaligenes Ferric Cytochrome c′. A Thunberg-type tube including the solution (pH 7.2) of *Al-*

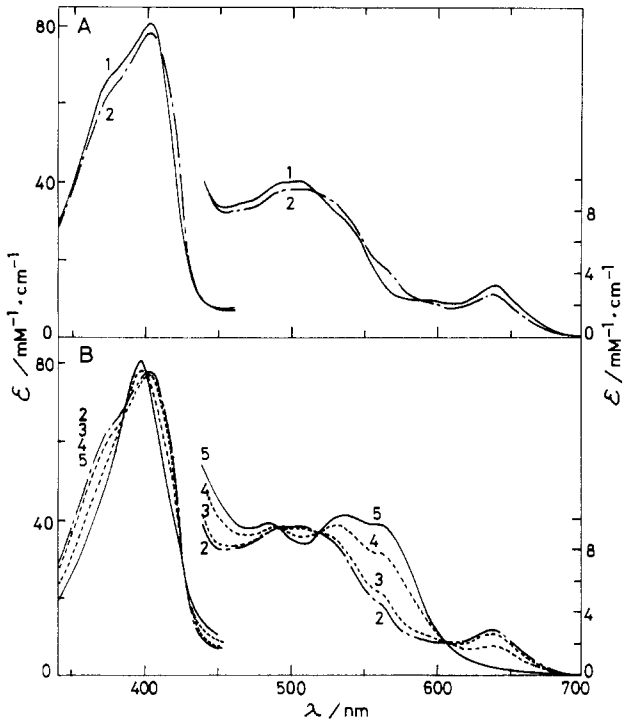

FIGURE 6: Reaction of NO with ferric cytochrome *c'* from *Alcaligenes* sp. NCIB 11015 at 20 °C and at pH 7.2 (50 mM phosphate buffer). The electronic spectral changes were traced with time: (1) before reaction with NO; (2) 5 min, (3) 1 h, (4) 25 h, and (5) 70 h after reaction with NO.

caligenes ferric cytochrome *c'* was filled with NO gas of slightly below 1 atm, and then the electronic and EPR spectral changes were traced with time.

As represented in Figure 6, the electronic spectral change appeared to proceed in two steps: the reaction in the first step was rapidly established and that in the second step was relatively slow. The characteristic of the first spectral change (Figure 6A) was an increase in absorption intensity at around 420 nm. The isosbestic points in the second spectral change (Figure 6B) were 385, 400, 427, 492, 520, and 627 nm, and the final spectrum thus obtained was quite identical with that (Figure 4) of NO–ferrous cytochrome *c'*.

Immediately after the reaction of NO with ferric cytochrome *c'*, the EPR spectral absorption based on ferric cytochrome *c'* was barley observed at the highest possible gain, while that of NO–ferrous cytochrome *c'* was readily observed. Subsequently, the former disappeared and the intensity of the latter increased finally to a usual intensity.

Discussion

Heme Axial Ligand of Alcaligenes Cytochrome c'. The probable heme fifth ligand is histidine residue 120 in the amino acid sequence of *Alcaligenes* cytochrome *c'*, and the vacant distal side of the heme can be surrouned by hydrophobic amino acid residues (Ambler, 1973; Ambler et al., 1981). Accordingly, NO can be coordinated to the vacant axial position trans to the proximal histidine.

This primary heme environment in NO–cytochrome *c'* resembles that in NO derivatives of hemoproteins such as hemoglobin, myoglobin, cytochrome *c*, and cytochrome *c* peroxidase (Kon, 1968; Yonetani et al., 1972). The heme iron of the latter NO–hemoproteins with histidine ligand at the axial position trans to the NO group is usually six-coordinated by four porphyrin nitrogens, a histidine N_ϵ, and a NO nitrogen. Several nitrosylhemoproteins with a five-coordinate nitrosylheme, however, have been demonstrated. They are the NO derivatives of hemoglobins in the presence of inositol hexakis(phosphate) [Palmer, 1983; Dickinson and Symons (1983) and references cited therein] or sodium dodecyl sulfate (Kon, 1968), of mutant hemoglobins M (Nagai et al., 1979), of catalase (Yonetani et al., 1972), and of cytochrome P-420 (O'Keefe et al., 1978).

The results of EPR and electronic spectroscopies for NO–*Alcaligenes* cytochrome *c'* at physiological pH show that the major component of nitrosylheme in a five-coordinate species and trace components are present as six-coordinate ones and also that the apparent ratio of five- to six-coordinate species at 20 °C and at 77 K is essentially identical. This suggests that the heme iron to proximal histidine bond for the major component of *Alcaligenes* cytochrome *c'* is cleaved upon coordination of the nitrosyl group to heme iron, while the same bond for the minor component is maintained.

It has been demonstrated that there are two different conformational states (A_1 and A_2) with slightly different heme environments in *Chromatium* ferric cytochrome *c'* at physiological pH, where the ratio of A_1 to A_2 is 40:1, and both the states have the heme iron with a mixed ($S = {}^3/_2, {}^5/_2$) spin state (Maltempo et al., 1974). Further, the electronic states of iron corresponding to A_1 would have the $S = {}^3/_2$ state lying below the $S = {}^5/_2$ state, while the state corresponding to A_2 would have the $S = {}^5/_2$ state lying below the $S = {}^3/_2$ state (Maltempo et al., 1974), suggesting that the heme iron to histidine bond of A_1 is weaker in strength than that of A_2 (Maltempo, 1974; Maltempo & Moss, 1976; Weber, 1982). The heme iron of *Alcaligenes* cytochrome *c'* at physiological pH is probably in a mixed ($S = {}^3/_2, {}^5/_2$) spin state (Yoshimura et al., 1985). Thus, the major and minor components of *Alcaligenes* cytochrome *c'* mentioned above may closely correspond to states A_1 and A_2 of *Chromatium* cytochrome *c'*, respectively.

At pH 1.5, *Alcaligenes* ferrous cytochrome *c'* is denatured, accompanying the protonation of coordinated histidine N_ϵ, and consequently the axial position trans to the nitrosyl group in NO–cytochrome *c'* may loosely interact with a solvent molecule to give an EPR spectrum with the characteristic features of five-coordinate nitrosylheme. This seems to be reasonable because in a ferric cytochrome *c* the protein is known to be denatured below pH 2.5 and the heme to be surrounded by solvent (Peisach et al., 1971).

The appearance of high- and low-spin absorptions in the EPR and electronic spectra of ferric cytochrome *c'* under high-alkaline conditions has been interpreted as due to the strengthening of heme iron to axial ligand bond (Maltempo & Moss, 1976; Weber, 1982; Yoshimura et al., 1985). The slight increase in intensity of EPR absorptions for six-coordinate nitrosylheme in NO–cytochrome *c'* at pH 11.0 and 13.4 may correspond to such strengthening of iron to axial ligand bond in the trans position of the nitrosyl group.

The resolution of the EPR spectrum arising from five-coordinate nitrosylheme was higher in NO–cytochrome *c'* than in model complexes. This may be explained on the basis of the interaction of the nitrosyl group with the amino acid residues surrounding the heme sixth coordination position.

Reductive Nitrosylation of Alcaligenes Ferric Cytochrome c'. As shown in Figure 6, the reaction of NO with *Alcaligenes* ferric cytochrome *c'* results in the formation of NO–ferrous cytochrome *c'*. The increase in intensity at around 420 nm in the first spectral change is probably attributed to the reduction of heme iron(III) by NO, taking into account the fact that the Soret band of *Alcaligenes* ferrous cytochrome *c'* is found at 426 nm (Iwasaki & Shidara, 1969) and NO functions as a reducing agent (Caulton, 1975). Accordingly, in the

Table I: Electronic Spectral Data for NO-Ferrous Hemoproteins and Their Model Complexes at Room Temperature[a]

	λ_{max} (nm) [ϵ ($mM^{-1}\cdot cm^{-1}$)]				
	Soret (γ)			β	α
NO-cyt c'					
Alcaligenes (pH 7.2)	396.5 (78.9)	415 sh	485 (9.8)	541 (10.4)	565 sh (10)
R. rubrum (pH 7.0)[b]	395 sh	417 (80)[i]	482	530 (11)[i]	560 sh
Rps. palustris (pH 7.0)[c]		420[i]		531[i]	567[i]
NO-cyt c[d]		411 (127)		538.5 (10.2)	567 (9.8)
NO-Mb[e]		420 (127)		548 (11.3)	579 (10.1)
NO-CCP[f]		421 (99)		542 (12.0)	572 (11.0)
NO-IDO[g]		418.5 (127)		544 (12.2)	574 (12.5)
Fe(PPIXDME)(NO)[h]	400.5 (81.6)		480 sh (11)	550 sh (10)	569.5 (10.5)
Fe(PPIXDME)(NO)(NMeIm)[h]		418.5 (118)		546.5 (11.5)	576.5 (10.6)

[a] Abbreviations used are described in the text. Extinction coefficients are the value per heme. [b] Taniguchi & Kamen, 1963. [c] De Klerk et al., 1965. [d] Butt & Keilin, 1962. [e] O'Keefe et al., 1978. [f] Yonetani et al., 1972. [g] Sono & Dawson, 1984. [h] Yoshimura & Ozaki, 1984; data in benzene. [i] Estimated from the figure in the reference.

reaction of NO with the ferric cytochrome c', NO first reduces the iron(III) and then coordinates to the iron(II) as a nitrosyl ligand. This is a typical case of reductive nitrosylation, which has been also found in the NO reaction of methemoglobin (Keilin & Hartree, 1937; Chien, 1969) and of iron(III)–porphyrin complexes (Scheidt & Frisse, 1975).

It is noted that NO–ferric cytochrome c' from *Alcaligenes* was not formed. On the other hand, in the reaction of NO with *R. rubrum* ferric cytochrome c', stable NO–ferric cytochrome c' is formed and distinguished from NO–ferrous cytochrome c' in electronic spectra (Taniguchi & Kamen, 1963). The redox potential (E_0' at pH 7) has been reported to be 132 mV for *Alcaligenes* cytochrome c' and −8 mV for *R. rubrum* cytochrome c' (Kamen et al., 1971). These suggest that the two cytochromes c' are different in the interaction of the unpaired electron on NO with ferric cytochrome c'.

Comparison with Other NO–Hemoproteins. The description in the preceding sections demonstrated that the heme iron to histidine bond in the major component of *Alcaligenes* ferrous cytochrome c' at physiological pH is very weak in strength and thus it is cleaved with the coordination of a nitrosyl group.

The NO derivatives of ferrous hemoproteins such as hemoglobin, myoglobin, peroxidase, indoleamine 2,3–dioxygenase, and cytochrome c have a six-coordinate nitrosylheme with a histidine at the axial position trans to the nitrosyl group, which has been confirmed mainly by EPR spectral results [Yoshimura (1983b) and references cited therein]. Their electronic spectral data are similar to those of a six-coordinate model nitrosylheme, Fe(PPIXDME)(NO)(N–base), as partly shown in Table I. The differences between six- and five-coordinate model nitrosylheme in electronic spectra can be distinctly found in the position of Soret band (six-coordinates, 415–419 nm; five-coordinates, 394–401 nm) and in the appearance of weak absorption at around 480 nm only for five-coordinates (Yoshimura & Ozaki, 1984).

The comparison of NO–cytochromes c' with model nitrosylheme in spectral data (Table I) indicates that NO–cytochromes c' from *Alcaligenes* and *R. rubrum* contain both five- and six-coordinate nitrosylheme, while that from *Rps. palustris* possibly contains only six-coordinate species. Further, it is evident that for NO–*Alcaligenes* cytochrome c' the five-coordinate species is much more than the six-coordinate species in relative amount and the reverse is found for *R. rubrum* cytochrome c'.

Therefore, the decreasing order of the Fe–N_ϵ bond strength among species is considered to be: *Rps. palustris* > *R. rubrum* > *Alcaligenes*. This suggests that the d_{z^2} orbital at the ground state of heme iron in cytochrome c' is stabilized in the same order and consequently the d_{z^2} orbital for *Alcaligenes* cytochrome c' is the most stable among the four cytochromes c'. It cannot be concluded, however, that the relative component ratio of $S = {}^3/_2$ in the mixed ($S = {}^3/_2$, ${}^5/_2$) spin state of *Alcaligenes* ferric cytochrome c' is highest among them, because the elucidation of in-plane ligand field in the heme remains ambiguous.

It has been estimated from magnetic properties that the mixed spin state of ferric cytochromes c' from *R. rubrum* and *Rps. palustris* consists of two thirds for $S = {}^5/_2$ and one-third for $S = {}^3/_2$ (Maltempo, 1974). Weber suggested that the heme iron(III) in the $S = {}^3/_2$ state is weaker in axial ligand field strength than that in the $S = {}^5/_2$ state (Weber, 1982). These suggestions may be related to the fact that NO–cytochromes c' from these two strains contains must larger quantities of six-coordinate nitrosylheme than those of five-coordinate species.

As shown in Table I, the α- and β-bands for the NO–cytochromes c' with six-coordinate nitrosylheme are in much shorter wavelength side than those for the other NO–hemoproteins and six-coordinate model nitrosylheme. Further, those for NO–*Alcaligenes* cytochrome c' at pH 5.3 which can contain only five-coordinate nitrosylheme are also in much shorter wavelength side than those for five-coordinate model nitrosylheme. These blue shifts of α- and β-bands may arise from unusual interaction of either the nitrosyl or heme group with the polypeptide chain in NO–*Alcaligenes* cytochrome c'.

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Registry No. Fe, 7439-89-6; N_2, 7727-37-9; ^{15}N, 14390-96-6; heme, 14875-96-8; cytochrome c', 9035-41-0.

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