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Electronic and Magnetic Circular Dichroism Spectra of Pentacoordinate Nitrosylhemes in Cytochromes *c'* from Nonphotosynthetic Bacteria and Their Model Complexes

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The NO derivatives of ferrous cytochromes *c'* from three nonphotosynthetic bacteria (*Alcaligenes* sp. NCIB 11015 and *Achromobacter xylosoxidans* GIFU 1055 and GIFU 1051) have been investigated by electronic absorption and magnetic circular dichroism (MCD) spectroscopy at room temperature. The electronic and MCD spectra of nitrosylcytochromes *c'* at pH 5.3 are substantially identical with those of the pentacoordinate nitrosyl(protoporphyrin IX dimethyl esterato)iron(II) complex [Fe(PPIXDME)(NO)] as a model complex for their nitrosylhemes. The pentacoordinate nitrosylhemes give the Soret bands at 395-400 nm, particularly exhibiting the characteristic Soret MCD spectra, which have a negative extremum in the same region. The hexacoordinate Fe(PPIXDME)(NO)(1-methylimidazole) complex displays a derivative-shaped MCD band probably assigned to a Faraday *A* term at 410-420 nm. On the other hand, the MCD patterns of pentacoordinate and hexacoordinate nitrosylhemes in the α- and β-band regions bear a resemblance to each other. Furthermore, the MCD spectrum of *Alcaligenes* nitrosylcytochrome *c'* at pH 7.2 indicates the coexistence of major pentacoordinate and minor hexacoordinate nitrosylhemes.

Introduction[2]

The cytochromes *c'* are widespread in a variety of photosynthetic and denitrifying bacteria.[3,4] They are usually dimeric and made up of identical subunits of about 14 000 daltons. Although their function is still unknown, their unique optical and magnetic properties have been noted by many investigators. Especially Maltempo et al. explained the unusual magnetic properties of ferric cytochrome *c'* from *Chromatium vinosum* as due to the existence of an intermediate-spin $^3/_2$ state with quantum-mechanical admixture of the more commonly encountered high-spin $^5/_2$ state.[5] The refined crystalographic structure of ferric cytochrome *c'* from *Rhodospirillum molischianum* reveals some features that related to the unusual mixed-spin-state character.[6]

The cytochrome *c'* of a denitrifying bacterium, *Alcaligenes* sp. NCIB 11015, was first isolated by Iwasaki et al.[7,8] The detailed spectroscopic characterization of the ferrous and ferric cytochromes *c'* has been recently carried out under the conditions of pH 1.5-13.[9] Moreover, the NO derivative of *Alcaligenes* cytochrome *c'* was prepared by the reaction of the ferrous cytochrome *c'* with nitric oxide, and the optical and EPR properties of the nitrosylheme were discussed.[10] The nitric oxide has so far been employed as a useful probe for elucidating the heme environments of hemeproteins.[11,12] The EPR and electronic absorption data of the nitrosylcytochrome *c'* indicated that the heme iron to axial histidine (Fe-N^ϵ) bond is readily cleaved upon the coordination of nitrosyl group to the vacant sixth coordination site of heme iron at physiological pH and pentacoordinate nitrosylheme is formed.

In this paper, we have characterized the electronic and MCD spectra of nitrosylcytochromes *c'* isolated from *Alcaligenes* sp. NCIB 11015, *Achromobacter xylosoxidans* GIFU 1055, and *Achromobacter xylosoxidans* GIFU 1051 and have compared them with those of the model complex, Fe(PPIXDME)(NO). Since it was recognized early that the magnetic optical activity of iron-containing porphyrins and hemoproteins is extremely sensitive to the redox state (Fe(II) and Fe(III)) of the iron, MCD spectra of these states have been examined in a significant number of hemoproteins and their derivatives.[13,14]. However, the MCD spectra of pentacoordinate ferrous nitrosylhemes are newly reported here.

Experimental Section

Cytochromes *c'* from *Alcaligenes* sp. NCIB 11015[7,8] and *Achromobacter xylosoxidans* GIFU 1055 (chemoheterotrophic nondenitrifying strain)[15] and GIFU 1051 (denitrifying strain)[16] were isolated and purified by the methods described already. The preparation of nitrosylcytochrome *c'* was carried out in a Thunberg type optical cuvette containing NO gas and cytochrome *c'* reduced with small amount of $Na_2S_2O_4$.[10] The reaction mixture was equilibrated with NO gas of slightly below 1 atm. The 50 mM sodium potassium phosphate buffer (pH 5.3) or 50 mM sodium phosphate buffer (pH 7.2) was employed as a solvent. The pentacoordinate Fe(PPIXDME)(NO) complex as a model complex of nitrosylcytochrome *c'* was obtained by treating Fe(PPIXDME)Cl with NO in a mixture of chloroform and pyridine.[17] The elementary analysis and the infrared spectrum demonstrated the absence of pyridine in the product. The dark purple crystals of Fe(PPIXDME)(NO) were dissolved in benzene under anaerobic conditions for spectroscopic measurements. The hexacoordinate Fe(PPIXDME)(NO)(*N*-MeIm) complex was prepared by the reductive nitrosylation of $[Fe(PPIXDME)(N\text{-MeIm})_2]^+$ with NO in DMeAc.[18] In the course of the reaction, the ferric form is automatically reduced to the ferrous form by NO, as found in the NO reaction of methemoglobin.[19]

The electronic and MCD spectra were measured at room temperature with a Shimadzu MPS-5000 spectrophotometer and a JASCO J-500A spectropolarimeter attached to an electromagnet (1.3 T) and a JASCO DP-501 data processor, respectively. Nitric oxide gas was purchased from Takachiho Trading Co. and purified by passing through a trap (-90

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(2) Abbreviations used: MCD = magnetic circular dichroism; EPR = electron paramagnetic resonance; Fe(PPIXDME)(NO) = nitrosyl(protoporphyrin IX dimethyl esterato)iron(II); *N*-MeIm = 1-methylimidazole; DMeAc = *N*,*N*-dimethylacetamide.
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Table I. Electronic Spectral Data for NO-Ferrous Cytochrome c' and Their Model Complexes at Room Temperature[a]

	absorption maxima, nm (ϵ, mM^{-1} cm^{-1})			
	Soret (γ)	β	α	ref
Alcaligenes NO-Cyt c'[b]	395.5 (76.6)	538 (10.4)	560 sh (10)	10
Achromobacter (GIFU 1055) NO-Cyt c'[b]	395 (90.3)	535 (11.8)	560 sh (11)	this work
Achromobacter (GIFU 1051) NO-Cyt c'[b]	395 (84.5)	533 (11.1)	560 sh (10.5)	this work
Fe(PPIXDME)(NO)[c]	400.5 (81.6)	550 (10)	569.5 (10.5)	20
Fe(PPIXDME)(NO)(*N*-MeIm)[d]	417.5 (132)	545 (13.1)	576 (12.4)	18

[a] Cyt c' = cytochrome c'. Millimolar extinction coefficients (ϵ) of NO-cytochrome c' were determined by using the alkaline pyridine hemochrome method (ϵ_{550nm} = 29.1: Drabkin, D. *J. Biol. Chem.* **1942**, *146*, 605–617). [b] In 50 mM sodium potassium phosphate buffer (pH 5.3). [c] In benzene. [d] In DMeAc.

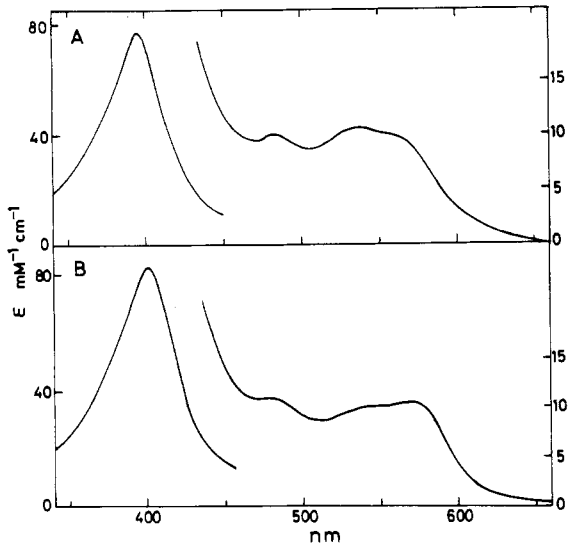

Figure 1. Electronic spectra of *Alcaligenes* NO-cytochrome c' in 50 mM sodium potassium phosphate buffer (pH 5.3) (A) and Fe(PPIXDME)(NO) in benzene (B).

°C) and a KOH column to remove N_2O and higher nitrogen oxides. All reagents used were of the highest grade commercially available.

Results and Discussion

The electronic absorption spectra of *Alcaligenes* NO-cytochrome c' (pH 5.3) and the Fe(PPIXDME)(NO) complex are given in Figure 1. The metal centers are NO-binding ferrous ions (low spin).[10] The absorption spectrum (A) of NO-cytochrome c' is closely analogous to that (B) of the pentacoordinate Fe(PPIXDME)(NO) complex in shape. Two *Achromobacter* NO-cytochromes c' also exhibit α, β, and Soret (γ) bands more similar to those of the pentacoordinate model complex than to those of the hexacoordinate Fe(PPIXDME)(NO)(*N*-MeIm) complex, as shown in Table I. The α-/β-band relative intensity ratios for NO-cytochromes c' are smaller than the ratio for the Fe(PPIXDME)(NO) complex. The NO-cytochromes display an extra band at about 485 nm, characteristic of the pentacoordinate nitrosylheme-like Fe(PPIXDME)(NO) complex.[20]

The MCD spectra of *Alcaligenes* and *Achromobacter* (GIFU 1055) NO-cytochromes c' at room temperature are illustrated in Figure 2. The NO derivative of *Achromobacter* cytochrome c' (GIFU 1051) also exhibits an MCD spectrum very similar to them (data not shown). The MCD band of *Alcaligenes* NO-cytochrome c' in the Soret region reveals a negative extremum at 395 nm. Further, a strong derivative-shaped band with a positive peak at 552 nm, a crossover at 563 nm, and a negative extremum at 578 nm appear in the α-band region and a 512-nm peak and a 530-nm trough are observed in the β-band region. A positive MCD band at 475 nm would be relative to the electronic absorption band near 485 nm, characteristic of pentacoordinate nitrosylhemes. The MCD spectra of two *Achromobacter* NO-cytochromes c' (GIFU 1055 and GIFU 1051) show extrema with the same wavelength values. Except for some minor wavelength and intensity differences, the MCD spectra in Figure 2 are essentially identical with that of the pentacoordinate Fe(PPIXDME)(NO) complex in Figure 3A. The MCD band of the model complex is observed with a negative extremum at 398 nm in the Soret-band region and two positive peaks at 514 and 558 nm and two negative extrema at 532 and 583 nm in the α- and β-band regions. The hexacoordinate nitrosylheme with *N*-MeIm as a sixth axial ligand,

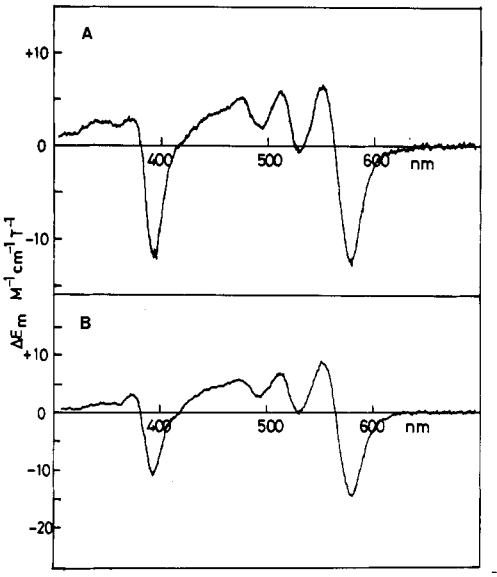

Figure 2. MCD spectra of *Alcaligenes* (A) and *Achromobacter* (GIFU 1055) (B) NO-cytochromes c' in 50 mM sodium potassium phosphate buffer (pH 5.3) at room temperature.

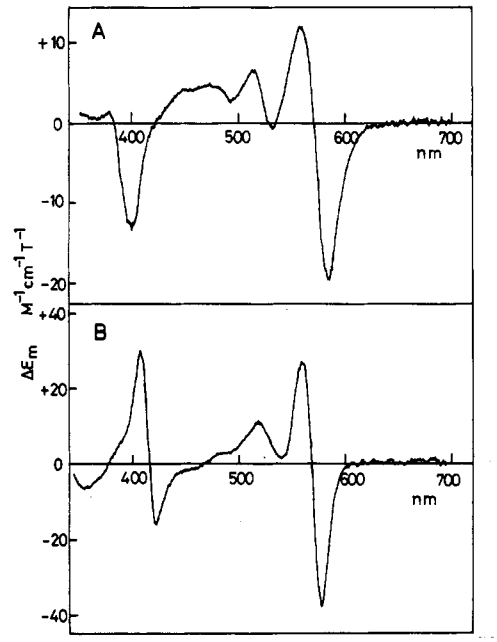

Figure 3. MCD spectra of Fe(PPIXDME)(NO) in benzene (A) and Fe(PPIXDME)(NO)(*N*-MeIm) in DMeAc (B) at room temperature.

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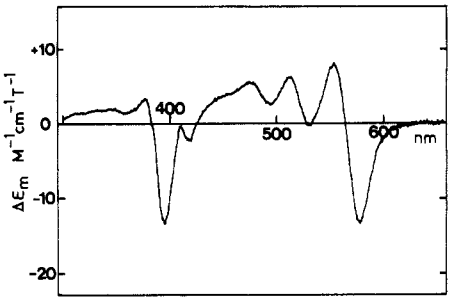

Figure 4. MCD spectrum of *Alcaligenes* NO-cytochrome *c'* in 50 mM phosphate buffer (pH 7.2) at room temperature.

Fe(PPIXDME)(NO)(*N*-MeIm), displays MCD extrema (520, 560, and 578 nm) in the α- and β-band regions similar to those of pentacoordinate cytochromes *c'* and their model complex (Figure 3B). In the Soret-band region a derivative-shaped MCD band observed with a peak at 408 nm, a crossover at 415 nm, and a negative extremum at 421 nm, however, is quite different from the Soret MCD bands of the pentacoordinate nitrosylhemes, which represent a negative peak alone. The hexacoordinate model complex appears to have the features of hexacoordinate ferrous nitrosylhemes (low spin) in the MCD spectra, which have been observed for the NO derivatives of ferrous hemoproteins:[21-23] in the α-band region the intensities of the derivative-shaped MCD bands of ferric hemoproteins having hexacoordinate nitrosylhemes ($\Delta\epsilon_m$ = +90 to −75 M^{-1} cm^{-1} T^{-1}) are higher than those for ferrous hemoproteins having hexacoordinate nitrosylhemes ($\Delta\epsilon_m$ = +25 to −35 M^{-1} cm^{-1} T^{-1}), though these MCD patterns are similar each other.[22,23] The MCD bands associated with the α, β, and Soret bands of the Fe(PPIXDME)(NO)(*N*-MeIm) complex are probably due to Faraday *A* terms, since all of the corresponding MCD bands for nitrosylhemoglobin and nitrosylmyoglobin are assigned to *A* terms.[21,24] The MCD spectra of pentacoordinate NO derivatives in the α- and β-band regions in Figures 2 and 3A are also proposed to consist of *A* terms, but the Soret MCD spectra are indicative of a *B* or *C* term.

We have already reported that the EPR spectrum of the NO derivative of *Alcaligenes* cytochrome *c'* at pH 5.3 demonstrates pentacoordinate nitrosylheme, which means that the heme iron to proximal histidine bond is cleaved by the binding of the NO group to heme iron.[10] The conspicuous *g* values of pentacoordinate nitrosylcytochrome *c'* were essentially identical with those of the Fe(PPIXDME)(NO) complex. The present conclusion concerning the heme environments in nitrosyl cytochromes *c'* by means of MCD spectroscopy is quite consistent with the previous results[10] obtained by EPR. Consequently, MCD measurements as well as the EPR are generally useful for the determination of geometries of nitrosylhemes. On the other hand, Scholler et al. undertook a joint resonance Raman and EPR study of pentacoordinate and hexacoordinate nitrosylheme model compounds and of some nitrosylhemoglobins, suggesting that EPR spectroscopy is not available as a test for the coordination state of a nitrosylheme.[25]

A significant difference of the MCD patterns between pentacoordinate and hexacoordinate nitrosylhemes exists in the region of Soret bands. It is concluded that the Soret MCD band is more sensitive to the structure of nitrosylheme than the MCD bands in the α- and β-band regions.

The MCD spectrum of *Alcaligenes* NO-cytochrome *c'* in 50 mM phosphate buffer (pH 7.2) is shown in Figure 4. Except for a weak negative extremum at 418 nm, the MCD spectrum resembles those of pentacoordinate nitrosylcytochromes *c'* at pH 5.3. Thus there are two forms of nitrosylheme in NO-cytochrome *c'* at pH 7.2. The major species is assigned to pentacoordinate nitrosylheme, exhibiting a negative 395-nm peak in the Soret-band region, and the minor one might be attributable to hexacoordinate nitrosylheme, which has a negative MCD band around 420 nm. This finding supports the previous proposal that the nitrosylheme in *Alcaligenes* NO-cytochrome *c'* at pH 7.2 consists of major pentacoordinate and minor hexacoordinate species, which were detected by optical and EPR measurements.[10]

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