The pH Dependence of the Stereochemistry around the Heme Group in NO–Cytochrome *c* (Horse Heart)

TETSUHIKO YOSHIMURA*

The Environmental Science Institute of Hyogo Prefecture, Yukihira-cho, Suma-ku, Kobe 654, Japan

and SHINNICHIRO SUZUKI

Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan

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Abstract

The electronic absorption, EPR and MCD spectra of NO derivatives of both ferrous and ferric cytochrome *c* (horse heart) have been measured in the pH region 2.0 to 12.9, in order to elucidate the pH dependence of the stereochemistry around the heme group. The reaction products of NO with ferrous cytochrome *c* in equilibrium were as follows: in the region $2.0 \leqslant pH \leqslant 5.3$, NO–ferrous cytochrome *c*; in the region $5.3 < pH \leqslant 11.0$, a mixture of NO–ferrous cytochrome *c* and native ferrous cytochrome *c*; at pH 12.9, NO–ferrous cytochrome *c*. At pH 2.0, the NO–ferrous cytochrome *c* contained a five-coordinate nitrosylheme as the major component and a six-coordinate species as the minor component, and at the other pH values it contained only the six-coordinate species. The reaction products of NO with ferric cytochrome *c* in equilibrium were as follows: in the region $2.0 \leqslant pH \leqslant 7.2$, NO–ferric cytochrome *c* with six-coordinate nitrosylheme; in the region $7.2 < pH \leqslant 11.0$, a mixture of NO–ferrous cytochrome *c* and native ferrous cytochrome *c*; at pH 12.0, NO–ferrous cytochrome *c*. Thus, the reaction of NO with ferric cytochrome *c* results in the formation of NO–ferrous cytochrome *c*, which is a typical case of reductive nitrosylation.

Introduction**

Nitric oxide is coordinated as a heme sixth ligand to heme iron in hemoproteins to give nitrosylhemoproteins and has an unpaired electron which delocalizes to the heme group and the *trans*-axial ligand through the heme iron. Accordingly, it has been widely employed as a useful probe for elucidating the symmetry around the heme iron and the electronic structure of the heme group in various hemoproteins [1]. In order to understand further the spectral results for the nitrosylhemoproteins, their model systems (nitrosyl(porphyrinato)iron(II) complexes under various conditions) have been extensively studied by various spectroscopic methods [2–5].

In the heme group buried into the hydrophobic protein interior in cytochromes *c*, the fifth ligand to the heme iron is provided by the histidine residue in which the $N_\delta H$ proton is hydrogen-bonded to the carbonyl oxygen in the proline residue [6, 7]. Nitric oxide may readily penetrate into such a hydrophobic heme crevice to interact with the heme iron, because it is a neutral small molecule and a powerful complexing agent. The study of the reaction of NO with cytochrome *c* has so far been carried out by several workers [8–13]. Butt and Keilin have demonstrated that NO–ferric cytochrome *c* is formed at physiological pH, while NO–ferrous cytochrome *c* is not formed at physiological pH but at high alkaline pH, indicating that the tertiary protein structure is more open in ferric– than in ferrous–cytochrome *c* [10]. Kon measured the EPR spectra of ^{14}NO and ^{15}NO derivatives of ferrous cytochrome *c* [2, 11]. The EPR spectrum of ^{14}NO–ferrous cytochrome *c* exhibited a triplet of triplets in the g_z absorption, which originates from the hyperfine interactions of the unpaired electron with the ^{14}N nucleus of the NO group and of the *trans*-axial histidine nitrogen, indicating the replacement of the sixth ligand methionine by NO [9]. Orii and Shimada investigated the reaction of cytochrome *c* with NO and nitrite as model systems for dissimilatory nitrite reductase and reported the pH dependence of the electronic spectra for the model systems [13]. These studies have revealed that the reaction product of cytochrome *c* with NO differs between the ferric and the ferrous forms and that it is affected by varying the pH values

*Author to whom correspondence should be addressed.

**Abbreviations: cyt, cytochrome; Hb, hemoglobin; Mb, myoglobin; HRP, horseradish peroxidase; IDO, indoleamine 2,3-dioxygenase; *Alcaligenes, Alcaligenes* sp. NCIB 11015; *R. rubrum, Rhodospirillum rubrum*; *Rps. capsulata, Rhodopseudomonas capsulata* B100; PPIXDME, dianion of protoporphyrin IX dimethyl ester; ImH, imidazole; Im^-, imidazolate; NMeIM, 1-methylimidazole; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; sh, shoulder absorption.

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of the medium. In these studies, however, the pH dependence of the stereochemistry around the heme group in NO–cytochrome *c* was not discussed. In the present work, the electronic absorption, EPR and MCD spectra of NO–ferrous and NO–ferric cytochrome *c* (horse heart) have been measured under various conditions, in order to obtain information about the pH dependence of the stereochemistry.

Experimental

Horse heart cytochrome *c* (Sigma, Type VI) was purchased and used without further purification. The oxidized form was obtained by the addition of 20 μl of 8.5 mM potassium ferricyanide solution to the protein solution. The reduced form was prepared by the addition of a minimum quantity of solid sodium dithionite to the ferric form under anaerobic conditions. The concentration of cytochrome *c* was determined spectrophotometrically by using the alkaline pyridine hemochrome method (ϵ_{mM} at 550 nm = 29.1 [14]).

Nitric oxide (99.5% minimum), purchased from Seitetsu Kagaku, was passed through a KOH column to remove higher nitrogen oxides. All other chemicals used were of the highest grade available.

The cytochrome *c* solution was carefully deoxygenated in a Thunberg-type tube with an optical cuvette (path length, 1 cm) or with an EPR tube and then reacted with NO gas of slightly below 1 atm in the reduced or oxidized form, as described previously [15].

The buffers used here were as follows: pH 2.0, 0.1 M sodium citrate/HCl; pH 3.4, 0.1 M CH_3COONa/CH_3COOH; pH 5.3, 0.05 M KH_2PO_4/Na_2HPO_4; pH values of 6.8 and 7.2, 0.05 M NaH_2PO_4/Na_2HPO_4; pH 9.8, 0.2 M $KHCO_3/Na_2CO_3$; pH values of 11.0 and 12.9, 0.1 M K_2HPO_4/KOH.

The EPR, electronic absorption and MCD spectral measurements at room temperature and at 77 K were carried out as described previously [15, 16].

Results and Discussion

Ferric cytochrome *c* has been found to be changed from a low-spin form at neutral pH to a high-spin form at low pH, suggesting that the iron-to-axial ligand bonds are cleaved with a decrease in pH values of the medium [6]. Furthermore, a 695 nm band in the electronic spectrum of ferric cytochrome *c*, which has been attributed to the presence of an iron-to-methionine bond [17], decreases in intensity as the pH value increases from neutral pH to high alkaline pH (pK_a values for horse heart cytochrome *c*, 9.1–9.3 [6, 18]). The disappearance of this band at high pH is interpreted as indicating the cleavage of the iron-to-methionine bond followed by replacement by the other amino acid residue, probably lysine [6, 17]. On the other hand, a pair of axial ligands, histidine and methionine, and the low-spin form in the ferrous cytochrome *c* are maintained over a wide range of pH values [15]. Thus, it has been demonstrated that the heme iron-to-methionine bond in cytochrome *c* is much weaker in the ferric form than in the ferrous form [6].

Accordingly, the pH dependence of the reaction of cytochrome *c* with NO is expected to be different between the ferrous and the ferric forms, which are separately discussed below.

Reaction of Ferrous Cytochrome c with NO

Ferrous cytochrome *c* at pH 2.0 reacted rapidly with NO to exhibit the electronic spectrum shown in Fig. 1 (solid line) and the EPR spectra shown in Fig. 2. The electronic spectrum was similar to that of a five-coordinate model nitrosylheme, Fe(PPIXDME)-(NO) [4] and an NO–ferrous cytochrome *c'* (*Alcaligenes* sp.) at pH 7.2 [15]. The shoulder absorption at around 410 nm was situated at the position of the Soret band of a six-coordinate model nitrosylheme with a nitrogenous base [4] and an NO–ferrous cytochrome *c* at pH 3.4 described later. The three *g* values (g_1', g_2' and g_3') of the EPR spectra at 77 K were essentially identical with those of five-coordinate model nitrosylheme in non-donor solvents [19, 20] and NO–ferrous cytochrome *c'* (*Alcaligenes* sp.) at pH 7.2 [15], and another two *g* values (g_1 and g_3) were also identical with those of six-coordinate model nitrosylheme [5] and NO–ferrous cytochrome *c* at higher pH (Table I). Accordingly, the NO–ferrous cytochrome *c* at pH 2.0 would contain a five-coordinate nitrosylheme as the major component and a six-coordinate species as the minor component. The content of the six-coordinate species can be estimated to be 20–30% from the electronic spectral intensity of the Soret band region.

When nitric oxide gas was introduced anaerobically into a Thunberg-type cuvette containing ferrous cytochrome *c* at pH 3.4, NO–ferrous cytochrome *c* was gradually formed, as shown in Fig. 3. At pH 5.3, the reaction of NO with ferrous cytochrome *c* proceeded in the same manner as that at pH 3.4, though a prolonged equilibration (90 min) with NO was required to obtain the final stable spectrum. These spectra share isosbestic points at 413, 430, 503, 530, 543 and 556 nm. The final electronic spectrum in Fig. 3 exhibits the spectral features characteristic of six-coordinate nitrosylheme with a nitrogenous base [4] and NO–hemoproteins (Table II). Figure 4 shows the EPR spectra of NO–ferrous cytochrome *c* at pH 5.3, which are essentially identical to those at pH 3.4. The overall pattern of EPR spectra at 77 K in Fig. 4 resembles that of the spectrum of NO–ferrous cytochrome *c* in 0.01 N NaOH

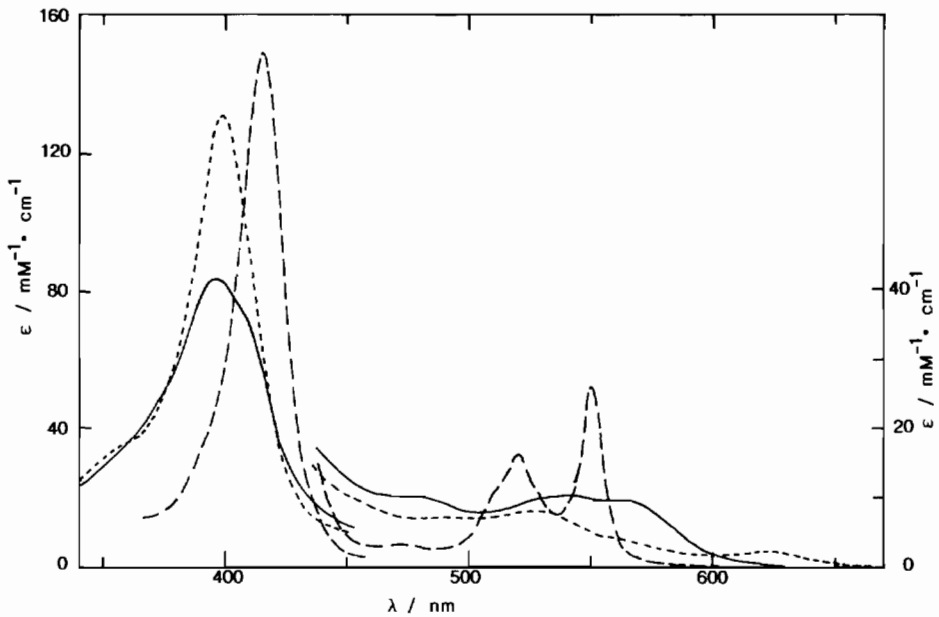

Fig. 1. Electronic spectra of cytochrome *c* (horse heart) at pH 2.0 and at room temperature: ----, ferric form; – – –, ferrous form; ——, NO–ferrous form.

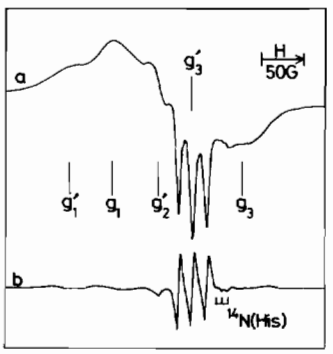

Fig. 2. EPR spectra of NO–ferrous cytochrome *c* (horse heart) at pH 2.0 and at 77 K: (a) first derivative; (b) second derivative ($g_1 = 2.07$, $g_3 = 1.98$; $g_1' = 2.10$, $g_2' = 2.035$, $g_3' = 2.010$, $a_3' = 16.6$ G). Instrument settings: modulation frequency and amplitude, 100 kHz and 3.2 G; microwave frequency and power, 9.172 GHz and 10 mW.

solution reported by Kon and Kataoka [2]. The spectrum exhibited a line shape with rhombic symmetry and the existence of three *g* values. In the g_2 absorption, which can be assigned to the g_z absorption [1], a triplet of triplets was readily observed (Fig. 4). As mentioned above, these results indicate that the sixth ligand methionine can be replaced by a nitrosyl group in NO–ferrous cytochrome *c*. At room temperature, the EPR spectrum gave a line shape of an almost symmetrical singlet with a peak-to-peak width of 70 G and a g_{iso} value of 2.018.

In the pH region from 7 to 11, the reaction of NO with ferrous cytochrome *c* proceeded at a slow rate. The electronic spectral changes from ferrous cytochrome *c* to the NO complex are shown in Fig. 5. The stable final spectrum was obtained after 19 h of NO addition. This spectrum remained unchanged after allowing the solution to stand for a day at 4 °C. As

TABLE I. EPR Spectral Data for NO–Ferrous Hemoproteins and their Model Complexes at 77 K[a]

	g Values				Coupling constants (G)			Reference
	g_1 (g_1')	$g_?$	g_2 (g_2')	g_3 (g_3')	$a_2(N_{NO})$	$a_2(N_{His})$	$(a_3(N_{NO})')$	
NO–cyt *c* at pH 7.2	2.068	2.037	2.0031	1.971	23.1	6.8		
NO–Hb [b]	2.075		2.005	1.975				1
NO–HRP [b]	2.080		2.004	1.955	20.5	6.5		1
NO–cyt *c'* (*Alcaligenes*)[b]	(2.106)		(2.033)	(2.010)			(16.0)	15
Fe(PPIXDME)(NO)(ImH)	2.072	2.030	2.0040	1.971	21.7	6.9		5
Fe(PPIXDME)(NO)(Im^-)	2.068	2.03	2.0032	1.966	22.2	6.8		5

[a]Abbreviations used are described in the text. [b]At neutral pH.

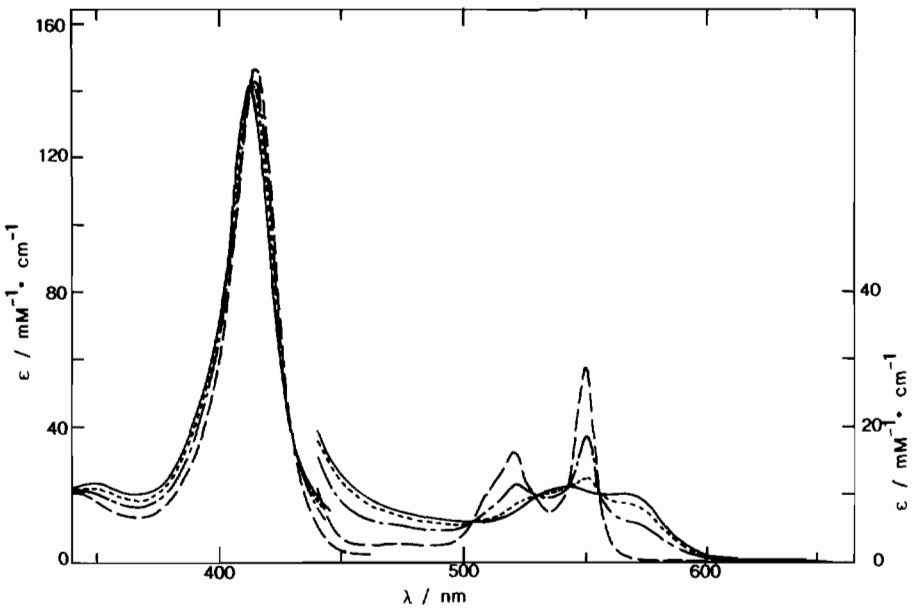

Fig. 3. Reaction of NO with ferrous cytochrome *c* (horse heart) at pH 3.4 and at room temperature: – – –, the spectrum measured before introduction of NO; –·– and ----, the spectrum after 10 and 15 min of NO addition respectively; ——, the final stable spectrum after 25 min of NO addition.

TABLE II. Electronic Spectral Data for NO–Ferrous Hemoproteins and their Model Complexes at Room Temperature[a]

	λ_{max} (nm) (ϵ (mM^{-1} cm^{-1}))						Reference
	Soret (γ)				β	α	
NO–cyt *c* (horse heart)							
pH 2.0		396.5(83.3)	410sh(70)	475(10.4)	539(10.9)	560sh(10.5)	
pH 3.4	349(23.6)		412(141)		541(11.5)	565(10.5)	
pH 5.3	349(24.1)		412(146)		538.5(11.6)	563.5(11.4)	
pH 12.9	358(23.3)		412(128)		540(11.2)	567.5(10.3)	
NO–cyt *c'* (*Alcaligenes*)[b]		396.5(78.9)	415sh	485(9.8)	541(10.4)	565sh(10)	15
NO–Mb [b]			420(127)		548(11.3)	579(10.1)	31
NO–IDO [b]			418.5(127)		544(12.2)	574(12.5)	32
NO–HRP [b]			421(110)		542(11.5)	570(10.5)	1
Fe(PPIXDME)(NO)		400.5(81.6)		480sh(11)	550sh(10)	569.5(10.5)	4
Fe(PPIXDME)(NO)(NMeIm)			418.5(118)		546.5(11.5)	576.5(10.6)	4

[a,b]See the footnotes of Table I.

shown in Fig. 5, the final spectrum has a hump at around 550 nm, unlike that of Fig. 3 (pH 3.4), which can be ascribed to the α-band of unreacted ferrous cytochrome *c*. Thus, it is probable that the sixth ligand methionine can neither readily nor completely be replaced by the nitrosyl group in this pH region because the heme iron-to-methionine bond is far more stable than that in other pH regions. In the EPR spectrum of the reaction product of NO with ferrous cytochrome *c* in this pH region, only a signal based on NO–ferrous cytochrome *c* was observed because ferrous cytochrome *c* is EPR silent.

At pH 12.9, ferrous cytochrome *c* reacted readily with NO to exhibit the electronic and EPR spectral line shape similar to those at pH values of 3.4 and 5.3 (Figs. 3 and 4).

The pH dependence of the reaction of NO with ferrous cytochrome *c* can be summarized as follows. Below pH 2.0, ferrous cytochrome *c* reacts rapidly with NO to form NO–ferrous cytochrome *c*, and the NO derivatives containing a five- and a six-coordinate nitrosylheme coexist in solution at equilibrium. In the region $2.0 < pH \leq 5.3$, ferrous cytochrome *c* reacts slowly with NO to form NO–ferrous cytochrome *c* with only six-coordinate nitrosylheme. In the region $5.3 < pH \leq 11.0$, the reaction proceeds very slowly to form NO–ferrous cytochrome *c* with a six-coordinate nitrosylheme, although a small

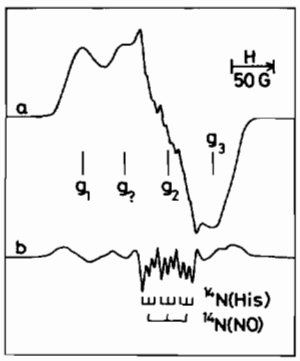

Fig. 4. EPR spectra of NO–ferrous cytochrome *c* (horse heart) at pH 5.3 and at 77 K: (a) first derivative; (b) second derivative ($g_1 = 2.067$, $g_? = 2.035$, $g_2 = 2.0033$, $g_3 = 1.971$; $a_2(N_{NO}) = 22.7$ G, $a_2(N_{His}) = 6.9$ G). Instrument settings: modulation frequency and amplitude, 100 kHz and 2 G; microwave frequency and power, 9.178 GHz and 10 mW.

portion of the ferrous cytochrome *c* remains unreacted in equilibrium. Above pH 11.0, ferrous cytochrome *c* reacts rapidly with NO to form NO–ferrous cytochrome *c* containing only six-coordinate nitrosylheme. These results suggest that although the iron-to-axial bonds of ferrous cytochrome *c* have been shown to be stable over a wide range of pH values [6], their strength is affected by the pH value of the medium.

It is interesting that the α-, β- and Soret-bands in the electronic absorption spectrum of NO–ferrous cytochromes with *c*-type heme are found at the shorter wavelength side than those of the other NO–hemoproteins and their model complexes with six-coordinate nitrosylheme (Table II). Furthermore, the electronic spectra of ferrous cytochrome *c* at 77 K (Fig. 6) are essentially identical with those at room temperature, although the resolution of α- and β-bands for the former is better than that for the latter.

The dependence of EPR parameters for model nitrosylhemes upon the electronic and the stereochemical environments surrounding the complexes has been extensively investigated [2, 3, 5, 21]. It has been demonstrated that the *g* values, in particular the g_2 value at 77 K, of six-coordinate nitrosylhemes with unhindered imidazoles vary linearly with the basicity of the base, while those with hindered imidazoles do not vary in such a manner because of the steric interaction with the porphyrin core [5]. As shown in Table I, EPR parameters of NO–ferrous cytochrome *c* at pH 7.2 and at 77 K are found to be close not to those of the Fe(PPIXDME)(NO) complex with imidazole but to those of the complex with imidazolate. This result may arise from the existence of a strong hydrogen bond between $N_\delta H$ of the fifth axial ligand histidine and the carbonyl of proline in cytochrome *c*.

The EPR parameters of NO–ferrous cytochrome *c* appear to be sensitive to pH values of the medium. It seems reasonable that the effect of pH values on EPR parameters is brought about by an indirect interaction of the medium with the heme moiety, on the basis of the following reasons: (a) the direct inter-

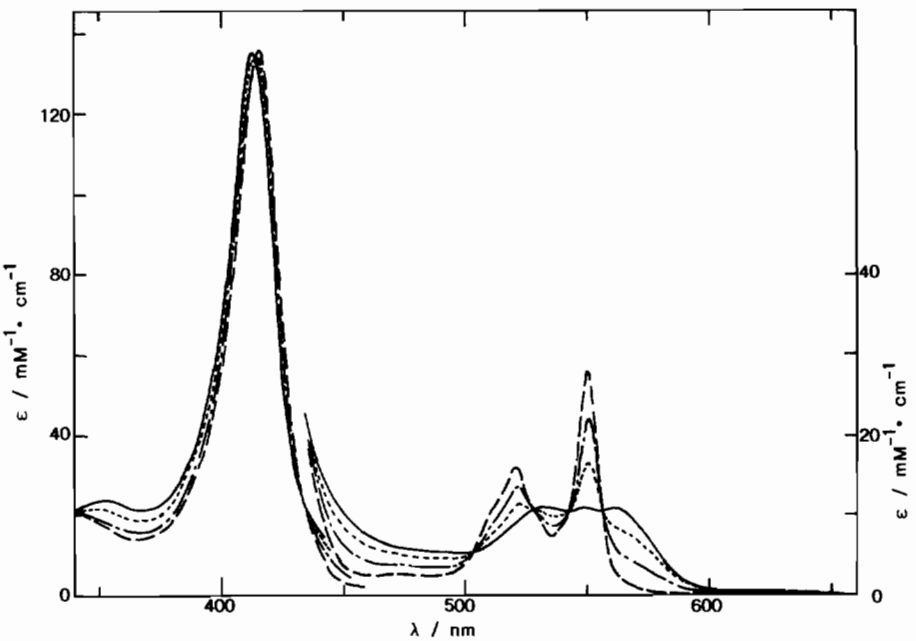

Fig. 5. Reaction of NO with ferrous cytochrome *c* (horse heart) at pH 7.2 and at room temperature: – – –, the spectrum measured before introduction of NO; –·– and ·–·–, the spectrum after 2 and 3 h of NO addition respectively; ——, the final stable spectrum 19 h after NO introduction.

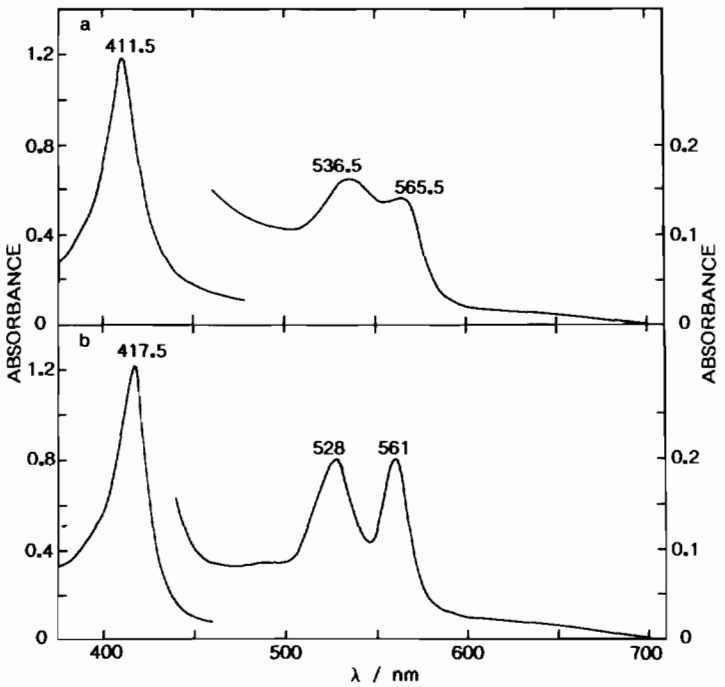

Fig. 6. Electronic spectra at 77 K of (a) NO–ferrous cytochrome *c* at pH 12.9 and (b) NO–ferric cytochrome *c* at pH 7.2.

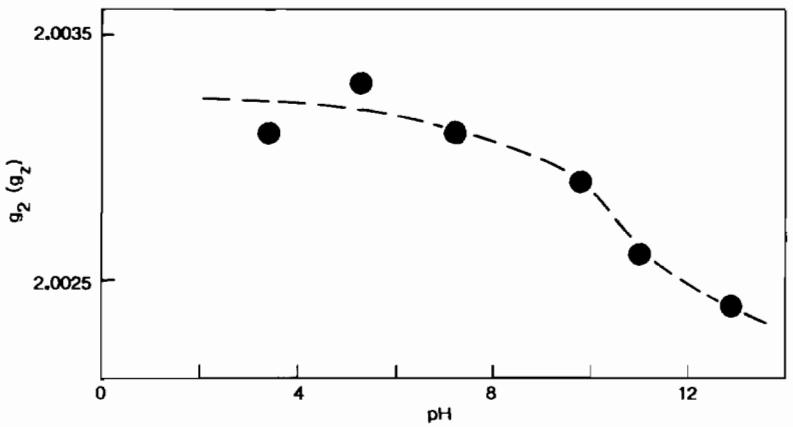

Fig. 7. Relationships between the g_2 (g_z) values (at 77 K) of NO–ferrous cytochrome *c* (horse heart) and the pH values of the medium.

action is hindered by the hydrophobic heme environments; and (b) the interaction of the medium with the hydrophilic exterior of the protein results in conformational changes to the protein followed by structural changes to the heme environments. Plots of the g_2 values against pH values of the medium are illustrated in Fig. 7. The pH value at an inflection point of the curve thus obtained is roughly estimated to be 9–10. This result suggests that, at high pH, the environment surrounding the sixth coordination position is rearranged also in ferrous cytochrome *c*, accompanying a strengthening of the hydrogen bond between the axial histidine $N_\delta H$ and the proline carbonyl, although the iron-to-methionine bond is maintained in ferrous cytochrome *c*. It has been demonstrated that the pH of buffer solutions can change on freezing [22, 23] and the EPR spectra of nitrosylhemoproteins and their model complexes vary markedly with temperature [21, 24, 25]. Thus, the finding obtained from EPR spectral results on frozen solutions of NO–cytochrome *c* should be considered from these standpoints, although electronic spectral results on the frozen solutions were essentially identical with those at room temperature, as mentioned above.

Reaction of Ferric Cytochrome c with NO

At pH 2.0, a weak absorption band at around 620 nm in the electronic spectrum of ferric cytochrome *c* (Fig. 1, dotted line) and an intense signal at around

$g = 6$ in the EPR spectrum at 77 K were observed, suggesting that a major portion of the heme iron(III) is in a high-spin state. At pH 3.4, the 695 nm band was found in a lower intensity than that at neutral pH and the high-spin EPR signal was also weak. Thus, it is inferred that the portion of N_ϵ in the fifth ligand histidine at these pH values is protonated and consequently the iron-to-histidine bond is cleaved. Ferric cytochrome *c* in the pH region from 2.0 to 3.4 reacted rapidly with NO to give an electronic spectra similar to that obtained at pH 7.2 (Table III; Fig. 8, broken line). These spectral features resemble those for several NO–hemoproteins reported previously (Table III), in which a *trans*-axial ligand is a histidine. These results suggest that the coordination of NO as a sixth ligand results in some stabilization of the iron-to-histidine bond in ferric cytochrome *c* at low pH.

The reaction of NO with ferric cytochrome *c* proceeded rapidly also in the pH region from 5.3 to 7.2 to give an NO–ferric cytochrome *c* analogous to that obtained at lower pH (Fig. 8). The electronic spectra of NO–ferric cytochrome *c* at room temperature were quite similar to those at 77 K (Fig. 6) and have absorption bands at slightly shorter wavelengths compared with those for the other NO–hemoproteins (Table III).

In the region $7.2 < \text{pH} \leqslant 11.0$, the reaction of NO with ferric cytochrome *c* proceeded rapidly to form NO–ferric cytochrome *c* (Fig. 9, chain line) which was subsequently converted into a mixture of ferrous cytochrome *c* as a minor component and an NO analogue as a major component (Fig. 9, dotted and solid lines); the EPR spectrum exhibited a signal characteristic of NO–ferrous cytochrome *c*. Figure 9

TABLE III. Electronic Spectral Data for NO–Ferric Hemoproteins at Room Temperature[a]

	λ_{max} (nm) (ϵ (mM^{-1} cm^{-1}))						Reference
	Soret (γ)				β	α	
NO–cyt *c* (horse heart)							
pH 2.0	362(29.2)	395sh	417.5(163)	485sh(3)	529(12.9)	562.5(12.4)	
pH 7.2	361(27.3)	395sh	417.5(159)	490sh(3)	529(13.1)	563(13.6)	
NO–cyt *c'* (*Rps. capsulata*)[b]			417.5(141)	490sh(4.4)	528.5(11.8)	562(11.2)	30
NO–cyt *c'* (*R. rubrum*)[b]			417	482sh	530	563	33
NO–HRP[b]			419(144)		533(13.0)	568(16.0)	1
NO–IDO[b]			418.5(146)		534	568	32

[a, b] See the footnotes of Table I.

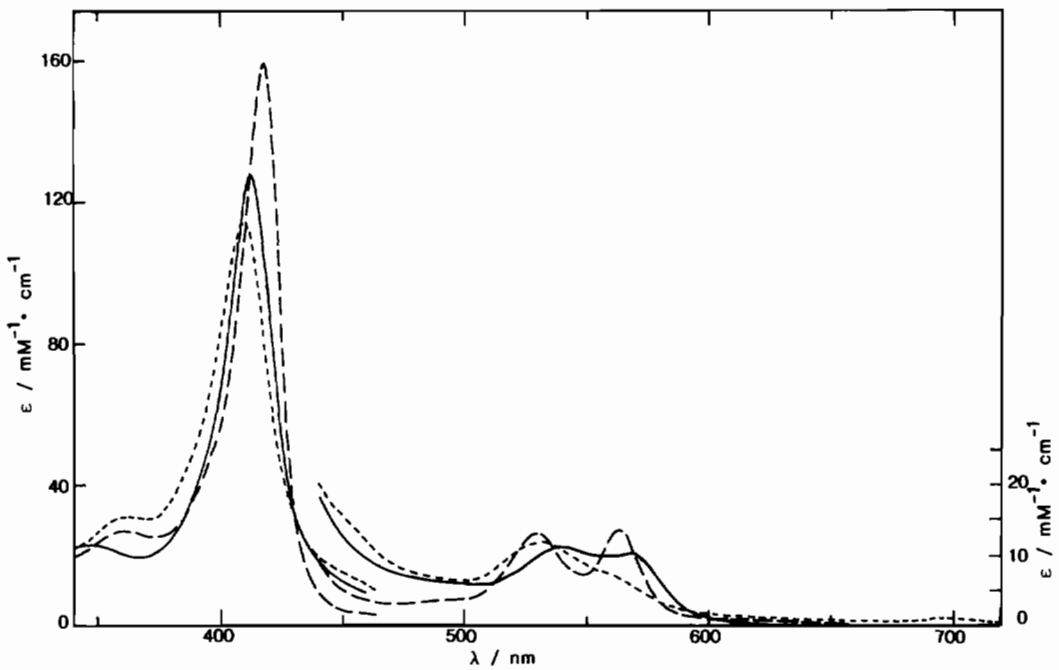

Fig. 8. Electronic spectra of cytochrome *c* (horse heart) at room temperature: ----, ferric form at pH 7.2; – – – and ——, reaction product of NO with ferric form at pH 7.2 and at pH 12.9, respectively. Details are given in the text.

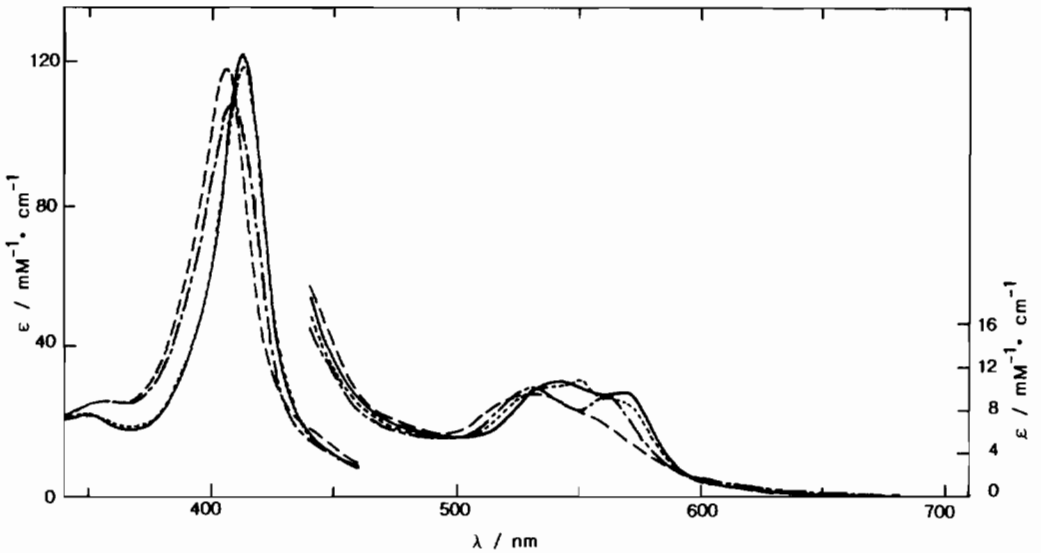

Fig. 9. Reaction of NO with ferric cytochrome *c* (horse heart) at pH 11.0 and at room temperature: – – –, the spectrum measured before introduction of NO; –·– and ····, the spectrum after 10 and 20 min of NO addition respectively; ——, the final stable spectrum after 5 h of NO addition.

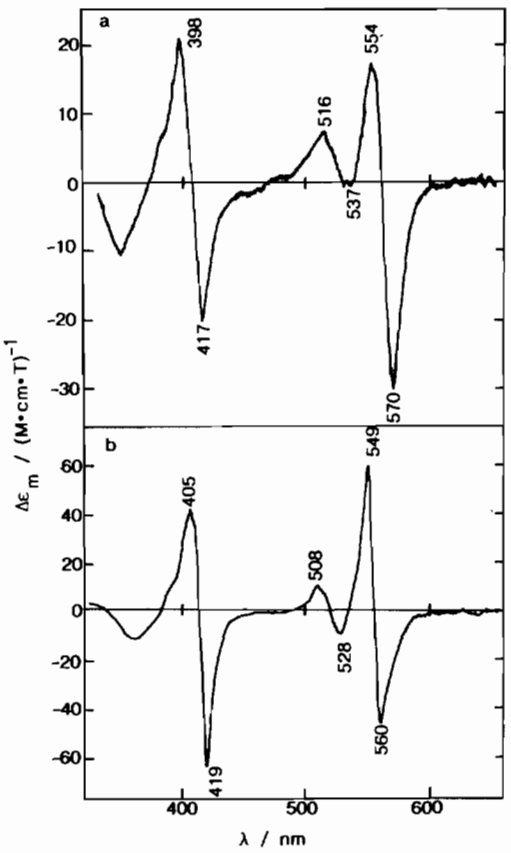

Fig. 10. MCD spectra for the reaction product of NO with ferric cytochrome *c* (a) at pH 12.9 and (b) at pH 6.8 at room temperature. Details are given in the text.

shows such electronic spectral changes at pH 11.0. The MCD spectrum of the solution thus obtained at pH 11.0 resembles that (Fig. 10) of NO–ferrous cytochrome *c* as a whole, but the weak peak at 540 nm and the relatively high intensity in the Soret band region are compatible with the existence of native ferrous cytochrome *c*.

At pH 12.9, ferric cytochrome *c* reacted rapidly with NO to exhibit electronic (Fig. 8) and EPR spectra quite similar to those of NO–ferrous cytochrome *c*.

The reaction products of ferric cytochrome *c* with NO in the pH region above 7.2 appear to be identical with that of ferrous cytochrome *c* at the same pH values because of the close similarity of the electronic and EPR spectral line shapes and parameters. Accordingly, in the reaction with ferric cytochrome *c* NO first coordinates to the iron(III) and then reduces it to iron(II), followed by coordination to the iron(II) as a nitrosyl ligand. In these reactions, NO functions as both a ligand and a reductant. Such a reductive nitrosylation reaction has been also found in the reaction of NO with methemoglobin [26, 27], ferric cytochrome *c'* [15] and iron(III) porphyrin complexes [28].

Figure 10a shows the MCD spectra of NO–ferrous cytochrome *c* at pH 12.9, which was obtained by the reductive nitrosylation of ferric cytochrome *c*; Fig. 10b shows those of NO–ferric cytochrome *c* at pH 6.8. These MCD spectral band positions and intensities closely resemble those of the NO derivatives for cytochrome *c'* in both the ferrous and ferric forms [29, 30]. In a similar manner to the electronic

spectra of NO derivatives, overall MCD spectral patterns of NO–cytochromes with *c*-type heme are similar to those of NO–hemoproteins with protoheme; the former have band positions at shorter wavelengths than those of the latter. It remains unclear whether the difference in spectral properties between NO–cytochromes with *c*-type heme and NO–hemoproteins with protoheme result from differences in the prosthetic group or in the heme environments.

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