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# Nuclear Magnetic Resonance Studies of *Rhodospirillum rubrum* Cytochrome *c'*†

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ABSTRACT: Cytochrome *c'* from *Rhodospirillum rubrum* has been studied by proton magnetic resonance (NMR) at 270 MHz. The pH and temperature-dependence properties as well as proton water relaxation enhancement and bulk susceptibility measurements were examined. We conclude that the fifth ligand to the iron is histidine. The pH-dependent shift of the heme methyl resonances of the ferric protein shows p$K_a$'s at 5.8 and 8.7. The low-pH equilibrium causes only minor changes in the properties of the protein. However, the high-pH equilibrium causes large changes throughout the NMR spectra which correlate with the reported visible spectral changes. These NMR spectral changes are compared with the low-temperature EPR and Mössbauer spectroscopic data. Analyses of the NMR data show that a second histidine, which is present in the sequence of *c'* from *R. rubrum* but is not conserved in other cytochromes *c'*, is not a "distal" histidine. The nature of the sixth ligand and the significance of the high-pH transition are discussed.

The cytochromes *c'* are an anomalous group of heme proteins whose precise biological function has not yet been established, although it has been suggested that they operate in electron-transport chains (Horio & Kamen, 1970; Kakuno et al., 1973; Lemberg & Barrett, 1973). These proteins are placed in the cytochrome *c'* class of heme proteins by virtue of their possession of a heme group covalently bound to the protein via thioether linkages and the high-spin nature of the heme iron. The sequence of nine cytochromes *c'* have been determined, and all possess heme binding units (Cys-x-y-Cys-His)[1] located near the C-terminus (Ambler, 1973; Meyer et al., 1975; Ambler et al., 1979a,b; R. P. Ambler, unpublished data). By analogy with other types of cytochromes *c*, the conserved histidine residue in this sequence probably provides the fifth ligand to the heme iron. The nature of the sixth ligand is not known. We shall address ourselves to the problem of the

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[1] Abbreviations used: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; IR, infrared; RHP, *Rhodospirillum* heme protein.

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ligands to the iron in this paper.

Initially, optical (Horio & Kamen, 1961), near-infrared (Kamen et al., 1973), Mössbauer spectroscopic examinations (Moss et al., 1968), and magnetic susceptibility measurements (Ehrenberg & Kamen, 1965; Tasaki et al., 1967) suggested that cytochromes *c′* are predominantly high spin, similar to myoglobin and hemoglobin. In recognition of this, the term "*Rhodospirillum* heme protein" (RHP) was used in preference to cytochrome (Bartsch & Kamen, 1958). However, later studies of the electron paramagnetic resonance (EPR) (Maltempto et al., 1974) and resonance Raman spectra (Strekas & Spiro, 1974) of cytochromes *c′* have led to the suggestion that the nature of the iron and the heme was different from those of myoglobin and hemoglobin, namely, that the spin state for cytochrome *c′* was a quantum mechanical admixture of $S = {}^5/_2$ and $S = {}^3/_2$ spin states (Maltempto, 1974). More recent studies of the Mössbauer (Emptage et al., 1977) and magnetic circular dichroism (MCD) spectra (Rawlings et al., 1977) have tended to swing the pendulum back to an emphasis on the similarities between cytochrome *c′*, the globins, and the peroxidases. However, it is possible that cytochromes *c′* from different species may have different physical properties. Recent (Reed et al., 1979; Goff & Shimomura, 1980) and future spectroscopic studies of the intermediate-spin ($S = {}^3/_2$) model compounds, ferric porphyrin perchlorate derivatives, may help to clarify these discrepancies.

Cytochrome *c′* from *Rhodospirillum rubrum* consists of a dimer ($M_r$ 27 500) with identical subunits (Meyer et al., 1975). The protein has a p*I* of 5.4 and the heme prosthetic group has a redox potential of 0.0 v at pH (Kamen et al., 1971). The ferric protein, in the pH range 6–10, undergoes changes in its visible (Horio & Kamen, 1961), EPR and Mössbauer (Emptage et al., 1977), MCD (Rawlings et al., 1977), and resonance Raman spectra (Kitagawa et al., 1977) with a p$K_a$ of near 8.5. In contrast, the ferrous protein reveals no detectable spectral changes over this same pH range.

In this paper we describe the nuclear magnetic spectral properties of *R. rubrum* cytochrome *c′* and compare our results with those obtained for myoglobin.

## Materials and Methods

*Rhodospirillum rubrum* (ATCC 11170) cytochrome *c′* was isolated and purified as described by Bartsch (1971). Myoglobin and horse heart cytochrome *c* (type IV) were obtained from the Sigma Chemical Co. Aqueous solutions of the purchased proteins were eluted through a small Sephadex G-25 column to remove low molecular weight impurities. The protein solutions were then lyophilized and dissolved in 300 μL of 0.1 M deuterated phosphate buffer to a final concentration of about $3 \times 10^{-3}$ M protein. The pH meter readings were uncorrected for isotope effects and are designated by pH*. Unbuffered protein solutions were used for the pH titrations. The pH was adjusted by addition of small aliquots of solutions of NaOD and DCl and was measured directly in the NMR tube using an Ingold microcombination glass electrode. Reduction of samples was carried out by the addition of a minimal amount of dry $Na_2S_2O_4$ under an atmosphere of argon. The reduced protein was then oixidized in steps by adding small aliquots of oxidized cytochrome under anaerobic conditions. After sufficient intermediate spectra were acquired, the solution was fully reoxidized by exposure to air. The spectrum of the reoxidized protein was essentially identical with previous spectra of ferricytochrome *c′*.

Proton NMR spectra were obtained using a Bruker 270-MHz spectrometer operation in the Fourier transform mode. 1,4-Dioxane was used as the internal standard, but all chemical shifts are quoted in parts per million (ppm) downfield from 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

The bulk magnetic susceptibility of ferricytochrome *c′* in 50 mM NaCl, pH* 6.5 and 10, and ferrocytochrome *c′*, pH* 6.5, was determined at several temperatures by the NMR method (Phillips & Poe, 1972).

The molar paramagnetic susceptibility, $\chi_M{}^P$, was calculated from

$$\chi_M{}^P = \frac{3}{4\pi}\frac{1000}{c}\left(\frac{\Delta\nu}{\nu}\right) - \chi_M{}^D$$

where $c$ is the concentration of the paramagnetic center, $\Delta\nu$ the frequency shift in hertz of a susceptibility marker (1,4-dioxane), and $\nu$ the frequency of the spectrometer. The diamagnetic molar susceptibility, $\chi_M{}^D = -1.7 \times 10^{-3}$ cgs, was estimated from a sample of reduced horse heart cytochrome *c*. The substitution of the value $\chi_M{}^D = -0.6 \times 10^{-3}$ cgs [obtained for hemin by Havemann et al. (1961)] decreases the calculated effective magnetic moment ($\mu_{eff}$) of cytochrome *c′* by 0.2 unit. The concentration of the cytochrome *c′* was determined using $\epsilon^{oxd}{}_{391nm} = 196$ mM$^{-1}$ for the dimer protein (Sletten & Kamen, 1967).

Proton water relaxation enhancements were measured by using a Bruker Minispec-20 spectrometer equipped with a temperature regulator which was fixed at 26 ± 0.1 °C. Passage through zero of the $H_2O$ signal with a 180°–$\tau$–90° pulse sequence was used to obtain $T_1$ values. Measurements were carried out by using 0.5 mL of a solution of protein with 0.05 M phosphate buffer. The change in volume after each adjustment of pH was compensated for upon calculation of $T_1$ values.

## Results

The NMR spectra of *R. rubrum* ferricytochrome *c′* at pH* 6.4 and pH* 10.2 are shown in Figure 1 (A and B, respectively). Very large isotropic shifts of the resonances arising from protons of the heme group are observed. The isotropic NMR shifts are due to both Fermi contact and pseudocontact (dipolar) interactions between the protons' nuclear spin and the electronic spin of the iron (Kurland & McGarvey, 1970; Walker & La Mar, 1973). The four peaks of three-proton intensity each and occurring between 50 and 90 ppm in all the spectra can only arise from the four heme methyl groups. Some of the single proton intensity peaks further upfield are assigned to protons of the heme propionate groups by analogy with model compounds (Walker & La Mar, 1973).

The NMR spectra of cytochrome *c′* were analyzed in the pH* range 4.5–11. Above pH* 11 the NMR spectra indicate that the tertiary structure of the protein is largely lost. This alteration in structure can be reversed by lowering the pH. Below pH* 5 the protein begins to precipitate. This is attributed to protein aggregation resulting from its low isoelectric point (5.4) and the high concentrations of protein used (3 mM). The titration data for the heme methyl resonances are presented in Figure 2. The data have been fitted to theoretical titration curves by assuming two pH-dependent equilibria with p$K_a$* values of 5.8 and 8.7. Similar shifts are observed for the single proton resonances (Emptage, 1978). However, because of their breadth and extensive overlapping, these signals are difficult to follow at intermediate pH and the p$K_a$* could not be accurately determined from these peaks. The transition of pH* 5.8 produces only minor shifts in the NMR spectra, whereas the transition at pH* 8.7 gave considerable changes throughout the entire spectral region. The downfield resonances, as well as those in the diamagnetic region, shift

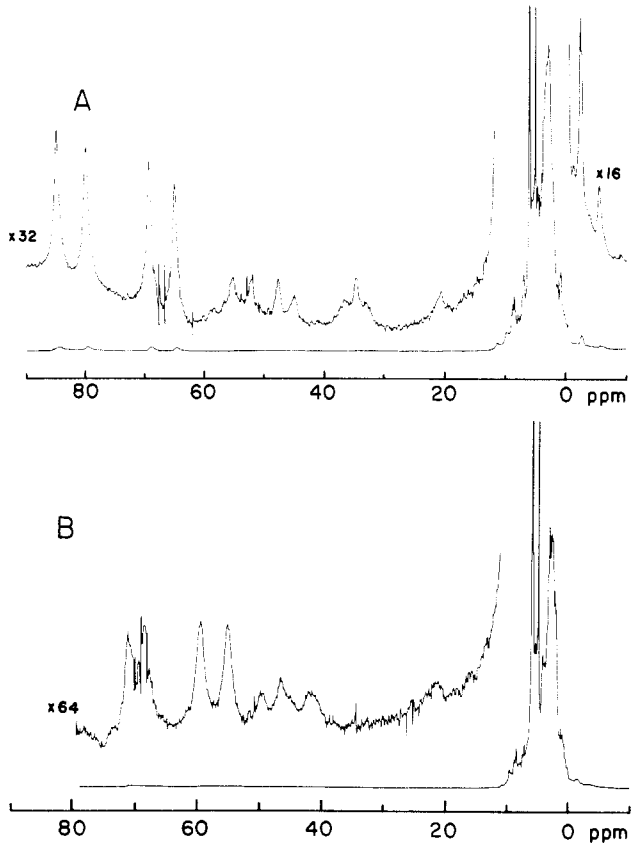


FIGURE 1: $^1H$ NMR spectral (270 MHz) of ferricytochrome $c'$ at pH* 6.4 (A) and pH* 10.2 (B) in $D_2O$. The spectra are the result of the accumulation of 2000 scans at ambient temperature. The protein concentration is approximately 3 mM. Chemical shifts are given in parts per million from DSS, using the resonance of 1,4-dioxane (3.76 ppm) as the internal standard. The magnetic field strength increases from left to right.

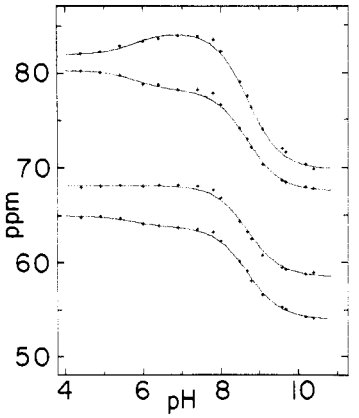


FIGURE 2: Titration curves of the position of the four heme methyl resonances at ambient temperature. The solid lines are computer-generated curves that assume two pH-dependent equilibria with $pK_a$'s of 5.8 and 8.7.

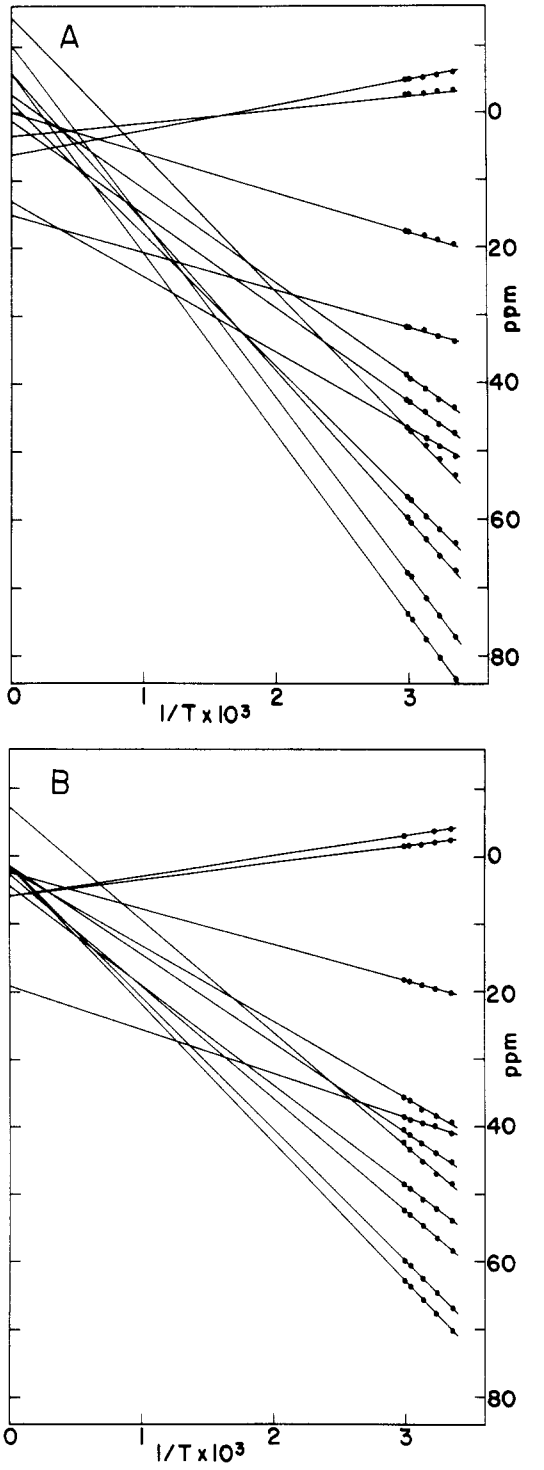


FIGURE 3: Temperature dependence plots of the isotropic shifted resonances of ferricytochrome $c'$ at pH* 6.7 (A) and pH* 10.3 (B). If the chemical shifts caused by isotropic interactions follow the Curie law, the resonances will have a $1/T$ dependence. The extrapolation of the data to $1/T = 0$ is done solely to indicate a further difference between the neutral and high pH species.

with pH but do not broaden during the transition. Thus, throughout the pH range studied, the rate of exchange between the neutral and pH 10 species must be greater than $5 \times 10^{-3}$ $s^{-1}$.

The temperature dependence of the resonances in the low- and high-field region of the NMR spectrum of cytochrome $c'$ at pH* 6.7 and 10.3 is shown in Figure 3 (A and B, respectively). These signals follow a $1/T$ dependence, obeying Curie's law. However, the apparent zero intercept of many of these signals falls beyond the diamagnetic region, as has been observed for other heme proteins and for model compounds (Wüthrich, 1970; La Mar et al., 1978a). This is much more apparent at pH* 6.7 than at pH* 10.3. A number of factors may contribute to these deviations. The zero-field splitting term $D$ is larger for the neutral pH species (Emptage et al., 1977), increasing the $1/T^2$ contribution to the dipolar shifts and producing a larger deviation for the zero intercept (Walker & La Mar, 1973). Another important factor could be the presence of low-lying excited states at neutral pH.

The water proton relaxation enhancement of $T_1$ with increasing pH for ferricytochrome $c'$ and metmyoglobin is given

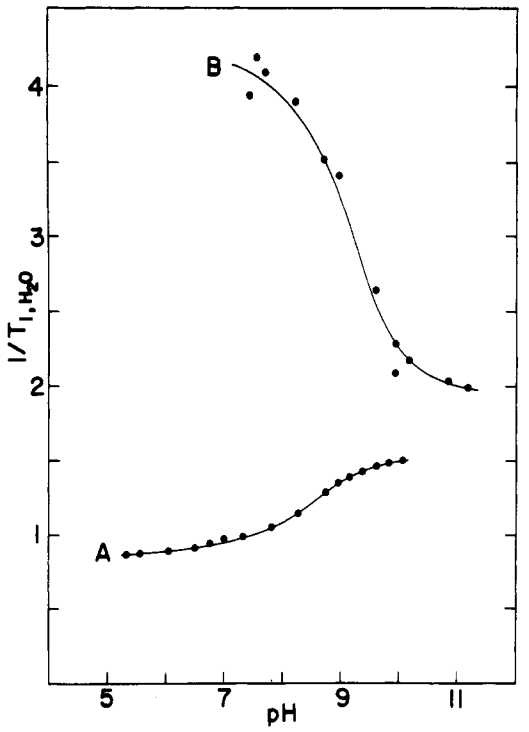


FIGURE 4: Plot of the pH dependence of the enhancement of the water relaxation time, $T_1$, for ferricytochrome $c'$ (A) and for metmyoglobin (B). The protein concentrations were 5 mM for myoglobin and 4 mM for cytochrome $c'$. The temperature was kept at 299 ± 0.1 K.

Table I: Magnetic Susceptibility Data for Cytochrome $c'$ [a]

| | $\chi_M^P$ (× $10^3$) | $\mu_{eff}$ |
|---|---|---|
| ferricytochrome $c'$, pH 6.5 | 12.3 ± 0.5 | 5.4 ± 0.1 |
| pH 10.1 | 14.4 ± 0.5 | 5.9 ± 0.1 |
| ferrocytochrome $c'$, pH 6.5 | 11.2 ± 0.5 | 5.2 ± 0.1 |

[a] Magnetic susceptibility measurements were done at 26 °C. The effective magnetic moment was calculated from the equation, $\mu_{eff} = [3kT\chi_M^P/N\beta^2]^{1/2}$. Here $k$ is Boltzmann's constant, $T$, the absolute temperature, $N$, Avogadro's number, and $\beta$, the Bohr magneton. Values for $\mu_{eff}$ were within the experimental error shown, as measured for two separate protein samples and after twice cycling the protein solution between pH* 7 and pH* 10.

in Figure 4. These curves exhibit p$K_a$*'s of 8.6 and 9.1, respectively. However, the sign and magnitude of the $T_1$ variation are quite different for the two proteins. Metmyoglobin shows a large decrease in relaxation rate with increasing pH, as has been previously reported (Fabry et al., 1971).

The magnetic susceptibility measurements for both the oxidized and reduced protein are given in Table I. Previous measurements of magnetic susceptibility were done either at low temperature (Tasaki et al., 1967) or at pH 7 only (Ehrenberg & Kamen, 1965). When the low-temperature electronic parameters of $c'$ and the equations of Kurland & McGarvey (1970) were used, the calculated values for $\mu_{eff}$ at room temperature are 5.9 at pH 10 and 5.8 at pH 7. While the measured value at pH* 10 (5.9) agrees with the predicted one, the pH* 7 value (5.4) is substantially lower. This difference can be accounted for by the presence of as little as 5–10% of the total spin population in a low-lying excited state with $S = {}^1/_2$. The detection of 5% of the low-spin species should prove to be extremely difficult. Another explanation for the low value of $\mu_{eff}$ at neutral pH would be the quantum mechanical admixture of spin states (Maltempo, 1974). However, other spectroscopic data, especially the EPR amd Mössbauer results of Emptage et al. (1977), are not compatible with the Maltempo model. A detailed study of the paramagnetic susceptibility over the temperature range 4.2–300 K should help to clarify this problem. The value of $\mu_{eff}$ for the reduced protein is 5.2 (Table I). This value is only slightly less than that obtained for deoxymyoglobin (Taylor, 1939).

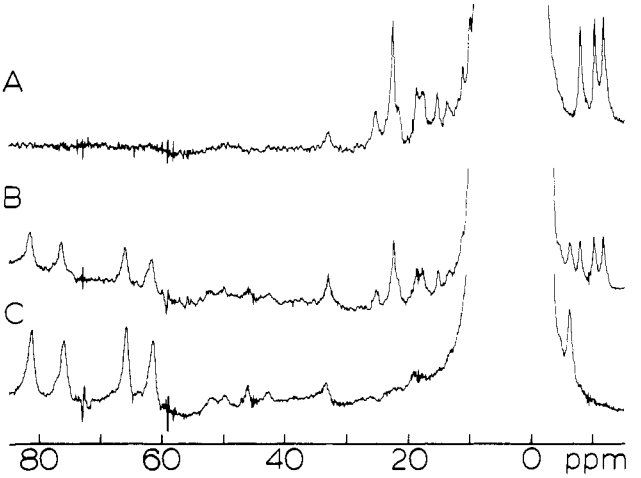


FIGURE 5: Spectra of cytochrome $c'$ of the high- and low-field regions at 30 °C when 100% reduced (A), 50% reduced (B), and 100% reoxidized (C). The protein solution was at neutral pH in $D_2O$.

Figure 5A–C displays the high-field and low-field regions of cytochrome $c'$ when 100% reduced, 50% reduced, and fully reoxidized, respectively. The presence of both oxidized and reduced cytochrome $c'$ resonances in the intermediate spectrum indicates that the electron exchange rate between centers is slow on the NMR time scale (less than $10^4$ $s^{-1}$). Four resonances of three-proton intensity and with similar line widths are seen at 23, -8, -10, and -12 ppm in the reduced cytochrome spectrum (Figure 5A). The chemical shifts for these resonances reveal a strong temperature dependence, (Emptage, 1978).

## Discussion

The NMR spectra of cytochrome $c'$ presented here are similar to those of other high-spin iron porphyrin species (Walker & La Mar, 1973; Goff & La Mar, 1977; Iizuka et al., 1976; Wüthrich et al., 1975). The physical properties of the cytochrome $c'$ are consistent with the coordination of a histidine as the fifth ligand to the iron, and we shall maintain this assumption in the subsequent discussion.

There is a broad resonance at 33 ppm in the spectrum of *R. rubrum* ferrocytochrome $c'$ (Figure 5A), and an analogous resonance appears in the spectrum of *Rhodopseudomonas palustris* ferrocytochrome $c'$ at 52 ppm (M. H. Emptage and A. V. Xavier, unpublished data). We assign this resonance to either the C-2 or C-4 proton of the histidine coordinated to the heme iron as the fifth ligand (i.e., His-120). This assignment is based on the work of La Mar et al. who have observed a similar resonance in spectra of model compounds (Goff, & La Mar, 1977) and deoxymyoglobin (La Mar et al., 1977). The spectrum of ferrocytochrome $c'$ (Figure 5A) has several features in common with *Aplysia* deoxymyoglobin (Wüthrich et al., 1975). Both proteins exhibit spectra with three resonances in the high-field region above +10 ppm which integrate for three protons. These spectra differ markedly from the spectrum of reduced sperm whale deoxymyoglobin (Shulman et al., 1970; La Mar et al., 1978b). Clearly, the four resonances must arise from protons which are close to the iron center, and since the four resonances have a similar $1/T$ dependence and line width, it is likely that they originate from the heme methyl groups. This unusual result implies that there is negative spin density on some of the peripheral carbon

atoms in the porphyrin ring (Wüthrich et al., 1975).

The X-ray-determined structure of sperm whale deoxymyoglobin shows that it has a 5-coordinate heme with histidine as the fifth ligand (Nobbs et al., 1966). This same structure is thought to be true for all the normal deoxymyoglobins and deoxyhemoglobins (Antonini & Brunori, 1971). The magnetic susceptibility, near-IR optical and CD spectra (Rawlings et al., 1977), and resonance Raman spectra (Kitagawa et al., 1977) are quite similar between those of sperm whale deoxymyoglobin and ferrocytochrome *c'*. These results, together with the NMR spectral similarities to *Aplysia* deoxymyoglobin, suggest that ferrocytochrome *c'* may be 5-coordinate as well, with histidine as the fifth ligand.

The spectrum of *R. rubrum* ferricytochrome *c'* is affected by two ionizations (Figure 2). The $pK_a$* values are 5.8 and 8.7. The $pK_a$* of 5.8 has only a small effect on the heme methyl resonances with shifts smaller than 2 ppm. The average isotropic shift for the four heme methyl resonances is maintained throughout this ionization, showing that the pertubations are not due to an alteration of the spin density in the porphyrin ring (La Mar et al., 1978c). This transition is not observed by optical (Horio & Kamen, 1961), MCD (Rawlings et al., 1977), or resonance Raman spectroscopy (Kitigawa et al., 1977) at room temperature. However, at low temperature (4.2 K) two species are resolved by Mössbauer spectroscopy at neutral pH (Emptage et al., 1977). Similar pH-dependent equilibria are observed in the NMR spectra of horseradish peroxidase (Morishima et al., 1977a) and deoxymyoglobin (La Mar et al., 1978b). *Rps. palustris* cytochrome *c'* contains only one histidine residue and shows no transition between pH 5 and pH 6 (M. H. Emptage and A. V. Xavier, unpublished data). Thus, the $pK_a$* of 5.8 probably arises from the ionization of a second histidine residue, although ionization of a heme carboxylate group cannot be excluded.

The ionization with $pK_a$* of 8.7 has been observed with a number of techniques. Pronounced changes in spectral properties occur upon this ionization (Horio & Kamen, 1961; Emptage et al., 1977; Kitagawa et al., 1977; Rawlings et al., 1977). This transition affects the entire NMR spectrum, and the large shifts of the heme methyl resonances indicates that there is a change in the spin-density distribution in the porphyrin ring. The fast NMR exchange rate indicates that the dissociation does not cause a significant change in the overall protein conformation. This is in agreement with previous circular dichroism studies of cytochrome *c'* (Imai et al., 1969).

We turn now to the presence of histidines, other than His-120, in the sequences of the cytochrome *c'*, and compare these with histidines in the sequences of myoglobins. The 28 myoglobins which have been sequenced are strongly homologous (Dayhoff, 1976), and only three of them lack distal histidine residues: those of *Aplysia*, *Glycera*, and *Chironomus*. The three-dimensional structures of *Glycera* and *Chironomus* myoglobins are available (Padlon & Love 1974; Huber et al., 1971; Dayhoff, 1976), and they are similar to that of sperm whale myoglobin. *R. rubrum* cytochrome *c'* contains two histidine residues although the NMR spectrum of its reduced form resembles that of the myoglobins which lack a distal histidine. Additionally, *Rps. palustris* and *R. molischianum* cytochromes *c'* contain only one histidine each, and their NMR spectra are similar to those of *R. rubrum* cytochrome *c'* (M. H. Emptage and A. V. Xavier, unpublished data; G. R. Moore and R. J. P. Williams, unpublished data). It seems clear that the second histidine residue of *R. rubrum* cytochrome *c'* is not distal to the iron.

X-ray diffraction (Kendrew et al., 1961), water proton relaxation rates (Mildvan et al., 1971), and hyperfine broadening of EPR signals by $H_2{}^{17}O$ (Vuk-Pavlović & Siderer, 1977) have established that water is the sixth ligand to the iron in metmyoglobin. The presence of a distal histidine changes the $pK_a$ for the water dissociation to about 8.5 (Antonini & Brunori, 1971). The three myoglobins which lack a distal histidine have lower $pK_a$'s. This influence of a second histidine is not seen in the cytochrome *c'* series of proteins. The optical transitions for two cytochromes *c'* which lack a second histidine residue, those from *Rps. palustris* and *Chromatium*, are 7.4 and 9.1, respectively (Miller, 1973), while for cytochromes *c'* which contain additional histidine residues the $pK_a$ ranges from 7.1 to 8.4 (Cusanovich et al., 1970); clearly the ionization is different for myoglobin and cytochromes *c'*. The possibility that the high $pK_a$ of *R. rubrum* ferricytochrome *c'* is caused by water coordinated to the iron is ruled out for the following reasons:

(1) The change to a hydroxide species at higher pH would be expected to reduce the magnetic susceptibility and show a low-spin component in the EPR spectrum. Neither change is seen.

(2) A marked decrease in the $1/T_1$ of myoglobin is caused by the ionization of the water ligand (Mildvan et al., 1971). By contrast, the small increase in the relaxivity of cytochrome *c'* at high pH (Figure 4) is due to the increase with pH of both $\mu_{eff}$ and $T_{1e}$ (Emptage, 1978) (and consequently the correlation time $\tau_c$) affecting the water molecules in the outer sphere. This is also reflected in a small increase of the line widths of the heme methyl resonances at high pH (Figure 1).

(3) Measurement of the EPR spectrum of metmyoglobin in 50% $H_2{}^{17}O$ shows hyperfine broadening due to iron-bound $H_2{}^{17}O$ (Vuk-Pavlović & Siderer, 1977). No such effect is seen in cytochrome *c'* at pH 7 or 10 (M. H. Emptage and W. H. Orme-Johnson, unpublished data).

(4) There are significant differences in the near-IR MCD between metmyoglobin and cytochrome *c'* (Rawlings et al., 1977).

Thus, there is no evidence for a water molecule bound to the iron of ferricytochrome *c'*; inner-sphere coordination of water as the sixth ligand does not occur. In fact, this position is known to be unavailable for coordination with charged species (i.e., $F^-$, $CN^-$, $N_3^-$) (Taniguchi & Kamen, 1963) while in metmyoglobin this position is available for such coordination (Antonini & Brunori, 1971). It is possible that the cytochromes *c'* are coordinatively unsaturated (i.e., 5-coordinate).

After our paper came back for revision we became aware of the recent determination of the X-ray structure of cytochrome *c'* from *Rps. molischianum* (Weber et al., 1980). The X-ray structure shows that the sixth site is unoccupied, as our NMR experience and recent resonance Raman data have suggested (Spiro et al., 1979). We initially favored the pH transition being caused by the coordination of a carboxylate group at the higher pH. This was supported by the large spectral changes observed, the shift to higher susceptibility, and the IR charge-transfer bands moving to higher energy (Kamen et al., 1971; Rawlings et al., 1977). However, examination of the sequence and structure of *c'* appears to show the absence of any charged group near enough to be a ligand at the sixth site. An inspection of the sequences of the cytochromes *c'* reveal that the Cys-x-x-Cys-His sequence is followed by a carboxylic acid, an aromatic residue, and a basic residue (R. P. Ambler, unpublished data). Because this sequence is conserved and there appear to be no charged residues near the hemin sixth site, it is likely that the ionization of one

of these groups results in the observed spectral changes.

Although the several cytochromes *c′* appear to have similar spectral properties at room temperature, they differ substantially in their $pK_a$'s and low-temperature EPR spectra (M. H. Emptage, unpublished data). In order to understand more fully these interesting heme proteins, a comparative study of their magnetic properties at both high and low temperatures should be undertaken.

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# Esterification of Terminal Phosphate Groups in Nucleic Acids with Sorbitol and Its Application to the Isolation of Terminal Polynucleotide Fragments†

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ABSTRACT: The exposure of mono- and polynucleotides to 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and high concentrations of sorbitol results in the esterification of their monosubstituted phosphate groups. The presence of the sorbitol moiety permits these derivatives to bind strongly at pH 8.7 to columns of chromatographic supports containing the dihydroxyboryl group and to be subsequently released by elution with buffers at pH 5.5. The procedure constitutes a method for the isolation of polynucleotide fragments arising from the terminals of nucleic acids. A new method for the preparation of the chromatographic supports involves the synthesis of the 1,3-propanediol cyclic ester of *m*-[[3-(*N*-succinimidoxycarbonyl)propanoyl]amino]benzeneboronic acid and its condensation with aminoethylcellulose or aminoethylpolyacrylamide. The reagent is readily prepared by reaction of *N*-[*m*-(dihydroxyboryl)phenyl)]succinamic acid with 1,3-propanediol to protect the boronate moiety followed by esterification with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide.

In earlier reports from this laboratory we described the preparation of cellulose derivatives containing the dihydroxyboryl group and the use of these supports as novel chromatographic materials for the fractionation of sugars, nucleotides, polynucleotides, and nucleic acids (Weith et al., 1970; Rosenberg et al., 1972). Separations of molecules belonging to these classes arise from differential stabilities of the complexes that they form with the immobilized dihydroxyboryl groups; the complexes are presumed to consist of cyclic boronate structures formed between such groups and pairs of contiguous hydroxyl groups that possess the appropriate conformation. The chromatographic supports were constructed by condensing *N*-[*m*-(dihydroxyboryl)phenyl]succinamic acid with an aqueous suspension of aminoethylcellulose, aminoethylpolyacrylamide, or amino-substituted glass in the presence of a water-soluble carbodiimide (Weith et al., 1970; Duncan and Gilham, 1975). The cellulose derivative, DBAE-cellulose,[1] has also been exploited in procedures for the isolation of 3′-terminal fragments from RNA molecules and for the purification of tRNA isoacceptors (Rosenberg and Gilham, 1971; Duncan and Gilham, 1975; McCutchan et al., 1975). The material is now commercially available and is in widespread use for these as well as other special separation problems such as the isolation of 5′-terminal fragments from eukaryotic messenger and viral ribonucleic acids that contain terminal 5′-linked nucleoside polyphosphate groups. One difficulty with the use of DBAE-cellulose prepared by the above method arises out of the apparently incomplete substitution of the amino groups on the cellulose. This necessitates the use of chromatographic solvents containing relatively high salt concentrations in order to minimize the binding of polynucleotide fragments through the residual anion-exchange capacity of the aminoethylcellulose (Rosenberg et al., 1972). The present work describes the development of two new experimental advances in this separation technique: a new method for the efficient preparation of DBAE-cellulose and DBAE-polyacrylamide that minimizes the ion-exchange problem, and a method for the introduction of "handles" into nucleic acids that permits the isolation of terminal polynucleotide fragments from DNA and RNA on DBAE supports.

*Synthesis of DBAE-cellulose and DBAE-polyacrylamide.* The compound chosen as a reagent for the preparation of the chromatographic supports has the structure *m*-[[3-(*N*-succinimidoxycarbonyl)propanoyl]amino]benzeneboronic acid, and the method used for the synthesis of its 1,3 propanediol cyclic ester (IV) is shown in Scheme I. *N*-[*m*-(Dihydroxyboryl)phenyl]succinamic acid (II) prepared from succinic anhydride and *m*-aminobenzeneboronic acid (I) is converted with 1,3-propanediol to the cyclic ester III to protect the dihydroxyboryl group during the subsequent activation step. The ester, upon condensation with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide, gives IV in 78% yield. In aqueous solution at pH values above 8 this activated carboxylic acid reacts rapidly with suspensions of chromatographic supports containing primary amino groups. The extent of the reaction, in each case, can be estimated by observing the absorbance changes in the reaction solution that are appropriate for the loss of the reagent's chromophore and for the appearance of the chromophore corresponding to the released *N*-hydroxysuccinimide. Thus, aminoethylcellulose and aminoethylpolyacrylamide can be substituted with di-

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[1] Abbreviations used: DBAE, *N*-[*N*′-[*m*-(dihydroxyboryl)phenyl]-succinamyl]aminoethyl; EPC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; DEAE, diethylaminoethyl.

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