Assignments of ^{15}N and ^{1}H NMR Resonances and a Neutral pH Ionization in *Rhodospirillum rubrum* Cytochrome c_2†

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ABSTRACT: The πNH proton and ^{15}N resonances of the ligand histidine of *Rhodospirillum rubrum* ferricytochrome c_2 are found at 14.7 and 184 ppm, respectively, contradicting the proposal that this proton is absent in the *R. rubrum* ferricytochrome. Substitution of the deuterium atom for this proton causes small upfield shifts of the π nitrogen in both oxidation states, indicating that the πNH–peptide carboxyl hydrogen bond is not substantially weakened by the substitution. The proton and ^{15}N resonances of the indolic NH group of the invariant tryptophan-62 and numerous proton resonances of the heme and extraheme ligands in the spectrum of the ferricytochrome are also assigned. An ionization in the ferrocytochrome occurring at neutral pH is assigned to the single nonligand histidine. This attribution is supported by the direct measurement of the ionization by NOE difference spectroscopy and by comparative structural arguments involving closely related cytochromes and chemically modified cytochromes.

Nuclear magnetic resonance spectroscopy has played an important role in characterizing electron-transfer proteins including cytochromes *c*. Subtle structural differences between the solution and crystal structures of mitochondrial cytochrome *c* have been demonstrated by NMR techniques (Moore et al., 1985; Williams et al., 1985a,b), as have conformational changes around the heme propionic acid groups and the Ω loop in *Rhodospirillum rubrum* cytochrome c_2 (Yu & Smith, 1988a,b). Even the stereochemistry of the iron coordination in different cytochromes *c* has been investigated and found to be different (Senn & Wuthrich, 1983a,b; Senn et al., 1983b). The influence of ionizations on the redox properties of cytochromes *c* has also been investigated by using NMR techniques (Moore et al., 1980, 1984; Leitch et al., 1984).

The primary problem in the structural interpretation using NMR techniques is that of resolving individual resonances and assigning them to individual atoms in the molecule. The assignments for the heme, axial ligand methionine and histidine, and other residues have been reported (Keller & Wuthrich, 1978a; Moore & Williams, 1980a–f, 1984). The proton NMR spectra of the mitochondrial cytochrome (horse) have recently been assigned (Wand et al., 1989; Feng et al., 1989). However, the assignments of important groups in both oxidation states of the bacterial cytochromes are incomplete, including several important resonances near the heme iron, such as the ligand histidine πNH proton and the C2 proton in the oxidized state. These resonances are particularly important in characterizing the state of the ligand histidine (Brautigan et al., 1977), and the structural difference between the oxidized and reduced cytochromes in terms of internal charge, hydrophobicity, exchange rates of exchangeable protons, hydrogen bonding, and local dynamics. They are also important in explaining the variation of redox potential with pH.

In the present paper, we describe the assignments of heme propionates, ligand histidine and methionine, and other related important resonances in both oxidation states of cytochrome c_2 from *Rhodospirillum rubrum*, and the assignment of a neutral pH ionization with a pK_a of 7.0 in this protein in the reduced state.

Experimental Procedures

Materials. $^{15}NH_4Cl$ was obtained from MSD Isotopes. The G-9 (blue-green) mutant of *R. rubrum* was kindly provided by P. F. Weaver.

Growth of Rhodospirillum rubrum. A facultative photoheterotroph *R. rubrum* can be grown photosynthetically under anaerobic conditions or fermentatively in air (Weaver, 1974). The growth media and conditions are described in a previous publication (Yu & Smith, 1988a). The cytochrome c_2 was purified from *R. rubrum* according to the methods described by Bartsch (1971) with minor modification (Smith, 1979). The purity of the protein was ≥99% as determined by using polyacrylamide gel electrophoresis.

Sample Preparation. The cytochrome c_2 was desalted by passage over Sephadex G-25 equilibrated with doubly deionized water and concentrated by lyophilization. The protein was dissolved in either 99.98% ^2H_2O or 15% ^2H_2O in H_2O, depending on the experiments to be carried out. The final cytochrome c_2 concentrations were about 3.5 mM. The sample pH was measured directly in the NMR tube. The pH was adjusted by using 0.1 N NaOH or 0.1 N HCl for samples in H_2O, or 0.1 N NaO^2H or 0.1 N ^2HCl for samples in ^2H_2O. The values reported are uncorrected pH meter readings. To adjust the oxidation states of the protein, sodium hydrosulfite was added as a reducing agent and $Co(phen)_3Cl_3$ or $K_3Fe(CN)_6$ was used as an oxidizing agent. Small ions and redox agents were removed by gel filtration over Sephadex G-25.

NMR Spectra. The ^{15}N NMR spectra were obtained with a GE NM-500 spectrometer at 50.7 MHz with a ^{15}N probe provided by Cryomagnet Systems, Inc. Chemical shifts are referenced to 1 N HNO_3 in ^2H_2O by taking the resonance of $^{15}NH_4Cl$ in H_2O to be 354.5 ppm. Sample temperature was usually controlled at 25 °C. The ^1H NMR spectra of the samples in H_2O were collected at 500 MHz on the same spectrometer equipped with a GE fixed-tuned probe, using either the 214-composite soft pulse (Redfield et al., 1975) or the 1331 (Hore, 1983) water-suppression pulse sequences. The chemical shifts for ^1H were measured from sodium 2,2-di-

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Table I: Individual Assignments and Chemical Shifts of ^{1}H NMR Resonances of Heme *c* in *Rhodospirillum rubrum* Cytochrome c_2 at pH 5.6

resonance assignments	chemical shifts (ppm) reduced	oxidized
heme		
meso proton α	9.22	
meso proton β	9.44	
meso proton γ	9.51	
meso proton δ	9.24	
ring methyl 1	3.51	10.8
ring methyl 3	3.25	29.9
ring methyl 5	3.34	15.0
ring methyl 8	2.13	33.2
thioether bridge 2		
methine	5.63	
methyl	2.36	
thioether bridge 4		
methine	6.28	
methyl	2.36	1.8
propionic acid 6		
α1 methylene	4.50	
α2 methylene	4.26	
propionic acid 7		
α1 methylene	3.86	6.0
α2 methylene	3.65	19.8
β2 methylene	3.79	

methyl-2-silapentanesulfonate as reference. Other parameters are listed in the figure legends. The heme numbering system used is the same as that used by Senn and Wuthrich (1983b).

Results and Discussion

NOE Experiments on Ferrocytochrome c_2. The assignment of resonances of ligand methionine of ferrocytochrome c_2 has been made in previous investigations (Smith, 1979; Senn & Wuthrich, 1983b) and confirmed by our COSY spectra (not shown). Also in the upfield region, the Leu-32 δ2 CH_3 protons (−2.28 ppm) were assigned in previous studies. NOE experiments and COSY spectra (not shown) led to assignment of the remaining side chain protons of Met-91 and the Leu-32 δ1 CH_3 protons and Leu-32 γ proton (see Table I). NOE's from these assigned resonances were used to assign several other resonances and to distinguish among heme protons. The Phe-93 ring 2,6 protons were identified by their proximity to the ligand methionine ϵCH_3 group. The heme meso δ proton and ligand histidine πNH proton (9.90 ppm at pH 6.8) were identified from NOE's from both Leu-32 methyl groups. The Phe-20 ring 2,6 protons and ring 3,5 protons received NOE's from the Leu-32 δ2 CH_3 only.

Irradiation of the ligand histidine πNH proton (9.81 ppm at pH 5.6), identified above and by its coupling to ^{15}N (Yu & Smith, 1988a), yielded strong NOE's at 0.29 and 3.29 ppm was well as to the Leu-32 side chain protons. The resonance at 0.29 ppm in the reduced state has been found to correspond to a resonance at −20.3 ppm in ferricytochrome c_2 by a saturation-transfer experiment (see below), indicating that this resonance must be very close to the paramagnetic center, and is assigned to the ligand histidine C2 proton. This assignment agrees with that made by Senn and Wuthrich (1983b) and is consistent with assignments of the mitochondrial cytochrome (Wand & Englander, 1985; Moore & Williams, 1980a). The resonance at 3.29 ppm is probably from the Pro-30 δ protons which are within 4.2 Å from the His-18 πN.

The assignments of the resonances of heme methyl groups of cytochrome c_2 (Smith, 1979; Senn & Wuthrich, 1983b) and mitochondrial cytochromes *c* (Keller & Wuthrich, 1978a) are the same. However, pH-dependent broadening and splitting arising from conformational heterogeneity in the heme pocket

Table II: Individual Assignments and Chemical Shifts of ^{1}H NMR Resonances of Axial Ligands and Amino Acid Residues in *Rhodospirillum rubrum* Cytochrome c_2 at pH 5.6

resonance assignments	chemical shifts (ppm) reduced	oxidized
axial Met ligand		
ϵ methyl	−2.90	−14.9
γ1 methylene	−3.58	−23.4
γ2 methylene	−1.21	−13.0
β1 methylene	−2.85	
β2 methylene	−0.11	
α proton	3.22	
axial His ligand		
C4H	0.00	23.9
C2H	0.29	−20.3
πNH	9.79	14.7
β1 methylene	0.39	15.2
β2 methylene		8.3
α proton		9.4
Leu-32		
γ proton	−0.40	
δ1 methyl	−0.72	
δ2 methyl	−2.30	
His-42		
C2H	8.22	
C4H	7.50	
Trp-62		
indole ring NH	10.75	10.4
C2H	7.80[a]	
C4H	7.10[b]	
C5H	6.12[b]	
C6H	5.89[b]	7.9
C7H	7.78[b]	8.0
Pro-30		
δ protons	3.29	
Phe-20		
3,5 protons	6.67	
2,6 protons	6.80	
Phe-93		
2,6 protons	7.00	

[a] From Yu and Smith (1988a). [b] From Smith (1979), but the order has been changed based on the NOE experiments.

have been observed around the heme 3-CH_3 in mitochondrial ferricytochromes *c* (Burns & La Mar, 1981) and around the heme 8-CH_3 in *R. rubrum* cytochrome c_2 (Yu & Smith, 1990). This raises the possibility of misassignment in either of the cytochromes as suggested in a previous investigation (Burns & La Mar, 1979). On the basis of a specific orientation of two methyl groups of Leu-32, the distinction between heme 1-CH_3 and heme 8-CH_3 resonances of the cytochrome c_2 could be made clearly based on an NOE effect at 2.13 ppm between the Leu-32 δ1 CH_3 and heme 8-CH_3 groups and an NOE at 3.51 ppm between the Leu-32 δ2 CH_3 and heme 1-CH_3 groups (trace B, Figure 2). The NOE results obtained from these experiments support the previous assignment of heme methyl resonances, which are listed in Tables I and II. Additional experiments on horse heart cytochrome *c* also support the previous assignment of heme methyl resonances. Therefore, the differences in pH-related behavior between the mitochondrial cytochrome *c* and the *R. rubrum* cytochrome c_2 must be due to a structural difference, which will be discussed in the following paper (Yu & Smith, 1990).

NOE's from the heme meso γ proton (9.54 ppm) can be used to assign the α1 and α2 protons of heme propionic groups 6 and 7, although the intensity is small because of *J* splitting. It is known that the α2 proton of the heme propionic group 7 resonates at 3.65 ppm from the saturation-transfer experiments (described below) and that the heme 8-CH_3 resonates at higher field than the heme 5-CH_3 in the reduced state, suggesting that the seven propionic α1 and α2 protons would also resonate at higher field (3.87, 3.65 ppm) than the six

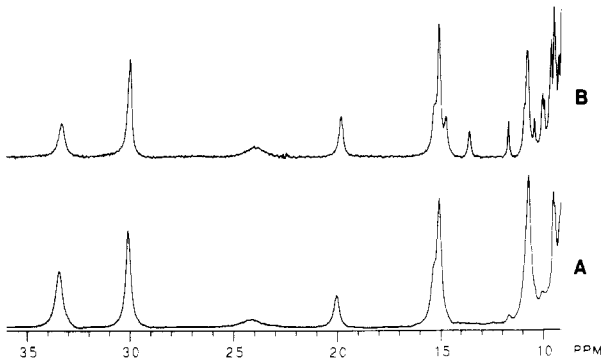

FIGURE 1: Comparison of ^1H NMR spectra (low-field region) of ferricytochrome c_2 in H_2O (trace B) with that in ^2H_2O for several days (trace A). Trace A was acquired with 5504 scans at p^{2}H 5.6, and trace B, 1024 scans at pH 5.7.

propionic α1 and α2 protons (4.49, 4.24 ppm). The assignment of the peak 7 propionate α2 proton at 3.65 ppm is confirmed by its pH dependence (Yu & Smith, 1990) and by NOE experiments on the ferricytochrome (see below). Resonances in the aromatic region at 7.40 and 6.97 ppm receiving NOE's from the meso γ H must arise from the Tyr-70 ring 3,5 protons and/or Tyr-52 ring 3,5 protons and/or Tyr-48 ring 3,5 protons.

Saturation-Transfer Experiments. Saturation-transfer experiments were performed in a solution of partially oxidized and partially reduced cytochrome c_2 to identify the corresponding resonances in the spectra of the reduced and oxidized proteins (Redfield & Gupta, 1971; Keller & Wuthrich, 1978a, 1981). When the resonances at 33.1, 30.0, 23.8, 19.9, 15.1, 14.7, and 10.8 ppm in the oxidized state were irradiated, the corresponding resonances in the reduced state were observed at 2.13, 3.25, 0.00, 3.65, 3.34, 9.79, and 3.51 ppm, respectively.

Although saturation-transfer experiments for the heme methyl groups have been reported (Senn & Wuthrich, 1983b; Smith, 1979), no such results have been reported for the oxidized-state resonance at 19.9 ppm. This resonance is assigned to the heme 7 propionic acid group α2 proton because it has a pH dependence characteristic of heme rings II and IV and maps to a resonance in the reduced state at 3.65 ppm. Other contact-shifted resonances, such as the His-18 C2 proton, have been accounted for, below. A similar resonance at about 20 ppm in the mitochondrial ferricytochromes *c* has also been assigned to one of the two α protons of the heme 7 propionic group based on the NOE data (Moore & Williams, 1984).

The broad resonance at 23.8 ppm in the ferricytochrome and its corresponding resonance at 0.00 ppm in the reduced state are assigned to the ligand histidine C4 proton, the remaining unassigned proton close to the paramagnetic center, which should be shifted upfield by at least 6 ppm in the reduced state by the ring-current shift (Giessner-Prettre & Pullman, 1971). This assignment is consistent with that made by Senn and Wuthrich (1983b) based on the comparison to low-spin ferric bisimidazole complexes of natural porphyrins. A resonance at 0.15 ppm in horse ferrocytochrome *c* has also been assigned to the ligand histidine C4 proton (Wand & Englander, 1985; Moore & Williams, 1980a).

The resonance at 14.7 ppm in the oxidized state is an exchangeable NH proton (Figure 1). When the closely spaced resonances at 15.1–14.7 ppm are irradiated, three corresponding resonances in the reduced state are observed, at 0.39, 3.34, and 9.79 ppm. The exchangeable resonance at 14.7 ppm corresponds to the exchangeable reduced-state resonance at 9.79 ppm of the ligand histidine πNH proton (Yu & Smith, 1988a). The total area of the two adjacent peaks around 15 ppm in the oxidized state corresponds to four protons in addition to the exchangeable NH proton. The methyl resonance at 15.2 ppm is already identified as the heme 5-CH_3 resonance leaving the single-proton peak at 15.1 ppm, which corresponds to the peak at 0.39 ppm in the reduced state.

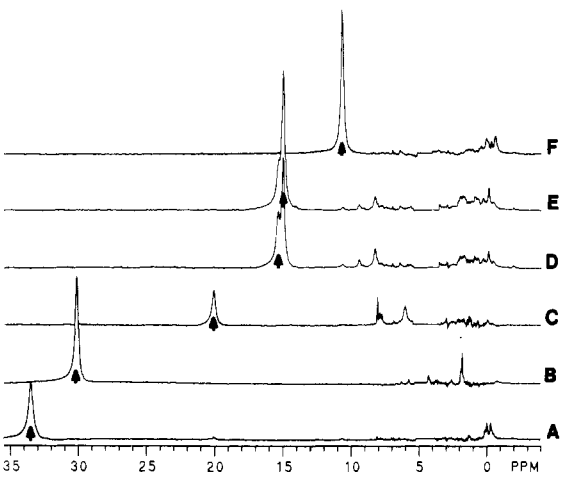

FIGURE 2: ^1H NMR NOE difference spectra of ferricytochrome c_2 in ^2H_2O at p^{2}H 5.7. Traces A–F show the differences between the spectra obtained with and without preirradiation during 0.1 s at positions indicated by arrows. These spectra were acquired with 5504 scans.

When the high-field resonances at −23.4, −20.3, and −13.0 ppm in the oxidized state are irradiated, the corresponding resonances in the reduced state were observed at −3.58, 0.29, and −1.21 ppm, respectively; these are already assigned (see Tables I and II). This is the first example of successful saturation-transfer experiments for these very broad resonances in the oxidized state. In support of the His-18 C2H assignment, this resonance is thought to occur at about −11 ppm in monocyanide complexes of cytochromes *c* (Behere et al., 1986), confirming the sign, if not the magnitude, of the shift.

NOE Experiments on Ferricytochrome c_2. Contrary to initial expectations, the successful application of NOE experiments to paramagnetically shifted resonances of hemoproteins has been reported (Johnson et al., 1983). NOE experiments involving the heme group are shown in Figure 2 and summarized in Table I. NOE's from heme 8-CH_3 (33.3 ppm) confirm the assignment of the α2 proton of the 7 propionic group (19.8 ppm) and the heme 1-CH_3 group (10.8 ppm). Other NOE's are observed at 0.00 and −0.30 ppm. The NOE's at 19.9 and 10.8 ppm are relatively small, but their signal-to-noise ratio is more than adequate to confirm the assignment of these resonances.

An NOE from the heme 3-CH_3 (29.9 ppm) can be used to assign the heme 4 thioether bridge CH_3 at 1.80 ppm. NOE's from the α2 proton (19.9 ppm) of the 7 propionic group lead to the assignment of its geminal proton at 6.00 ppm and the C7 proton of Trp-62 at 8.00 ppm. The latter receives a small NOE from the heme 8-CH_3 ~4.7 Å away (trace A, Figure 2). The resonance at 7.87 ppm receiving an NOE from the α2 proton then could be assigned to the Trp-62 indole C6 proton.

NOE's from the resonances near 15.0 ppm (traces D and E, Figure 2) are observed at 8.26 and 9.41 ppm from the resonance at 15.2 ppm and at −0.11 ppm from the heme 5-CH_3 group (15.0 ppm). The resonance at 15.2 ppm in the oxidized-state mapping to 0.39 ppm in the reduced state by saturation transfer must be situated perpendicular to the heme plane and very close to the heme center, to be shifted upfield by the heme ring current in the reduced state and downfield by a paramagnetic effect in the oxidized state. The resonance

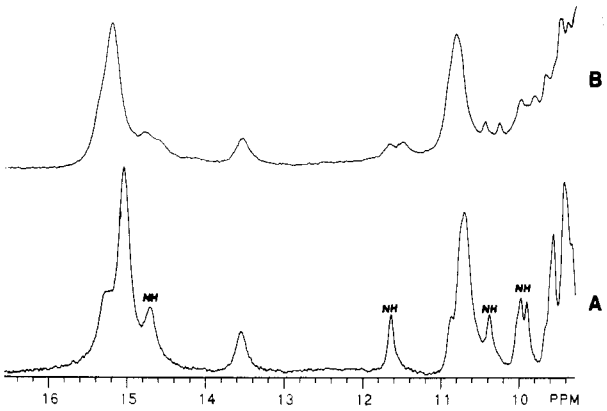

FIGURE 3: Comparison of ^1H NMR spectra (low-field region) of ferricytochrome c_2 at natural abundance of ^{15}N (trace A) with that of ^{15}N-enriched ferricytochrome c_2 (trace B) in H_2O. The spectra were collected by using the 214 sequence with a recycle time of 1 s. Trace A was acquired with 1024 scans at pH 5.7, and trace B, 2048 scans at pH 5.8.

at 15.2 ppm is assigned to a ligand histidine β proton, presumably the $\beta 1$ proton because it is closer to the heme center. The broad resonances at 8.26 and 9.41 ppm are assigned to the $\beta 2$ proton and α proton, respectively, by virtue of their proximity to the heme and the His-18 $\beta 1$ proton.

^{15}N and Exchangeable ^1H Resonances in Ferricytochrome c_2. ^1H NMR spectra of ferricytochrome c_2 dissolved in H_2O (Figure 1) revealed the resonances of exchangeable protons are at 14.71, 13.56, 11.65, 10.39, 9.99, and 9.89 ppm at pH 5.7, which could arise from peptide NH protons, tryptophan ring NH protons, histidine ring NH protons, or a tyrosine phenol OH proton. The exchange rates for these protons are similar, having half-lives of less than hours, except the proton at 11.65 ppm, which exchanges slightly more slowly. A comparison of the ^1H spectra of ^{15}N-enriched and natural-abundance samples (Figure 3) clearly indicates that the resonances at 14.71, 11.65, 10.39, 9.99, and 9.89 ppm at pH 5.7 are NH protons with exchange rates of less than $0.35J = 35$ s^{-1} as evidenced by their appearance as doublets with $^1J_{NH}$ of 95 Hz. However, the resonance at 13.56 ppm at pH 5.7 is either a slowly exchangeable OH proton or a NH proton with an exchange rate between 35 and 1000 Hz because it is a singlet and has not merged with the solvent water resonance.

The corresponding ^{15}N resonances for these NH protons are found by conventional spin decoupling experiments (not shown) as follows: The proton resonances at 11.65, 10.80, 10.39, 9.99, and 9.89 ppm are J-coupled to ^{15}N resonances at 255.0, 246.2, 249.2, 253.8, and 256.1 ppm at pH 5.7, respectively. There is an additional NH proton resonance beneath the heme 1-CH_3 resonance (10.8 ppm), which is coupled to the ^{15}N resonance at 246.2 ppm at pH 5.7. At pH 6.5, the 1-CH_3 resonance appears to be split into a doublet, covering the exchangeable protons at 10.80 and 10.39 ppm (Yu & Smith, 1990). Because of the interesting NH proton resonances beneath these peaks, the proton-decoupled ^{15}N difference spectra were collected at higher pH to determine whether this splitting is due to the abnormal behavior of these NH protons. The selective irradiation experiments clearly indicate that these exchangeable protons are still present and that the splitting must be caused by other mechanisms (Yu & Smith, 1990).

No ^{15}N decoupling was observed upon irradiation of the exchangeable NH proton at 14.7 ppm in the oxidized state. However, ^{15}N-decoupled ^1H NMR spectra successfully revealed that the proton resonance at 14.7 ppm is J-coupled to the ^{15}N resonance at 184 ppm, which is itself too broad to exhibit splitting. Thus, this experiment has unequivocally identified the ligand histidine πN resonance in ferricytochrome c_2 at 184 ppm at pH 5.6. This assignment is critical to understanding the effect of changes in pH and hydrogen bonding upon the NMR spectra and midpoint potential of cytochromes c (Yu & Smith, 1990).

Isotope Effects on the Chemical Shift of the Ligand Histidine π Nitrogen. When the ^{15}N-enriched ferricytochrome is dissolved in ^2H_2O, the ligand histidine πNH proton is easily exchanged for deuterium, unlike the ferrocytochrome. The ^{15}N NMR spectrum of the ferricytochrome in ^2H_2O revealed that the substitution causes an upfield shift of the π nitrogen of 2.5 ppm. This sample was reduced, and the isotope effect on the chemical shift of this same resonance in the ferrocytochrome was accurately determined to be 1 ppm, using internal calibration.

Assignment of a Neutral pH Ionization in Ferrocytochrome c_2. Several ionizations affecting the midpoint potential and other properties of *R. rubrum* cytochrome c_2 are thought to occur with pK_a values of 6.2, 7.0, and 8.4 as determined by ^1H NMR studies and redox potential measurements (Smith, 1979; Pettigrew et al., 1978). Recently, an ionization with a pK_a of 6.8 for the ferrocytochrome c_2 (in H_2O) has also been observed from ^1H and ^{15}N NMR studies. This neutral pK_a is thought to correspond to one that has been assigned to the His-42 imidazole group in the reduced state (Yu & Smith, 1988a).

A controversy concerning this ionization has existed in the literature for some time. The possible ionizing groups have been discussed by Pettigrew et al. (1976, 1978). In the study of *Pseudomonas* cytochromes c_{551} by Leitch et al. (1984) and Moore et al. (1980), the neutral pK_a's between 6.2 and 8.5 are assigned to the heme 7 propionic group based on the chemical modification of histidines and ^1H NMR studies. For the following discussion, it is useful to classify the cytochromes (mitochondrial or photosynthetic low-spin monoheme) according to structural patterns near the heme 7 propionate. In class I, there are no positively charged groups near the heme 7 propionic acid group. In class II, there is one positively charged group (either arginine or lysine) close to the heme 7 propionic acid group, but no histidine. In class III, there is one histidine residue ion-paired with and/or hydrogen-bonded to the heme 7 propionic acid group at neutral to low pH, but there are no other positively charged groups nearby. In class IV, there are both histidine and arginine or lysine residues close to the heme 7 propionic acid group. In cytochromes c, the heme 7 propionic group is usually situated in a very hydrophobic environment, and any residual charge on this group is unfavorable. Therefore, in class I, the heme 7 propionic group would have an apparent pK_a below 7 (Falk, 1964), depending upon the degree of hydrophobicity in that area. In classes II, III, and IV, it would have an apparent pK_a lower than that in class I depending on the orientation of the side changes because the ion-pair is favored.

The cytochromes c_{551} from *Pseudomonas mendocina*, *Pseudomonas stutzeri* strain 221, and *Ps. stutzeri* strain 224 and cytochrome c_2 from *R. rubrum* belong to class III; and the cytochrome c_{551} from *Pseudomonas aeruginosa* belongs to class II, because the distance between the charged Arg-47 and the heme 7 propionic group is 2.6 Å (Matsuura et al., 1982). The assignment of the pK_a's of above 7 in the oxidized state and above 8 in the reduced state in the cytochromes c_{551} from *P. mendocina* and *P. stutzeri* and the neutral pK_a's of cytochrome c_2 from *R. rubrum* to the heme 7 propionic group (Leitch et al., 1984; Moore et al., 1984) is unconvincing. Since the pK_a of *N*-(ethoxyformyl)histidine is 3.4 (Melchior &

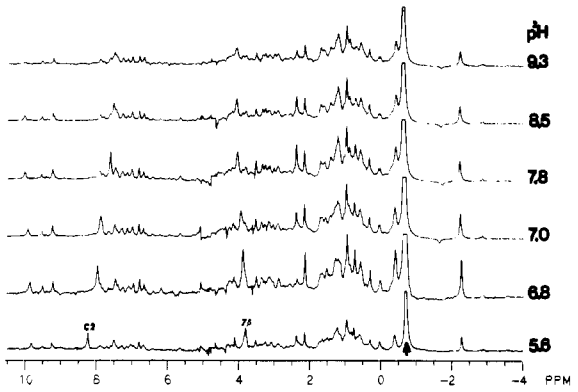

FIGURE 4: ^1H NMR pH titration spectra (NOE differences) of ferrocytochrome c_2 in ^2H_2O. Difference spectra were obtained by subtracting the NOE spectra with irradiation at Leu-32 $\delta 1$ CH_3 (arrow) from the control spectrum with irradiation away from the resonance at different pH values as indicated. The His-42 C2 proton is labeled C2, and the heme 7 propionic $\beta 2$ proton is labeled 7β. The spectra represent about 3584 scans. The Leu-32 $\delta 1$ CH_3 resonance itself is pH dependent with a pK_a of 7.0. Its chemical shift is −0.72 ppm at p^{2}H 5.6 and −0.65 ppm at p^{2}H 9.3.

Fahrney, 1970) and the ethoxyformic anhydride (EFA) modified histidine residues in these cytochromes have pK_a's < 4.4 (Leitch et al., 1984), after chemical modification by EFA, the pK_a for the heme 7 propionic group would be expected to be higher than in the native protein because of the loss of ion pairing in the hydrophobic environment. In contrast, a pK_a value 2 pH units lower was observed (Leitch et al., 1984). From these arguments, it is apparent that there is some contradiction in these assignments. Assigning the neutral pH ionization in the native proteins to the histidine residue would make good chemical sense. In the native proteins, because of ion pairing between the positively charged imidazole groups and the negatively charged heme 7 propionic COO^- in the neutral to low-pH range, the propionic acid group would have a pK_a near 5, which is not detected under the experimental conditions, and the histidine imidazole group would have a pK_a near 7, which is observed in the cytochromes *c* belonging to class III. Because of the low pK_a of the chemically modified imidazole group, the EFA-modified cytochromes are converted to class I cytochromes, and the ionizations with p$K_{a_{ox}}$ of 5.5 and p$K_{a_{red}}$ of 6.3 in this case (Leitch et al., 1984) arise from the heme 7 propionic group.

Previously, we assigned the ionization occurring with a pK_a of 6.8 in *R. rubrum* ferrocytochrome c_2 in H_2O to the His-42 imidazole group (Yu & Smith, 1988a). ^{15}N NMR failed to confirm this assignment because of chemical exchange (tautomerization) broadening of the imidazole nitrogens. The histidine C2 and C4 ^1H resonances were not unequivocally identified in a previous investigation (Smith, 1979). Even NMR difference spectra taken at different pH values (Campbell et al., 1975) were unsuccessful in identifying the resonances of the histidine C2 and C4 protons because a pH-induced conformational change (Yu & Smith, 1990) and the large number of resonances in this region obscure the effect. However, the Leu-32 $\delta 1$ CH_3 group, whose resonance is resolved, pH-invariant, and far away from the aromatic region, appears to be within 4.3 Å from the C2 proton of the His-42 ring. Therefore, NOE difference spectroscopy with irradiation of the resonance of this methyl group should select a volume element within the protein containing the His-42 C2. A pH titration employing NOE difference spectra (Figure 4) revealed a single resonance (7.98 ppm at pH 6.8) with a chemical shift variation ($\Delta\delta$ = 0.77 ppm) typical of a histidine C2 proton (Markley, 1975). A pK_a of 7.0 (in ^2H_2O) was obtained from the Hill equation protein dissolved. The only nonligand histidine in this protein is His-42. Thus, the previous assignment of both the resonance and the pK (Yu & Smith, 1988a) is confirmed. Other assignments obtained from this experiment included the heme 7 propionic $\beta 2$ proton (3.89 ppm at pH 6.8). The assignments made so far for *R. rubrum* cytochrome c_2 are summarized at pH 5.6 in Tables I and II.

The assignment of ionizations or pK_a's to inidividual groups from redox potential (E_m) measurements is difficult because these ionizations may be coupled and because all the ionizations occurring in each oxidation state are not necessarily observable in the E_m vs pH measurements. Even NMR measurements do not always allow the direct observation of the ionizing group itself. On the basis of this and previous investigations, it is proposed that the neutral pK_a's for the cytochromes *c* belonging to class I are from the heme 7 propionic acid group, and the neutral pK_a's for the cytochromes *c* belonging to classes III and IV and having the two propionic acid groups on the different side of heme (that is, one above and the other below the heme plane) are from the his imidazole group which is ion-paired with and/or hydrogen-bonded to the heme 7 propionic COO^-. However, the neutral pK_a's for the cytochromes *c* belonging to classes III and IV and having the two propionic acid groups on the same side of the heme plane are from both the histidine imidazole group and the heme 7 propionic acid group, which are probably coupled to form a broad transition. The pK_a's of the heme 7 propionic acid group for the cytochromes *c* belonging to class II but with their two propionic acid groups on the same side of the heme would be high and observable in the neutral pH range because of electrostatic repulsion. The high "neutral" pK_a of *Ps. aeruginosa* cytochrome c_{551} (Moore et al., 1980) may result from these effects (Matsuura et al., 1982). The pK_a's of the heme 7 propionic acid group for the cytochromes of class II with their two propionic acid groups on different sides of heme would be lower and may be not observable under the experimental conditions, as in the case of horse cytochrome *c*.

Registry No. Cytochrome c_2, 9035-43-2.

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