Borgström, B., & Donnér, J. (1975) *J. Lipid Res. 16*, 287–292.
Borgström, B., & Donnér, J. (1976) *Biochim. Biophys. Acta 450*, 352–357.
Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1973) *J. Magn. Reson. 11*, 172–181.
Campbell, I. D., Dobson, C. M., Moore, G. R., Perkins, S. J., & Williams, R. J. P. (1976) *FEBS Lett. 70*, 96–100.
Charles, M., Erlanson, C., Bianchetta, Y., Joffre, J., Guidoni, A., & Rovery, M. (1974) *Biochim. Biophys. Acta 359*, 186–197.
Charles, M., Sari, H., Entressangles, B., & Desnuelle, P. (1975a) *Biochem. Biophys. Res. Commun. 65*, 740–745.
Charles, M., Astier, M., Sauve, P., & Desnuelle, P. (1975b) *Eur. J. Biochem. 58*, 555–559.
Cozzone, P. (1976) *FEBS Lett. 69*, 153–156.
Donnér, J. (1977) Ph.D. Thesis, University of Lund, Sweden.
Donnér, J., Spink, C. H., Borgström, B., & Sjöholm, I. (1976) *Biochemistry 15*, 6513–6517.
Dwek, R. A. (1973) in *Nuclear Magnetic Resonance in Biochemistry*, Oxford University Press, London.
Erlanson, C., & Borgström, B. (1972) *Biochim. Biophys. Acta 271*, 400–412.
Erlanson, C., Fernlund, P., & Borgström, B. (1973) *Biochim. Biophys. Acta 310*, 437–455.
Erlanson, C., Charles, M., Astier, M., & Desnuelle, P. (1974) *Biochim. Biophys. Acta 359*, 198–203.
Erlanson, C., Barrowman, J. A., & Borgström, B. (1977) *Biochim. Biophys. Acta 489*, 150–162.
Hofmann, A. F., Szczepanik, P. A., & Klein, P. (1968) *J. Lipid Res. 9*, 707–713.
Kalk, A., & Berendsen, H. J. C. (1976) *J. Magn. Reson. 24*, 343–366.
Mayliê, M. F., Charles, M., Grache, C., & Desnuelle, P. (1971) *Biochim. Biophys. Acta 229*, 286–289.
Navon, G., & Lanir, A. (1972) *J. Magn. Reson. 8*, 144–151.
Norman, A. (1955) *Ark. Kemi 8*, 331–342.
Patton, J. S., Albertsson, P.-Å., Erlanson, C., & Borgström, B. (1978) *J. Biol. Chem. 253*, 4195–4202.
Robinson, N. C., & Tanford, C. (1975) *Biochemistry 14*, 369–378.
Small, D. M., Pankett, S. A., & Chapman, D. (1969) *Biochim. Biophys. Acta 176*, 178–189.
Werbelow, L. G., & Marshall, A. G. (1973) *J. Am. Chem. Soc. 95*, 5132–5134.
Wieloch, T., & Falk, K.-E. (1978) *FEBS Lett. 85*, 271–274.
Woessner, D. E. (1962) *J. Chem. Phys. 36*, 1–4.
Wütrich, K. (1977) in *NMR in Biological Research: Peptides and Proteins*, North Holland/American Elsevier, New York.
Wütrich, K., & Wagner, G. (1975) *FEBS Lett. 50*, 265–268.

Proton Nuclear Magnetic Resonance Studies of *Rhodospirillum rubrum* Cytochrome c_2†

Gary M. Smith*

ABSTRACT: *Rhodospirillum rubrum* cytochrome c_2 was studied by proton nuclear magnetic resonance at 220 MHz. Assignments were made to the resonances of heme *c* by double-resonance techniques and by temperature-dependence studies. The aromatic resonances of Trp-62 and Tyr-70 of ferrocytochrome c_2 were identified by spin-decoupling experiments. The resonances of the Met-91 methyl group of the ferri- and ferrocytochromes were assigned by saturation-transfer experiments. The assignments are compared to those made for cytochromes *c*. A pH titration showed that the methionine methyl resonance of ferricytochrome c_2 shifted with a p*K* of 6.25 and disappeared above pH 9. No histidine CH resonances that titrated normally over the neutral pH range were observed in the spectrum of either oxidation state of the protein. The possible origins of the ionizations at pH 6.25 and 9 are discussed.

The cytochromes c_2 of photosynthetic bacteria are small, momeric, monoheme proteins that are structurally similar to the well-known mitochondrial cytochromes *c*. In the nonsulfur purple photoheterotroph *Rhodospirillum rubrum*, the cytochrome c_2 is believed to function as the electron donor to bacteriochlorophyll (Smith et al., 1973).

The heme group of cytochrome c_2 is attached to the polypeptide chain via condensation of the two-carbon side chains with the sulfur atoms of cysteines-14 and -17. The heme iron is involved in coordinate bonds with two other amino acid residues, His-18 and Met-91 (Salemme et al., 1973). The iron exists in either of two oxidation states, diamagnetic Fe^{2+} or low-spin Fe^{3+}. The midpoint potential at neutral pH is 310 mV (Pettigrew et al., 1978), some 60 mV higher than that of the mitochondrial cytochrome. Despite the striking similarity of the X-ray structure of the mitochondrial and *R. rubrum* cytochromes, they differ not only in midpoint potential but also in their reactivity with purified preparations of mitochondrial cytochrome oxidase and reductase (Davis et al., 1972; Errede & Kamen, 1978) and *Pseudomonas aeruginosa* nitrite reductase (Yamanaka, 1972).

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093, and the Molecular Biology Section, University of Southern California, Los Angeles, California 90007. *Received November 3, 1978.* Reprint requests should be addressed to Dr. M. D. Kamen, Marine Biology Building A-002, Scripps Institution of Oceanography, UCSD, La Jolla, CA 92093. Financial support was provided by grants to Dr. M. D. Kamen from the National Institutes of Health (GMS-18528) and the National Science Foundation (BMS-75-13608). The NMR facility at UCSD was supported by a grant from the National Institutes of Health (RR-00708).

* Present address: Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111.

0006-2960/79/0418-1628$01.00/0

Cytochrome *c* has been the subject of numerous investigations because of its interesting and informative optical spectra and its fundamental biological importance. In addition, it has been found to be well suited to investigations employing proton nuclear magnetic resonance (^{1}H NMR) because of its small size ($M_r \simeq 12\,500$) and its interesting shift and relaxation mechanisms. Furthermore, factors affecting the parameters measured by ^{1}H NMR (e.g., electron delocalization) may be closely related to the structural and functional properties of cytochromes.

Some of the resonances in the ^{1}H NMR spectra of ferro- and ferricytochrome c_2 may be assigned to the structural groups of the protein simply from a knowledge of its crystal structure, by comparison to published studies of the mitochondrial cytochrome or by comparison to the spectra of other heme compounds. Such assignments have been presented in a preliminary report of this work (Smith & Kamen, 1974). I now present supporting evidence for these assignments and results of experiments designed to allow a more detailed interpretation of the ^{1}H NMR spectra of ferro- and ferricytochrome c_2 of *R. rubrum*.

Materials and Methods

Cytochrome c_2 was prepared from *R. rubrum* according to the method of Bartsch (1971) with the following modifications. The cells used were the malate-fed G-9 mutant (a gift of Dr. P. F. Weaver). The frozen cells were thawed and suspended in three volumes of 1 mM Tris-HCl, pH 8, 3 mM in EDTA, for 15 min at 4 °C. The unbroken cells were removed by centrifugation, washed with one volume of 1 mM Tris-HCl, pH 8, and recentrifuged. The combined supernatant fractions were brought to 25% saturation with ammonium sulfate and centrifuged. The supernatant solution was desalted by passage over Sephadex G-25. The procedure outlined by Bartsch was then followed, beginning at the stage of the first DEAE-cellulose chromatography. Some of the cytochrome used in this study was donated by Dr. R. G. Bartsch.

The protein for NMR experiments (1–5 μmol) was dissolved in about 2 mL of H_2O. To adjust its oxidation state, it was treated with dithioerythritol for reduction or $Co(phen)_3Cl_3$ (gift of J. V. McArdle) for oxidation. Small ions and redox agents were removed by gel filtration over Sephadex G-25. The samples were lyophilized, then twice lyophilized from D_2O (99.8% D), and taken up in the desired volume (usually 0.3 mL) of D_2O (99.97% D; Aldrich). Samples were used with no added salt or with 0.1 N NaCl at pH 6, unless specified otherwise. The pH of the samples was adjusted by addition of 0.1 N NaOD or DCl. The pH of the solutions in D_2O was measured with a 3-mm o.d. Ingold electrode; the values given represent uncorrected meter readings.

Proton nuclear magnetic resonance spectra were collected by using a Varian HR-220 spectrometer modified for pulse-Fourier transform operation with equipment supplied by Nicolet Instruments Corporation.

The ambient temperature of the probe was determined from the chemical shift of ethylene glycol to be 20 (±1) °C. For experiments at higher temperatures, a Varian variable-temperature controller was employed.

Data from saturation-transfer experiments (Redfield & Gupta, 1971) were collected as the difference of free induction decays (FID's) by alternate addition and subtraction of transients obtained with the auxiliary irradiation on and off resonance. The irradiation was applied during delay times and gated off during acquisition.

Spin-decoupling data were acquired in a similar manner except that the FID's acquired with the irradiation on and off resonance were stored separately and the subtraction was done by computer at the end of each experiment. The time-shared spin-decoupling method (Campbell et al., 1975) was used.

Convolution-difference spectra were obtained as described by Campbell et al. (1973). Parameters for convolution-difference spectra are listed in the figure legends.

Chemical shifts were measured from sodium 2,2-dimethyl-2-silapentanesulfonate as reference. Shifts to low field were assigned positive values.

Calculations of ring-current contribution to chemical shift were done by computer by using the empirical equation given by Shulman et al. (1970). The shifts calculated by this approximate method were found to be equivalent to those reported by Giessner-Prettre & Pulman (1971) except for those of protons very close to the heme. For aromatic amino acid residues, the agreement was excellent. The coordinates of the protons of cytochrome c_2 were calculated by the program HMAKER (written by Stephen Dempsey, University of California, San Diego, 1973).

The nomenclature of the heme pyrrole rings is that used previously (Smith & Kamen, 1974).

Results

The resolved resonances of the heme ring methyl (33.9, 30.1, 15.1, and 10.8 ppm), ligand methionine methyl (−15.2 ppm), and thioether methyl (−1.9 ppm) groups of ferricytochrome c_2 have been assigned as such by comparison to spectra of model compounds and of mitochondrial ferricytochrome (Smith & Kamen, 1974). Likewise, the ligand methionine methyl (−2.9 ppm) and leucine-32 methyl (−2.2 ppm) resonances were assigned similarly and by calculation of the ring-current contribution to chemical shift.

Calculations of chemical shift have allowed the identification of the resonance of the second methyl group of Leu-32 (−0.7 ppm at 20 °C), as it is the only other methyl group expected to resonate upfield of reference. At 35 °C, both Leu-32 methyl resonances appear as doublets in convolution-difference spectra. Spin decoupling experiments intended to locate the resonance of the single proton to which the methyl protons are coupled were unsuccessful, probably because of the multiple splitting interactions and low intensity of the single-proton resonance.

The temperature dependence of the chemical shift of contact-shifted resonances in paramagnetic compounds has been exploited by many authors. Wuthrich (1971) has published temperature-dependence data for several cytochromes *c*. Although direct interpretation of such data may be difficult in cases in which the simple Curie law is not obeyed, the information may be used in a comparative manner. A comparison of the temperature-dependence data for ferricytochrome c_2 shown in Figure 1 with that of Wuthrich for other cytochromes shows that, with the exception of the Met-91 resonances, the chemical shifts of analogous resonances show similar dependences on temperature. It is particularly useful to note that the resonances of the *R. rubrum* cytochrome assigned to two of the heme ring methyl groups show inverse temperature dependence. An inverse temperature dependence was also found for the corresponding resonances of the mitochondrial cytochrome.

The data obtained from saturation-transfer double-resonance experiments allow the location of the unresolved ring methyl resonances of the ferrocytochrome (see Table I). They also give additional evidence for the assignments to the spectrum of the ferricytochrome. It is noteworthy that the resonance assigned to the Met-91 methyl group of the ferricytochrome can be firmly assigned despite the large difference in chemical

Table I: List of Assignments for *R. rubrum* Cytochrome c_2 and Comparison to Shifts of Analogous Protons of Horse Cytochrome *c*

R. rubrum, chemical shift (ppm)			method[a]		horse, chemical shift (ppm)		
reduced[d]	oxidized	assignment	reduced	oxidized	reduced	oxidized	ref[b]
3.47	10.8	heme ring CH_3 1			3.5	10.3	
3.30	30.1	heme ring CH_3 2	1, 2	1, 2, 3, 6	2.1	34.0	A
3.18	15.1	heme ring CH_3 3			3.4	7.2	
2.10	33.9	heme ring CH_3 4			3.8	31.3	
2.34[c]	?	thioether CH_3 1	5	–	1.53[c]	−2.1[c]	
2.36[c]	−1.9 ?	thioether CH_3 2	5	2	2.59[c]	3.1[c]	B
6.29[c]	−2.3 ?	thioether CH 1	5	2	6.34[c]	–	
5.66[c]	?	thioether CH 2	5	–	5.24[c]	–	
9.81, 9.51 9.45, 9.44	–	heme meso CH	2	–	9.32, 9.59 9.62, 9.04	–	B
−2.90	−15.2	Met-91 CH_3	1, 2 3, 4	1, 3, 6	−3.3	−15.3	A
−2.85, −0.35	−13.3	Met-91 $C_\gamma H$	2, 4	2	−2.59, −0.17	–	D
−3.60, −1.20	−23.0, −20.1	Met-91 $C_\beta H$	2, 4	2	−3.8, −1.87	−27.4	A, D
−2.2 −0.7[c]	–	Leu-32 CH_3	2, 3, 4	–	−0.45	–	D
8.27	–	His-42 C_2H	6	–	–	–	
7.78[c]	–	Trp-62 C_4H	5, 4	–	7.60[c]	7.57	
7.10[c]	–	Trp-62 C_7H	5, 4	–	7.07[c]	7.37	D
6.12[c]	–	Trp-62 C_6H	5, 4	–	6.68[c]	6.31	
5.89[c]	–	Trp-62 C_5H	5, 4	–	5.74[c]	6.54	
5.99	–	Tyr-70 ring C-3, 6 H	5, 4	–			
6.71	–	Tyr-70 ring C-2, 5 H	5, 4	–			
7.27	–	Phe-33, -36	4	–			

[a] The methods indicated are the following: (1) saturation-transfer double resonance; (2) comparison to other cytochromes; (3) temperature dependence; (4) calculation of chemical shift; (5) spin decoupling; and (6) pH behavior. [b] References for the mitochondrial cytochrome are (A) Redfield & Gupta, 1971; (B) Keller & Wuthrich, 1977; (C) Moore & Williams, 1975; and (D) McDonald & Phillips, 1973. [c] Indicates that the temperature was higher than 20 °C; see text. [d] The chemical shifts of the methyl resonances of heme rings 1–4 measured relative to the broad, temperature- and pH-dependent resonances of the ferricytochrome in difference spectra of saturation-transfer experiments were 3.57, 3.38, 3.37, and 2.23 ppm, respectively. The values in Table I, measured from convolution-difference spectra of the ferrocytochrome at 35 °C, are more accurate.

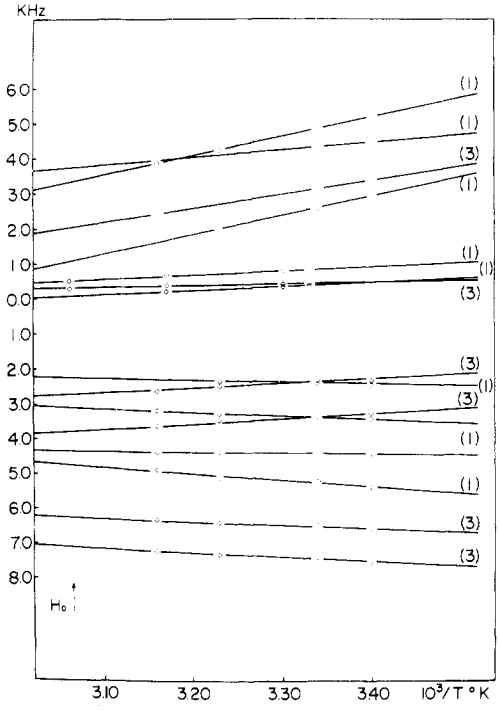

FIGURE 1: The temperature dependence of the chemical shifts of resolved resonances of ferricytochrome c_2. The numbers in parentheses represent the number of protons in each resonance.

shift and temperature dependence from those of the analogous resonance of the mitochondrial ferricytochrome. Keller et al. (1977) have found that the methionine methyl chemical shift of *Euglena gracilis* cytochrome c_{552} is even more dissimilar to that of the horse cytochrome (−3.1 ppm).

The single resolved methyl resonance of ferricytochrome c_2 that is tentatively assigned to one of the heme thioether methyl groups was not linked to a resonance of the ferrocytochrome by this technique. The meaning of the failure of these experiments is not clear and will be discussed below.

It has been demonstrated that homonuclear spin decoupling at 270 MHz (Moore & Williams, 1975) and 360 MHz (Keller & Wuthrich, 1977) can lead to unequivocal assignment to a particular kind of amino acid because each amino acid exhibits a unique spin-coupling pattern. The resolution obtained at 220 MHz is not sufficient to allow complete interpretation of the aromatic region of the spectrum of ferrocytochrome c_2. However, some resonances may be assigned on the basis of spin-decoupling experiments carried out at elevated temperature. Kinetic studies have shown that cytochrome c_2 is active in the temperature range. It is clear that the protein is not significantly denatured at 35 °C from the fact that the Met-91–iron bond remains intact as judged by the chemical shift of the methionine methyl resonance.

Irradiation of the two-proton doublet at 5.99 ppm caused decoupling of a resonance at 6.71 ppm. Irradiation of 6.71 ppm decoupled only the resonance at 5.99 ppm. It can be concluded that the pair of resonances arise from the ring protons of a tyrosyl residue. It is assumed that the two protons ortho to the hydroxyl group are equivalent as are the two meta protons because of rapid rotation about the C_β–C_γ bond axis, accounting for the simplicity of the spectrum in this region. Calculations of chemical shift due to the ring-current mechanism indicate that the aromatic protons expected to resonate most upfield are the *o*-CH's of Tyr-70, the closest to the heme, and the resonances are tentatively assigned accordingly.

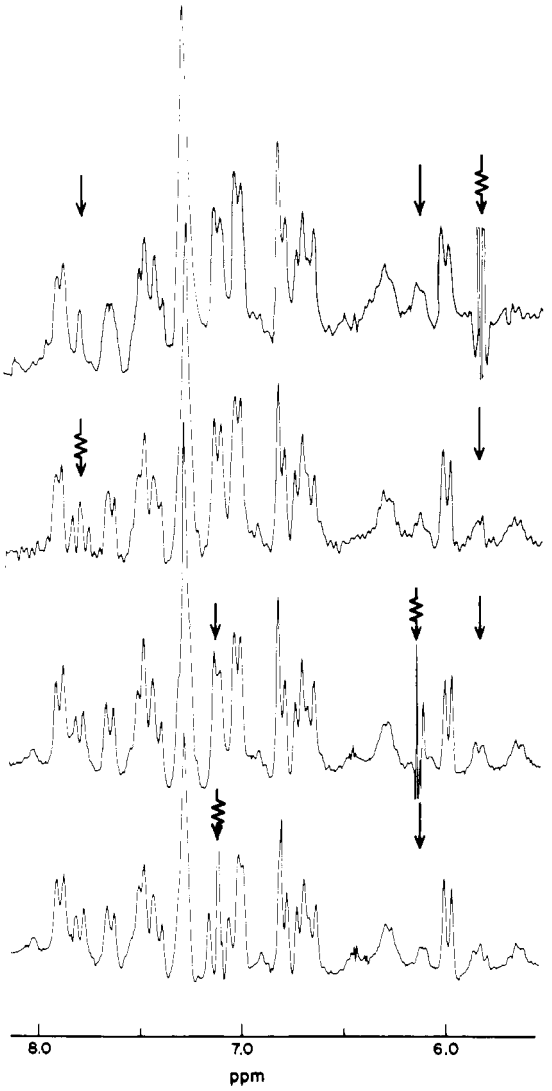

FIGURE 2: Spin decoupling of the benzenoid protons of tryptophan-62 at 37 °C. A spectrum broadened by 5 Hz was multiplied by 0.9 and subtracted from a spectrum broadened by 0.7 Hz to produce the convolution-difference spectra. Assignments are given in Table I.

The resonances of the four benzenoid protons of the single tryptophan residue of ferricytochrome c_2 are expected to appear as two doublets and two triplets, assuming approximately equal coupling. Figure 2 shows the results of experiments leading to the identification of these resonances. The triplets at 6.12 and 5.84 ppm were found to be coupled to each other, and each was also coupled to a doublet (7.78 and 7.10 ppm, respectively).

The only protons resonating in the aromatic region of the spectrum of ferrocytochrome c_2 that are expected to be coupled to methyl resonances in the aliphatic region are the single protons of the thioether CH of the heme side chains. The single proton quartets at 5.66 and (incompletely resolved) at 6.29 were found to be coupled to doublets at 2.36 and 2.34 ppm, respectively (Figure 3).

On the basis of spin-decoupling and nuclear Overhauser effect data, Moore & Williams (1975) have assigned a one-proton quartet at 6.36 ppm and a three-proton doublet at 2.27 ppm in the spectrum of horse ferrocytochrome *c* to the side-chain protons of Thr-78. Keller & Wuthrich (1978), using similar data, assigned these resonances to the thioether methine and methyl protons, respectively. Our assignment to the resonances at 6.27 and 2.34 ppm supports that of Keller and Wuthrich because the *R. rubrum* cytochrome contains

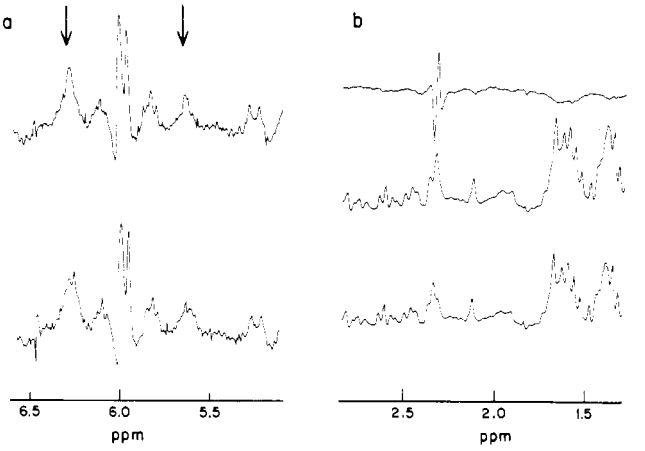

FIGURE 3: Spin decoupling of thioether methyl and methine protons of ferrocytochrome c_2. (a) Irradiation at 2.35 ppm produced the decoupling shown by the arrows. The bottom trace is the control spectrum. (b) Bottom spectrum was taken with irradiation at 5.66 ppm (decoupling observed at 2.36 ppm); middle spectrum was taken with irradiation at 6.20 ppm (decoupling observed at 2.34 ppm). The top trace shows the difference between the middle and lower spectra. Conditions were the same as in Figure 2.

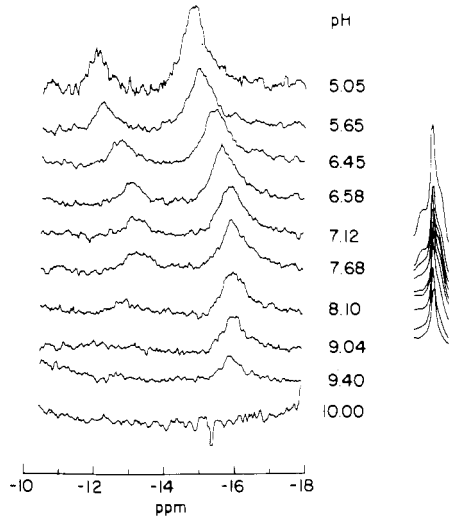

FIGURE 4: The resonances of methionine-91 protons of ferricytochrome c_2 at several pH values. The reference signal (shown on the right) did not shift appreciably.

serine at a position spacially and sequentially homologous to Thr-78 of the horse protein.

The intense resonance (10–12 protons) at 7.31 ppm (shown in Figure 2) corresponds to the chemical shift of the ring protons of phenylalanine. The ring protons of Phe-33 and -36 are near the surface of the protein and as calculated are not expected to receive shifts from the ring-current mechanism. It is thus reasonable to expect that the protons of Phe-33 and Phe-36 account substantially for the resonance intensity at this position.

pH titrations of cytochromes *c* have allowed the identification of histidine C-2 and C-4 proton resonances and the determination of the p*K* values for ionization of the imidazole moiety of the residue (Cohen et al., 1974; Cohen & Hayes, 1974). Other than the ligand His-18, *R. rubrum* cytochrome c_2 contains only one histidyl residue, at position 56. However, pH titrations of neither ferro- nor ferricytochrome c_2 caused shifts in aromatic resonances characteristic of His C-2 or C-4 protons. The spectrum of ferrocytochrome c_2 contained a single-proton resonance at 8.27 ppm that disappeared above pH 5.

A pH titration of ferricytochrome c_2 revealed that the contact-shifted resonances decreased in intensity when the pH was raised above about 9 (Figure 4). In addition, their chemical shifts decreased (i.e., moved upfield) with a pK of about 6.25.

Discussion

Table I summarizes the assignments that have been made to the ^{1}H NMR spectra of ferro- and ferricytochrome c_2 and lists the criteria used. The assignments for mitochondrial cytochrome c from horse heart are also listed for comparison.

The benzenoid proton resonances of Trp-62 were unequivocally identified by spin-decoupling experiments. The distinction between the pair of triplets and between the pairs of doublets shown in Table I was made, at least tentatively, from calculations of ring-current shifts. These assignments are not consistent with those made by Moore & Williams (1975) for the mitochondrial cytochrome. The order of the chemical shifts of the Trp-62 C-5 and C-6 protons is reversed from that found in the mitochondrial cytochrome. In cytochrome c_2 the inner doublet–triplet pair is coupled as are the members of the outer pair, whereas, in the horse cytochrome, the first and third resonances are coupled. According to ring-current calculations, this difference in shift arises from the interaction with Tyr-52, Tyr-70, and Phe-77. The homologous aromatic residues of the horse cytochrome, Tyr-67 and Tyr-74, are farther from the Trp protons. The heme ring current contributes little to the shifts of the Trp C-5 and C-6 protons.

The chemical shifts of the thioether methyl protons of ferrocytochrome c_2 are decidedly different from those of the horse cytochrome. Keller and Wuthrich attribute the pronounced upfield shift of the resonance of the methyl group on heme ring 1 from its expected position to the proximity of the aromatic ring of Phe-82. The homologous residue in the bacterial cytochrome, Phe-89, appears to be positioned differently in the X-ray model. The α-carbon atom of Phe-89 is closer to the center of the heme, and the aromatic ring is more symmetrically placed between the thioether methyl groups.

The pH dependence of physical and chemical properties of cytochromes has been studied by measurements of the near-IR spectrum (Pettigrew et al., 1975, 1978), the NMR spectrum (Gupta & Koenig, 1971; Morishima et al., 1977), the redox potential (Pettigrew et al., 1975, 1978; Wilson & Greenwood, 1971), and kinetic parameters (Wood et al., 1971). In all cases, the horse ferricytochrome was found to exhibit ionizations with apparent pK values of about 3.5 and 9. Below pH 3.5, the horse ferricytochrome exists in the high-spin state with only five intramolecular ligands. Above pH 9, the cytochrome remains low spin, but the methionine is not thought to be coordinated to the iron. *R. rubrum* cytochrome c_2 also exhibits ionizations with pK values near 3 and 9 (Pettigrew et al., 1978). The loss of intensity of the methyl resonance of methionine-91 at alkaline pH occurs with a pK of about 9.2. However, the relation between pH and intensity of the resonance is complex and cannot be explained by a single ionization. Pettigrew et al. (1978) found an ionization with a pK of 8.4 from a study of midpoint potential and a pK of 9.1 from near-IR data for the *R. rubrum* cytochrome. Wood et al. (1977) have suggested similar pK values from their kinetic data.

Although the origin of the pK at 8.4 remains totally obscure, there has been much speculation concerning the ionization at pH 9 found in all c-type cytochromes that have been studied. The methionine ligand is apparently replaced by a strong-field ligand, as judged by the line width of the contact-shifted resonances at high pH in ferricytochromes c and c_2 and the failure to observe an absorption band at 630 nm in the visible spectrum of ferricytochrome c. It has been proposed that the amino group of lysine is the strong-field ligand at high pH from EPR evidence (Brautigan et al., 1977) as well as other information. The lysine modification work of Wilgus & Stellwagen (1974) has indicated that a lysine in the carboxyl half of the horse cytochrome molecule acts as the high-pH ligand. The lysine residue that is most suitably positioned for this function is lysine-79 (horse cytochrome numbering), the residue immediately preceding the neutral-pH ligand. *R. rubrum* cytochrome c_2 also has a lysine in that position. However, Pettigrew et al. (1975) have found that *Rhodopseudomanas capsulata* (strain 2.3.1) cytochrome c_2, which exhibits an alkaline isomerization with a pK of 8.6 (measured as loss in intensity of the 695-nm absorption), does not contain a lysine at this position.

It has been suggested that the measured pK of 9 for the alkaline isomerization does not reflect the actual ionization but instead reflects an ionization with a lower pK, followed by a conformational change that is independent of the ionization (Czerlinski & Bracokova, 1973). This argument is supported to some degree by the observation that the EPR spectrum of carboxymethylmethionine ferricytochrome c at neutral pH resembles the spectrum of native ferricytochrome c at high pH (Brautigan et al., 1977). Thus, it may be that the measured pK for the alkaline isomerization of a particular cytochrome does not depend on the identity of the high-pH ligand.

The ionization of ferricytochrome c_2 that occurs near pH 6.25 measured in this study has also been observed by titration of the midpoint potential (Pettigrew et al., 1978) and by kinetic experiments (Wood et al., 1977) on the *R. rubrum* protein. It has not been observed in the mitochondrial cytochrome. Brautigan et al. (1977) have characterized several ionized forms of the horse and *R. rubrum* ferricytochromes by measurement of the principal values of their g tensors at low temperature. They interpret their observations of multiple low-spin forms of the cytochromes in the neutral-pH range in terms of the ionization or hydrogen-bonding state of the ligand His-18 N-1 atom. The predominant form of the mitochondrial cytochrome (assumed to have an un-ionized His-18) exhibits g values of 3.06, 2.25, and 1.25. A minor component, at pH 7.5, has g_1 and g_2 values of 3.2 and 2.08. The major form of *R. rubrum* cytochrome c_2 had g_1 and g_2 values of 3.17 and 2.05. These forms were thought to contain a His-18 imidazole that was ionized or participated as a proton donor in a hydrogen bond. From the similarity of the g values for the minor form of the mitochondrial cytochrome c and the major form of cytochrome c_2, it is tempting to draw the inference that the predominant protonated form of the horse cytochrome is not accessible to the bacterial cytochrome at pH 7.5. According to this interpretation, the ionization at pH 6.25 might represent the transition between protonated and unprotonated (or hydrogen-bonded) imidazole of cytochrome c_2. This hypothesis is not sufficient to explain all of the pH titration data available. At lower pH, the midpoint potential of cytochrome c_2 rises toward 360 mV, becoming more dissimilar to the mitochondrial cytochrome.

The propionic acid side chain of heme ring 3 has been suggested as another possible ionization site (Smith, 1976). The carboxyl groups of the heme appear to be engaged in hydrogen bonds with Tyr-46, Tyr-48, Ser-49, and Trp-62 in the *R. rubrum* cytochrome. The observation that the horse

cytochrome contains a Phe homologous to Tyr-46 cannot be used to explain the differences in behavior at pH 6.25 because the cytochromes c_2 from *Rhodopseudomonas sphaeroides* and *Rhodomicrobium vannielii* contain a phenylalanine at that position yet exhibit ionizations at pH 6.2 (Pettigrew et al., 1975).

Titrations of proteins over the neutral-pH range have been used extensively for the assignment of resonances of histidine protons [reviewed by Markley (1975)]. The resonances of His C-2 and C-4 protons move upfield as the nitrogen-bound proton is removed at increasing pH. Although cytochrome c_2 contains one histidine, His-42, other than the ligand His-18, no resonances that titrate in a manner characteristic of solvent-accessible histidines have been observed in conventional or convolution-difference spectra of the ferri- or ferrocytochrome. The observation of such a resonance in the spectrum of the ferricytochrome is difficult because of the movement of resonances associated with the ionization at pH 6.25 and the alkaline isomerization. However, a singlet at 8.27 ppm of one-proton intensity in the spectrum of ferrocytochrome c_2 is found to disappear when the pH is raised above 6. Coincident with the disappearance of this resonance was the appearance of a resonance at 7.95 ppm. Poor resolution in this region makes exact integration impossible. It is reasonable to assign these resonances to the C-4 proton of His-42, at least tentatively, to account for the absence of normally titrating histidines. The multiplicity of the resonance at 8.27 ppm may be used as supporting evidence. From the X-ray studies, His-42 is on the surface of the protein and would be expected to titrate normally. If the resonances at 8.27 and 7.95 ppm arise from the same proton in two different environments, the residue must be in slow exchange between the two sites. Such a slow-exchange situation could be created by a conformational change involving the rupture of a hydrogen bond at low pH. The residue would therefore be unavailable for titration over the neutral-pH range.

The resonance of ferricytochrome c_2 at −2.9 ppm is tentatively assigned to one of the thioether methyl groups of the heme. In a previous publication (Smith & Kamen, 1974), it was held that because *both* thioether methyl resonances were thought to be visible upfield of reference in the spectrum of the mitochondrial ferricytochrome but only one was observed in the spectrum of ferricytochrome c_2, the stereochemistry of the asymmetric carbon atom at the point of condensation was inverted from that in cytochrome *c*. This proposal was consistent with the X-ray (Salemme et al., 1973) and magnetic resonance data (Redfield & Gupta, 1971) available at the time. The possibility that the electronic *g* tensor of the bacterial cytochrome might be different from that of the mitochondrial cytochrome was not ruled out. It has since been established that the stereochemical configuration at the heme attachment sites is the same for both cytochromes (Slama et al., 1977). It has also been shown that the principal values of the *g* tensors of the two cytochromes are slightly different (Brautigan et al., 1977). Recently, Keller and Wuthrich have found that only one thioether methyl resonance is visible upfield of reference in the spectrum of the mitochondrial ferricytochrome (Keller & Wuthrich, 1978). The second methyl resonance in this region arises from the side chain of an amino acid residue.

Although the resonances of both thioether methyl groups of ferrocytochrome c_2 were assigned, we were unable to pair the resonance of ferricytochrome c_2 at −2.9 ppm with either of them by double-resonance experiments. These experiments failed, probably because of the proximity of the irradiated peak to the bulk of the aliphatic resonances and the lack of specificity of our secondary irradiation frequency. The possibility remains, however, that the resonance at −2.9 does not arise from the thioether methyl group.

R. rubrum cytochrome c_2 exhibited another property that has not been reported for the mitochondrial cytochrome. The width of the ring methyl resonance at 33.9 ppm increased from 40 to 200 Hz upon aging of the cytochrome. The protein became less stable at elevated temperature (>30 °C) as evidenced by precipitation and a decrease in resolution in the NMR spectrum. Removal of dimer by gel filtration did not restore the smaller line width nor did treatment with EDTA or electrodialysis. Rechromatography of the protein on DEAE-cellulose, followed by recrystallization, could be used to decrease the line width in samples showing moderate broadening (<200 Hz). Although the cause of this phenomenon was not determined, it was found that the broadening could be prevented by storing the cytochrome in the crystalline state at −20 °C. The published spectra of several cytochromes [e.g., Pettigrew et al. (1975)] show that the resonance of the analogous methyl group is slightly broader than that of the second low-field heme ring methyl resonance. However, several spectra of the mitochondrial cytochrome [e.g., Keller & Wuthrich (1977)] show a narrower resonance compared to the second low-field resonance. The interpretation of these observations is not obvious because the ring methyl groups are all expected to be the same distance from the paramagnetic center.

Acknowledgments

The author gratefully acknowledges the interest in and support of this work by Professor M. D. Kamen. In addition, the author thanks Dr. John M. Wright and Richard Freisen who donated considerable time to the modification of the spectrometer for the double-resonance experiments.

References

Bartsch, R. G. (1971) *Methods Enzymol. 23*, 344.
Brautigan, D. L., Feinberg, B. A., Hoffman, B. M., Margoliash, E., Peisach, J., & Blumberg, W. E. (1977) *J. Biol. Chem. 252*, 574.
Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Xavier, A. V. (1973) *J. Magn. Reson. 11*, 172.
Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1975) *Proc. R. Soc. London, Ser. B 189*, 503.
Cohen, J. S., & Hayes, M. B. (1974) *J. Biol. Chem. 249*, 5472.
Cohen, J. S., Fisher, W. R., & Schechter, A. N. (1974) *J. Biol. Chem. 249*, 1113.
Czerlinski, G., & Bracokova, V. (1973) *Biochim. Biophys. Acta 295*, 480.
Davis, K., Hatefi, J., Salemme, F. R., & Kamen, M. D. (1972) *Biochem. Biophys. Res. Commun. 49*, 1329.
Errede, B. J., & Kamen, M. D. (1978) *Biochemistry 17*, 1015.
Giessner-Prettre, C., & Pullman, B. (1971) *J. Theor. Biol. 31*, 287.
Gupta, R. K., & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1134.
Keller, R. M., & Wuthrich, K. (1977) *Biochim. Biophys. Acta 491*, 416.
Keller, R. M., & Wuthrich, K. (1978) *Biochim. Biophys. Acta 533*, 195.
Keller, R. M., Wuthrich, K., & Schejter, A. (1977) *Biochim. Biophys. Acta 491*, 409.
Markley, J. R. (1975) *Acc. Chem. Res. 8*, 70.
McDonald, C. C., & Phillips, W. D. (1973) *Biochemistry 12*, 3170.

Moore, G. R., & Williams, R. J. P. (1975) *FEBS Lett. 53*, 334.
Morishima, I., Ogawa, S., Yonezawa, T., & Iizuka, T. (1977) *Biochim. Biophys. Acta 495*, 287.
Pettigrew, G. W., Meyer, T. E., Bartsch, R. G., & Kamen, M. D. (1975) *Biochim. Biophys. Acta 430*, 197.
Pettigrew, G. W., Bartsch, R. G., Meyer, T. E., & Kamen, M. D. (1978) *Biochim. Biophys. Acta 503*, 509.
Redfield, A. G., & Gupta, R. K. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 405.
Salemme, F. R., Freer, S. T., Xuong, N. H., Alden, R. A., & Kraut, J. (1973) *J. Biol. Chem. 248*, 3910.
Shulman, R. G., Wuthrich, K., Yamane, T., Patel, D. J., & Blumberg, W. E. (1970) *J. Mol. Biol. 53*, 143.
Slama, J. T., Willson, C. G., Grimshaw, C. E., & Rapport, H. (1977) *Biochemistry 16*, 1750.
Smith, G. M. (1976) Ph.D. Thesis, University of California, San Diego, CA.
Smith, G. M., & Kamen, M. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4303.
Smith, W. R., Sybesma, C., Licthfield, W. J., & Dus, K. (1973) *Biochemistry 12*, 2665.
Wilgus, H., & Stellwagen, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2892.
Wilson, M. T., & Greenwood, C. (1971) *Eur. J. Biochem. 22*, 11.
Wood, F. E., Post, C., & Cusanovich, M. E. (1977) *Arch. Biochem. Biophys. 184*, 586.
Wuthrich, K. (1971) in *Probes of Structure and Function of Macromolecules and Membranes*, Vol. II, Academic Press, New York.
Yamanaka, T. (1972) *Adv. Biophys. 3*, 227.

Isolation of Eukaryotic Ribosomal Proteins: Purification and Characterization of S25 and L16†

Alan Lin, Tatsuo Tanaka, and Ira G. Wool*

ABSTRACT: Proteins were extracted from rat liver ribosomal subunits with ethanol and ammonium chloride. The extract from the 40S subunit contained mainly S25, but smaller amounts of a number of other proteins were found as well; the extract from the 60S subparticle had L16 in addition to P1, P2, S25, and several other proteins. S25 and L16 had not been purified before. The former was isolated from the ethanol–ammonium chloride extract by stepwise elution from carboxymethylcellulose with LiCl, chromatography on phosphocellulose, and filtration through Sephadex G-75; L16 was purified by elution from carboxymethylcellulose with LiCl (in steps). The molecular weight of the two proteins was estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate; the amino acid composition was determined also.

Eighty proteins have been isolated from rat liver ribosomes and characterized (Collatz et al., 1976b, 1977; Tsurugi et al., 1976–1978). The proteins that were purified comprise a number that were not in the original classification (Sherton & Wool, 1972). Moreover, several proteins that were included in the initial group were not isolated; that subset includes the 40S subunit protein S25, and L16 from the 60S subparticle. S25 was identified in group D40 after the initial fractionation of the 40S subunit proteins (Collatz et al., 1976a, 1977), but it could not be purified because there were only small amounts. L16 occurs only occasionally on two-dimensional polyacrylamide gel plates when 60S ribosomal subunit proteins are analyzed (Sherton & Wool, 1972) and it was not encountered during the purification procedures.

Acidic proteins can be extracted from ribosom subunits with ethanol and ammonium chloride (Reyes et al., 1977). The extract from 40S subunits contains relatively large amounts of S25, and that from the 60S subunit has L16 even though the two proteins are basic. S25 and L16 have been resolved from ethanol–ammonium chloride extracts of ribosome subunits by chromatography and the purified proteins characterized.

Experimental Procedure

Preparation of Ribosomes and Ribosomal Subunits. Subunits were prepared from rat liver ribosomes (Martin & Wool, 1969) on a large scale by centrifugation in a zonal rotor (Sherton et al., 1974).

Ethanol–Ammonium Chloride Extraction of Ribosomal Subunit Proteins. The procedure used to extract ribosomal proteins was modified in minor ways from that described by Hamel et al. (1972) and Reyes et al. (1977). Ribosome subunits, either 40 S or 60 S, were suspended (100 A_{260} units per mL) in buffer (1 M NH_4Cl, 20 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 mM imidazole hydrochloride, adjusted to pH 7.4 with HCl[1]) and 0.5 volume of 95% ethanol was added. The suspension was shaken gently in an ice bath for 15 min; then a second 0.5 volume of 95% ethanol was added and shaking continued for another 5 min. The final concentration of NH_4Cl was 0.5 M, of ethanol 47.5%. The extracted proteins (referred to as EA40 or EA60[2]) were separated from the core particles by centrifugation (15 min at 27000*g*). The core particles were extracted a second time

† From the Department of Biochemistry, University of Chicago, Chicago, Illinois 60637. *Received December 1, 1978.* The expenses of the research were met by grants from the National Institutes of Health (GM-21769 and CA-19265).

[1] The pH of solutions and buffers was determined at 20 °C.

[2] Abbreviations used: EA40 and EA60, ethanol–ammonium chloride extract of the 40S or 60S ribosomal subunits, respectively; EA40 and EA60 core, the core particle remaining after extraction of 40S or 60S ribosomal subunits with ethanol–ammonium chloride, respectively; TP40 and TP60, the total proteins of the 40S and 60S ribosomal subunits, respectively.

0006-2960/79/0418-1634$01.00/0