Proc. Nat. Acad. Sci. USA
Vol. 71, No. 11, pp. 4303–4306, November 1974

Proton Magnetic Resonance Spectra of *Rhodospirillum rubrum* Cytochrome c_2

(nuclear magnetic resonance assignments/cytochromes c/comparative structures)

GARY M. SMITH AND MARTIN D. KAMEN

Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037

Contributed by Martin D. Kamen, August 6, 1974

ABSTRACT **Nuclear magnetic resonance spectra of ferro- and ferricytochrome c_2 from the facultative photoheterotroph, *Rhodospirillum rubrum*, obtained with a 220-MHz spectrometer, are presented. Assignments to protons of various important structural groups in contact with or near the prosthetic heme group are given. These include (*a*) in the ferro-form, methyl, c_β and c_γ protons of the extraplanar ligand residue, methionine 91, and methyl protons of the residue leucine 32; and (*b*) corresponding protons in the ferri-form with the addition of methyl protons from the condensation of a heme vinyl group with cysteine 17, a single proton from the condensation involving cysteine 14, and protons from the ring methyls. These resonances are compared with those corresponding to the same groups in horse heart cytochrome *c*.**

The *c*-type cytochrome c_2 of the photoheterotrophic bacterium, *Rhodospirillum rubrum* (1), functional in anaerobic photosynthetic electron transport (2, 3), is closely homologous in primary (4) and tertiary (5) structure to the mammalian mitochondrial cytochromes *c* (6–8) but shows altered activities in the mammalian cytochrome *c* reductase and oxidase systems (9). Thus, it provides an excellent opportunity to compare structural parameters as related to function in *c*-type cytochromes—particularly with regard to relative resonance positions of protons associated with crucial groups that interact with or are close to the prosthetic group. It is well established that assignments to such protons can be made relatively unambiguously because of large shifts in resonance positions arising from interactions with ring currents in the diamagnetic ferro-state and also paramagnetic effects caused by the unpaired electron in the low-spin ferri-state (10, 11).

We present proton magnetic resonance spectra obtained at 220 MHz for both ferri- and ferrocytochrome c_2, compare these with those reported for the horse heart protein, and comment on similarities and differences that arise from known structural features of the two cytochromes *c*.

METHODS

R. rubrum cytochrome c_2 was kindly supplied by Dr. R. G. Bartsch (12). Samples were reduced with dithioerythritol and oxidized with $Co(phen)_3^{+++}$ (13), with subsequent removal on a Sephadex G-25 column. Concentrations of the samples, which were exchanged in D_2O several times by lyophilization and solution in D_2O (99.97% D) together with the reference material (see below), were about 10–12 mM in a total volume of 0.3 ml. A Teflon plug was used in a 5-mm nuclear magnetic resonance (NMR) tube to prevent vortexing.

Spectra were recorded on a Varian Associates HR-220 NMR spectrometer, operated by irradiating with the first upfield 20-kHz sideband of the 220-MHz frequency. By using this large separation between sidebands, we avoided the production of spurious peaks, observed when instruments with frequency modulated at 10 kHz are used, that occur at the point where protons are brought into resonance with a higher order sideband or the main band (14). Signal averaging, as needed, was performed on a Fabri-Tek 1074. The probe temperature was 20° unless noted otherwise.

Chemical shifts are reported in parts per million (ppm) from the methyl resonance of 2,2-dimethyl-2-silylpentane-5-sulfonate (DSS), with the convention that positions downfield are assigned positive values.

RESULTS

Reduced State. It is established that in mitochondrial cytochromes *c*, Met 80 is directly bonded to the heme iron as an extraplanar ligand (6–8, 15, 16). Hence, the methionyl methyl, c_β and c_γ protons close to the heme have resonances shifted upfield by ring current interactions. The methyl group resonates at −3.3 ppm.

The residue in *R. rubrum* cytochrome c_2 homologous to Met 80 is Met 91 (4, 21). Its proton resonances (shown in Fig. 1) appear in the same region as those of Met 80 of cytochrome *c*. The methionyl methyl resonance is found at −2.9 ppm. Integration of this resonance shows it to arise from four protons, indicating that a Met 91 c_β proton resonance contributes. This c_β proton may be partially resolved with difficulty at elevated temperature (Fig. 1). The c_γ protons resonate at −3.6 and −1.2 ppm. The second c_β proton at −0.35 ppm is resolved at a probe temperature of 60°.

Temperature effects on the ferrocytochrome resonances result from changes in diamagnetic interactions, the largest of which is expected to arise from expansion or partial unfolding of the three-dimensional structure of the polypeptide chain. We assign methionine proton resonances on the basis of their relative temperature independence as they are not expected to be significantly displaced as long as the iron-ligand bond remains intact.

We confirm the observation by Krejcarek *et al.* (17) that a second methyl group resonates in this region, and assign this resonance at −2.2 ppm to one of the two methyl groups of Leu 32. From coordinates of ferricytochrome c_2 furnished by Drs. F. R. Salemme and J. Kraut, and from calculations of ring current contribution to chemical shift (18), we conclude that this methyl resonance should be subject to a 3 to 4 ppm upfield shift from its usual position at about +0.9 ppm (19, 20). McDonald and Phillips (20) have assigned a resonance at about −0.6 ppm to Leu 32 of horse heart cytochrome, indicating that Leu 32 is farther from the heme group in the mammalian cytochrome. As the position of Leu 32 in models

Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silylpentane-5-sulfonate.

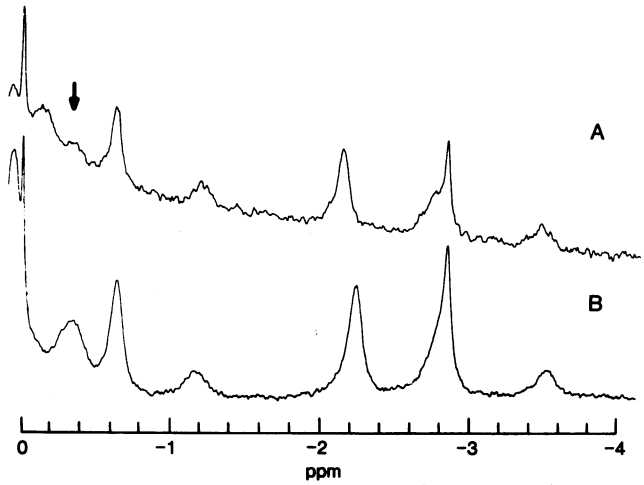

FIG. 1. Ferrocytochrome c_2 at (A) 60°; (B) 20°. Vertical scale of trace A is 0.7 times that of B. Computer average of 16 scans. Assignments are as follows: Met 91 protons: −0.35 (arrow, trace A) and −2.8 ppm (shoulder, trace A), $c_\beta H$; −1.2 and −3.6 ppm, $c_\gamma H$; −2.9 ppm, methyl. Leu 32 methyl: −2.2 ppm.

of ferricytochromes c and c_2 are very similar, the different chemical shifts of the Leu 32 methyl in spectra of the ferro forms may reflect a conformational change that occurs in the region of the peptide chain surrounding Leu 32 upon reduction of the mitochondrial ferricytochrome c. No corresponding conformational change is likely upon reduction of ferricytochrome c_2, because oxidized state coordinates suffice to predict the correct chemical shift of the Leu 32 methyl in the reduced state. This observation is consistent with x-ray data on mitochondrial c (in particular, see Figs. 10 and 11 of ref. 8) and the bacterial c_2 (21).

Oxidized State. In the far upfield region (Fig. 2), the spectrum of ferricytochrome c_2 differs from that of the mammalian cytochrome. Three single protons and a methyl group resonate between −13 and −24 ppm. The Met 91 c_γ protons are separated by only three bonds from the paramagnetic center and should experience a greater contact shift than the c_β protons, which are five bonds distant. We therefore assign the resonances at −23.0 and −20.1 ppm to Met 91 c_γ protons. The single-proton resonance at −13.3 ppm is assigned to a methionine c_β proton. The three-proton resonance at −15.2 ppm belongs to the methyl group of Met 91 by analogy to cytochrome c (e.g., ref. 11).

We note that the Met 91 methyl experiences a smaller contact interaction with the paramagnetic iron than the corresponding protons of Met 80 of the mammalian cytochromes (chemical shift, −24.2 ppm). This observation may reflect

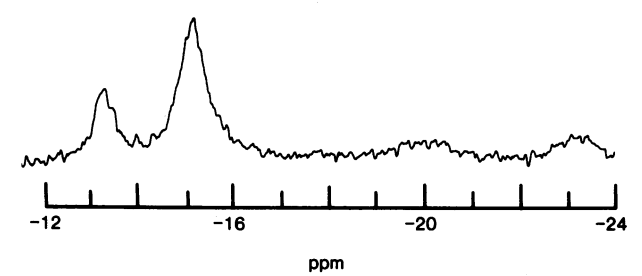

FIG. 2. Highfield spectral region of ferricytochrome c_2. Assignments: Met 91 protons: −23.0 and −20.1 ppm, $c_\gamma H$; −13.3 ppm, $c_\beta H$; −15.2 ppm, methyl.

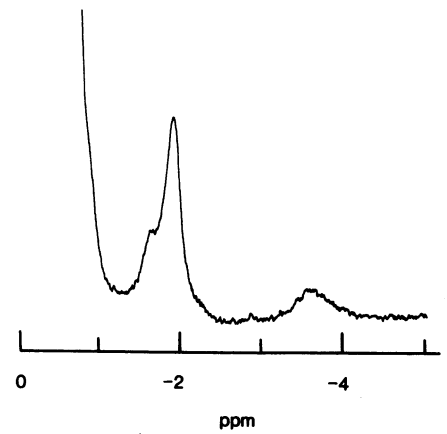

FIG. 3. Near upfield region of ferricytochrome c_2. Assignments: −2.0 and −3.3 ppm, methyl and C-H of Cys 17 linkage; −1.5 ppm (shoulder), C-H of Cys 14 linkage.

the condition observed by Salemme *et al.* (5, 21) in which the methionyl sulfur appears to be pulled away from the His 18 N-Fe axis, whereas there is no published evidence that the mitochondrial proteins show such distortion of ligand positions. A decrease in orbital overlap or a slight change in the hybridization of the sulfur orbitals can reduce the coupling constants involved so that the Met 91 methyl experiences a smaller hyperfine contact interaction, and might also contribute to the observed difference in midpoint potential between cytochrome c_2 and the mitochondrial cytochrome (21). Among the few methionine-containing c-type cytochromes that have been studied by NMR, there appears to be a qualitative correlation between midpoint potential and chemical shift of the methionyl methyl in the spectrum of the oxidized cytochrome. For example, the *Pseudomonas aeruginosa* cytochrome c-551, which has a midpoint potential ($E_{m,7}$ = +286 mV) (22) between those of *R. rubrum* cytochrome c_2 and the horse heart protein, exhibits a Met 61 methyl resonance at −16.8 ppm (23), a position intermediate to those of the corresponding resonances of the horse and *R. rubrum* proteins. To determine how general this quantitative relationship between the paramagnetic shift of the methionyl methyl resonance and the midpoint potential of a cytochrome may be will require extension of these studies to several cytochromes c that have different midpoint potentials.

The resonances associated with Met 91 exhibit the same pH dependence as those of Met 80 of mammalian cytochromes (24). Upon titration with NaOD, the resonance intensities decrease with a pK of about 9, corresponding to the loss of absorbance at 695 nm (25). The optical absorbance spectrum retains low-spin character, indicating that a strong field ligand still occupies the sixth coordination position although the methionine is no longer coordinated. The inference is that the same strong field ligand participating in the high pH complex is present in both cytochromes c and c_2. Lysine 79 (c_2 homolog Lys 90, see refs. 4 and 21) has been suggested as the high pH ligand by Gupta and Koenig (24), although hydroxide cannot yet be ruled out because it is expected to be a strong field ligand also under these conditions (26).

The region of the spectrum between −1 and −6 ppm, usually associated with linkage of the prosthetic group to the protein, is shown in Fig. 3. Only one resolved methyl resonance and two single proton resonances can be seen in this region. The spectrum of horse heart ferricytochrome c (not

shown) contains two methyl group resonances at −2.4 and −2.7 ppm and two resonances of one proton each at −4.0 and −6.3 ppm. McDonald and Phillips (20) have assigned these as thioether bridge methyls and heme meso protons, respectively. However, a study by Keller *et al.* (27) of cytochrome c_{557} from *Crithidia oncopelti*, which lacks Cys 14, demonstrated that the single protons are the α protons of the thioether condensation, in agreement with earlier work by Redfield and Gupta (11).

Concerning the direction of maximum unpaired electron density in the heme based on the chemical shifts of the heme ring methyls (see below), Redfield and Gupta (11) found that the resonance positions of each of the thioether bridge methyls moved the same distance upfield from their positions in the reduced state upon oxidation, a result consistent with the suggestion that the methyl groups were symmetrically disposed with respect to the direction of maximum electron delocalization. Only an *R* configuration for both carbons of the methyls at the two sites of condensation is consistent with proper positioning.

The thioether bridge methyls of ferricytochrome c_2 do not show comparable shifts to those of mammalian cytochrome *c;* indeed, only one methyl resonance is resolved. It is apparent that the chemical shifts in the oxidized state reflect a structural difference from the mammalian cytochrome. We conclude that, if the electronic g-tensor of the heme iron of c_2 is similar to that of *c*, the stereochemistry at the thioether condensations must be different. According to the electron density map of Salemme *et al.*, the stereochemistry at both the Cys 14 and Cys 17 condensations appears to be *S* (see Fig. 4).

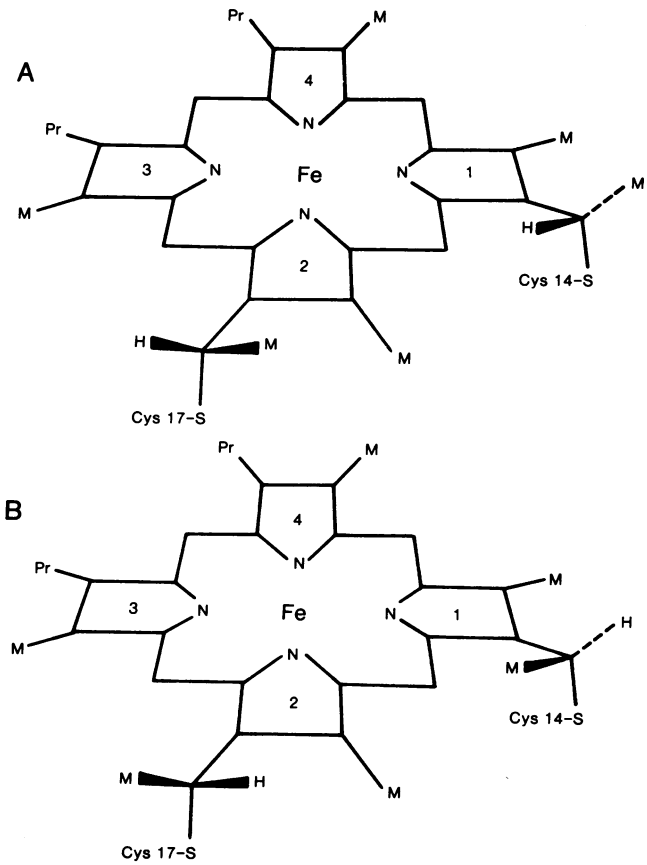

FIG. 4. Heme *c* showing proposed stereochemistry for (A) cytochrome c_2 and (B) cytochrome *c* (see *text*). Pr, propionic acid; M, methyl.

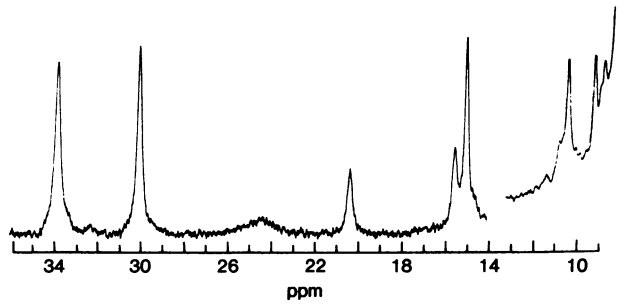

FIG. 5. Extreme lowfield region of ferricytochrome c_2 spectrum. Ring methyls of rings 2 and 4 resonate at 33.9 and 30.1 ppm. Other ring methyl resonances are shown at 10.6 ppm and possibly 15.0 or 9.0 ppm. Vertical scale of 9–13 ppm region is 0.75 that of 14–36 ppm region.

4). On this basis, the methyl and one-proton resonances at −2.0 and −3.3 ppm are those of the pyrrole ring 2-Cys 17 thioether linkage, and the single proton −1.5 ppm is that of the ring 1-Cys 14 linkage.

The heme ring methyl resonances have been found to experience a large downfield contact shift in the NMR spectra of various ferric heme proteins (e.g., refs. 10 and 28). Resonances from these methyls appear in the spectrum of ferricytochrome c_2 at +33.9, 30.1, 10.6, and possibly at 15.0 ppm (Fig. 5).

Two ring methyls of horse cytochrome *c* resonate at 35.4 and 32.9 ppm. These farthest-shifted resonances have been assigned by Redfield and Gupta (11) to the methyl substituents of pyrrole rings 2 and 4, respectively, by examining interactions in the reduced state and correlating the reduced state resonances with oxidized state resonances by a double resonance experiment.

The important conclusion drawn from this assignment is that the unpaired electron density in the heme ring is localized for the most part along the pyrrole N4-Fe-pyrrole N2 axis of the heme. Mailer and Taylor (29) have measured the electronic g-tensor in a single crystal of horse heart cytochrome. Their measurements are consistent with the results of the double resonance assignment (C. P. S. Taylor, personal communication). This model of unpaired electron density has been invoked in mechanistic arguments (21) and in further magnetic resonance studies (30). It is therefore of interest to examine whether it may apply to cytochrome c_2.

The farthest shifted methyl group at 33.9 ppm can indeed be assigned to the pyrrole ring 2 methyl by a study of the interaction between cytochrome c_2 and exogenous paramagnetic ions. We have observed broadening (Fig. 6) of the 33.9 ppm resonance in the presence of Co(II)(phen)$_3$, the product of oxidation of reduced cytochrome by Co(III)(phen)$_3$ (13). We have been able to observe no complex formation, and the broadening is eliminated by gel filtration over Sephadex G-25.

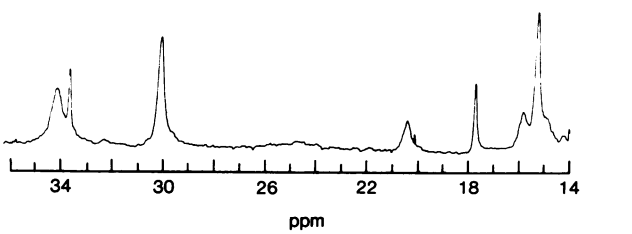

FIG. 6. Ferricytochrome c_2 + Co(II)(phen)$_3$. Spectrum shows broadened methyl resonance at 34 ppm. Sharp resonances at 33.6 and 17.7 ppm arise from the cobalt complex (see *text*). Average of 16 scans.

TABLE 1. *Correlations of hyperfine contact shifts of ring methyl groups with ligand character in heme compounds*

Ferric heme	Ligands	Chemical shift of farthest shifted ring methyls (ppm)	Ref.
Cytochrome c_2	His, Met	33.9, 30.1	
Cytochrome *c* (horse heart)	His, Met	35.4, 32.9	11,20
Cytochrome c_{577} (*Crithidia oncopelti*)	His, Met	32.5, 30.8	27
Cyanoferricytochrome *c*	His, CN^-	22.9, 21.1	28
Azidoferricytochrome *c*	His, N_3^-	17.3, 16.1	31
Cytochrome *c*, pH $\simeq$ 9	His, Lys NH_2?	$\simeq$24, $\simeq$21	24
Cytochrome c_3 (*Desulfovibrio vulgaris*)	His, His?	$\simeq$29	32
Cytochrome b_5 (protoheme)	His, His	21.5, 14.0	33
Cyanoferrimyoglobin (protoheme)	His, CN	$\simeq$24, $\simeq$18	14

We therefore conclude the interaction is on the surface of the cytochrome. As the ring methyl that is most exposed is that of pyrrole ring 2, it is reasonable to identify it as associated with the 33.9 ppm resonance. It appears to be an acceptable working hypothesis, then, that in cytochrome c_2, the unpaired electron resides in an iron-porphyrin molecular orbital that is comprised largely of the iron d_{xz} orbital with the *x*-axis defined as the Fe-pyrrole 2N direction and the *z*-axis normal to the heme plane. This suggestion has been put forward previously by Salemme *et al.* in their rationalization of a mechanism for oxidation and reduction of cytochrome c_2 (21).

It may be of interest to compare the chemical shifts of resolved ring methyl resonances of methionine-containing *c*-type cytochromes with those of other heme proteins, modified cytochromes, and model compounds. From Table 1, which lists several low-spin ferric heme systems together with their extraplanar ligand and chemical shifts of the most downfield ring methyls, it is apparent that the electron density at the edges of the pyrrole rings is larger in systems in which methionine is a ligand. This effect may be plausibly interpreted in terms of the energy levels of the iron d-orbitals. For the case of D_{4h} symmetry (an over-simplification, but one customarily used; e.g., ref. 34), splitting into a lower-lying b_{2g} orbital and an upper pair of e_g orbitals effected by a strong field ligand leaves the b_{2g} orbital filled. The cytochrome has lower symmetry than D_{4h} and the e_g orbitals are further split, leaving one filled and one containing an unpaired electron. The greater the splitting, the greater is the contribution of the porphyrin molecular orbital to the total molecular orbital—in other words, the more unpaired electron density is concentrated in the ring system. Methionine sulfur, because of its bulk and relatively rigid orbital orientation, can be particularly effective in maintaining electron density on the heme periphery, in contrast to smaller or less rigid ligands.

An extension of this argument to the case of the multiheme cytochromes c_3, in which methionine is absent, can account for the relatively low chemical shifts observed (Table 1), although the alternative proposal of heme "stacking" made by others (32) may be a contributing factor.

We thank Drs. J. Kraut and R. E. Dickerson for permission to examine their models of cytochromes c_2 and *c*, Dr. R. G. Bartsch for the cytochrome c_2 samples, Mr. J. McArdle for the Co(phen)$^{+++}$, and Dr. J. Wright for guidance in use of the NMR spectrometer. This work was supported by grants from the National Science Foundation (GB-36019X) and the National Institutes of Health (GM-18528). G.S. is a USPHS Predoctoral Trainee (GM-01045).

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