One- and Two-Dimensional NMR Characterization of Oxidized and Reduced Cytochrome *c'* from *Rhodocyclus gelatinosus*†

Ivano Bertini,*,‡ Gabriele Gori,‡ Claudio Luchinat,§ and Alejandro J. Vila‡

Department of Chemistry, University of Florence, via G. Capponi 7, 50121 Florence, Italy, and Institute of Agricultural Chemistry, University of Bologna, via Berti Pichat 10, 40127 Bologna, Italy

Received June 30, 1992; Revised Manuscript Received October 20, 1992

ABSTRACT: 1D and 2D NMR spectra of both the reduced and oxidized forms of cytochrome *c'* from *Rhodocyclus gelatinosus* have been recorded. The analysis of the pH dependence of the ^{1}H NMR spectrum of the ferric form has been performed, and two main ionizing groups have been identified. By comparison of the pH dependence of the available spectra of cytochromes *c'*, an ambiguity remaining from previous studies on related cytochromes *c'* has been solved. By means of 2D spectra, an assignment of all the paramagnetically shifted signals is proposed both for the ferrous and for the ferric forms.

Cytochromes *c'* are a group of electron-transport heme proteins found in photosynthetic, denitrifying, and nitrogen-fixing bacteria (Bartsch, 1978; Meyer & Kamen, 1982; Cusanovich et al., 1988). They possess a heme moiety covalently bound to two cysteinyl residues by means of thioether links (Figure 1) (Cusanovich, 1971; Kennel et al., 1972). They are usually found as dimers although a monomeric cytochrome *c'* has been also isolated and studied (Dus et al., 1967). The heme binding sequence pattern (-Cys-X-Y-Cys-) is similar to that of cytochrome *c*, but in this case, the iron atom is pentacoordinated and high spin, instead of being hexacoordinated and low spin (Weber et al., 1980, 1981; Weber, 1982). The fifth ligand of the iron is a histidine residue exposed to the solvent.

In general, cytochromes *c'* may be considered as anomalous members of the heme proteins, and not only by the notorious difference with the well-known cytochromes *c*. The absence of a sixth ligand is a common feature with other high-spin heme proteins, like globins and peroxidases. Nevertheless, cytochromes *c'* exhibit a low affinity for the binding of small ligands, like CO and CN^- (Taniguchi & Kamen, 1963; Gibson & Kamen, 1966; Cusanovich & Gibson, 1973; Kassner et al., 1985; Kassner, 1991). On the other hand, the hydrogen bonding of the Nδ1 proton of the coordinated histidine is a key feature for characterizing the function and properties of the different high-spin heme proteins. In the case of cytochromes *c'*, the axial histidine is exposed to the solvent and is not able to form hydrogen bonds through its Nδ1 proton (Weber et al., 1981; Weber, 1982). The specific biological role of cytochromes *c'* has not been established, but their ubiquity in nature suggests that they could play an essential function (Bartsch, 1991).

Cytochromes *c'* have been studied by different spectroscopic methods, for example, by EXAFS (Korszun et al., 1989), Mössbauer (Emptage et al., 1977), EPR (Ehrenberg, 1965; Maltempo, 1974), MCD (Rawlings et al., 1977), Raman (Strekas & Spiro, 1974; Spiro et al., 1979), and NMR spectroscopy (La Mar et al., 1981, 1990; Emptage et al., 1981; Jackson et al., 1983; Bertini et al., 1990; Banci et al., 1992).

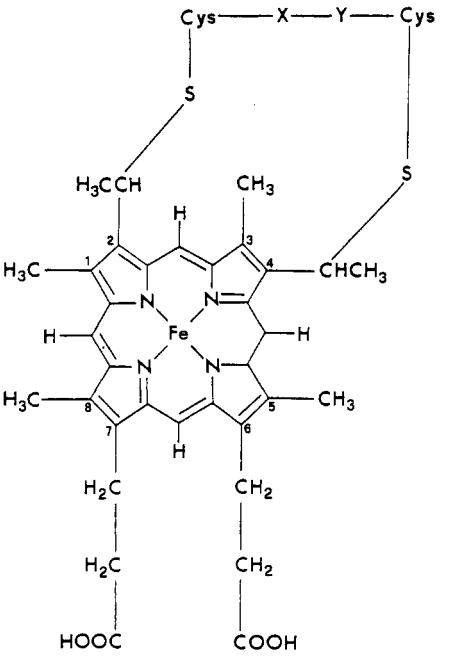

FIGURE 1: Schematic drawing of the heme moiety in cytochromes *c'*.

Some anomalies in their spectroscopic properties led Maltempo to propose that Fe(III) was present in a quantum mechanical admixture of a high ($S = {}^5/_2$) and an intermediate ($S = {}^3/_2$) spin state (Maltempo, 1974, 1976; Maltempo et al., 1974). Nevertheless, NMR results seem to indicate the presence of an essentially high-spin iron at room temperature. Regarding the crystallographic studies, only the X-ray structure of the cytochrome *c'* from *Rhodospirillum molischianum* (*R. molischianum*)[1] is available (Weber et al., 1981), and a preliminary report of the crystallization of the *Chromatium vinosum* protein has appeared (Mc Ree et al., 1990).

Cytochromes *c'* display pH-modulated transitions that can be monitored by means of NMR, as already shown for different

† A.J.V. received a postdoctoral fellowship from the ICGEB (International Centre for Genetic Engineering and Biotechnology) and was further supported by the Fundación Antorchas.

* Address correspondence to this author.
‡ University of Florence.
§ University of Bologna.

[1] Abbreviations: *R. molischianum*, *Rhodospirillum molischianum*; *C. vinosum*, *Chromatium vinosum*; *R. rubrum*, *Rhodospirillum rubrum*; *R. palustris*, *Rhodopseudomonas palustris*; *R. gelatinosus*, *Rhodocyclus gelatinosus*; WEFT, water-eliminated Fourier transform; NOE, nuclear Overhauser enhancement; TPPI, time proportional phase incrementation; NOESY, nuclear Overhauser effect spectroscopy.

0006-2960/93/0432-776$04.00/0

Fe(III) cytochromes *c'* (La Mar et al., 1990). By carefully comparing the pH dependencies of the ^{1}H NMR spectra of different cytochromes, it might be possible to recognize the residues which are responsible for the different pK_as. Until now, the pH behaviors of three dimeric cytochromes *c'* [from *C. vinosum* (Bertini et al., 1990; La Mar et al., 1990; Banci et al., 1992), *Rhodospirillum rubrum* (Emptage et al., 1981), and *R. molischianum* (La Mar et al., 1990)] and of a monomeric one [*Rhodopseudomonas palustris* (Jackson et al., 1983)] have been studied by means of NMR.

2D NMR studies, which have been applied in the last years to paramagnetic heme proteins (Yamamoto et al., 1989; Satterlee & Erman, 1991; Emerson et al., 1990; Wu et al., 1991; Banci et al., 1991c,d), are difficult to perform in high-spin Fe(III) systems (Banci et al., 1992; de Ropp et al., 1991). The slow electron relaxation displayed by this metal ion (Bertini & Luchinat, 1986; Banci et al., 1991a) induces fast-relaxing and broad proton signals so that some cross-peaks may be lost in the 2D maps (de Ropp et al., 1991; Banci et al., 1991a). Recently, the first 2D NMR study of a cytochrome *c'* has been reported providing a partial assignment of the heme moiety and showing the feasibility and usefulness of 2D methods for this class of protein (Banci et al., 1992).

On the other hand, little attention has been given to the study of the paramagnetic ($S = 2$) reduced ferrous cytochromes *c'*. Only the spectra of the Fe(II) forms of the *C. vinosum* (Bertini et al., 1990) and *R. rubrum* (Emptage et al., 1981) have been reported but without performing any assignment in spite of the interesting shift pattern observed for the heme methyl groups.

One primary objective in the study of a new cytochrome *c'* is the characterization of the ionizable groups by comparison with the previously studied cytochromes *c'*. Another goal of such an investigation is to complete the assignments of the paramagnetically shifted signals in the oxidized form and to study the relatively unexplored reduced species.

Here we report a biophysical study of both the reduced and oxidized forms of cytochrome *c'* from *Rhodocyclus gelatinosus* by means of 1D and 2D NMR spectroscopy. This cytochrome is a 28-kDa dimeric protein containing two identical subunits each with a covalently bound heme moiety (Ambler et al., 1979, 1981). This work describes the first paramagnetic 2D NMR investigation of a high-spin Fe(II) heme protein; it confirms the previous findings on the Fe(III) form of the cytochrome *c'* of *C. vinosum* (Banci et al., 1992) and extends the previous assignments. The analysis of the pH dependence of the hyperfine shifts in comparison with those already reported allows the identification of the group responsible for the low-pH ionization. The final aim is not restricted to the structural elucidation of a particular cytochrome, but we intend to compare these findings with the previous ones on different cytochromes *c'* in order to derive general conclusions on this class of protein and explain some differences in the series.

EXPERIMENTAL PROCEDURES

Cytochrome *c'* from *R. gelatinosus* was isolated and purified as previously reported (Bartsch, 1971). The samples were ca. 2 mM in protein in unbuffered solutions. Reduction of the Fe(III) cytochrome was performed by adding small quantities of solid sodium dithionite under nitrogen, but after some time, the reduced species was prone to autoxidation.

The ^{1}H NMR spectra were recorded on Bruker MSL 200 and AMX 600 spectrometers. The ^{1}H NMR spectra at 200 MHz were performed using the super WEFT pulse sequence (Inubushi & Becker, 1983) to eliminate the water signal, while

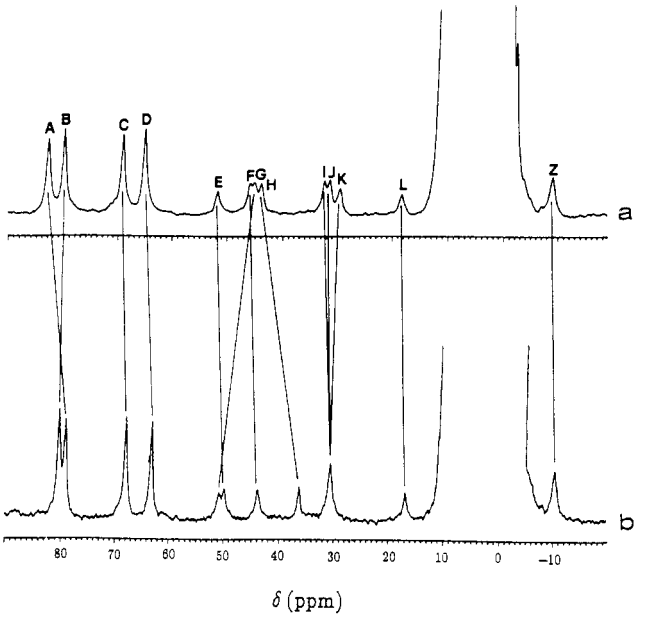

FIGURE 2: 600-MHz ^{1}H NMR spectra of the ferricytochrome *c'* from *R. gelatinosus* at 300 K in H_2O at (a) pH 4.0 and (b) pH 7.4.

at 600 MHz a presaturation of the residual solvent signal was applied during the relaxation delay. The absence of hyperfine-shifted exchangeable signals in H_2O samples was checked on spectra recorded using the $\overline{1331}$ pulse sequence (Hore, 1983). T_1 measurements were performed by using the inversion recovery method (Vold et al., 1968). In the case of the oxidized cytochrome *c'*, the transmitter offset was positioned in the frequency range of the paramagnetic signals in order to reduce imperfections in the 90° and 180° pulses. The pH titration was performed in a H_2O solution with 10% of D_2O, and the pH of the solution was adjusted by small aliquots of HCl or NaOH solutions. As no exchangeable paramagnetic signals were detected in this spectral window, the other experiments were performed in D_2O solutions. Reported pH values are not corrected for the isotopic effect.

Steady-state NOE and saturation-transfer experiments were performed at 600 MHz using a previously described technique (Banci et al., 1989, 1991b; Lecomte et al., 1991). 2D spectra were recorded in D_2O using presaturation to eliminate the residual HDO signal. Phase-sensitive TPPI (Marion & Wüthrich, 1983) NOESY (Gordon & Wüthrich, 1978) experiments were performed with mixing times ranging from 7 to 50 ms for the Fe(III) cytochrome *c'* with relaxation delays from 50 to 250 ms. For the Fe(II) cytochrome *c'*, mixing times from 20 to 80 ms were employed. The time-domain data were multiplied in both dimensions by sine-squared bell-window functions and zero-filled as indicated in the figures.

RESULTS

Fe(III) Cytochrome c'. The 600-MHz ^{1}H NMR spectra performed at pH 4.0 and 7.4 of the oxidized cytochrome *c'* of *R. gelatinosus* are shown in Figure 2. The signals are correlated by means of the pH titration (Figure 3). The spectra show a set of broad signals which experience large isotropic shifts typical of a high-spin Fe(III) heme system. An additional very broad upfield signal (ca. −40 ppm) is detected only at low pH, which is at the limit of detectability at 600 MHz since a considerable line broadening is induced by Curie relaxation. All the hyperfine-shifted signals observed in the H_2O spectra were also detected in D_2O, allowing us to rule out the presence of exchangeable signals, as previously found

for other cytochromes *c'* (La Mar et al., 1981, 1990; Jackson et al., 1983).

The spectrum at pH 5.0 consists of four strongly downfield-shifted signals each of intensity three (which are due to the four heme methyl groups) and eight downfield signals each of one-proton intensity. According to previous assignments on other cytochromes *c'*, these signals are due to the two α-CH protons establishing the heme thioether bridge, to the four α-CH_2 protons of the propionate heme side chains, and to the two β-CH_2 of the proximal histidine bound to the iron atom. The broad upfield signal observed in the low-pH species (data now shown) is attributable to a meso proton. Meso protons are known to experience a large contact shift. Moreover, it has been proposed that their shifts may be useful to predict the spin state and coordination number of the iron atom. In this case, the observed upfield shift is characteristic of a five-coordinated high-spin Fe(III) (La Mar et al., 1990; La Mar & Walker, 1978; Budd et al., 1979; Rajarathnam et al., 1991). The presence of these resonances in the low-pH species was also observed in the cytochromes *c'* from *R. rubrum*, *R. palustris*, and *R. molischianum*, while in that from *C. vinosum* the meso proton signals were detected at high pH (La Mar et al., 1990).

A further sharper signal (Z) is observed upfield. This signal has an intensity sizably larger than 1, although lower than 2. Accurate integration is prevented by its being close to the diamagnetic envelope. Under no condition (pH, temperature) is the possible accidental degeneracy of two protons resolved. In other cytochromes *c'*, a three-proton intensity signal has been detected in the high-field region (Bertini et al., 1990; La Mar et al., 1990), tentatively attributed to the methyl group of a methionine residue located in a proximal position, but at such a distance (4 Å) that no bond is formed. In this cytochrome *c'*, this methionine is lacking (Ambler et al., 1979, 1981) so that this signal might arise from proton(s) of a phenylalanine which substitute(s) methionine in the *R. gelatinosus* protein. No dipolar connectivities were found between this resonance and other hyperfine-shifted signals in the 2D experiments.

The analysis of the pH dependence of the paramagnetically shifted signals has proved useful in the study of the acid–base equilibrium of cytochromes *c'* (La Mar et al., 1990; Banci et al., 1992). Figure 3 shows the pH dependence of the shifts in the 4.1–10.6 pH range in H_2O. Signals J and K begin to broaden when raising the pH and then disappear at pH 7.5–8. These two signals reappear at pH values over 10 as shown in Figure 3. The behavior of signals G and H is very similar to that observed for a similar pair of signals in *C. vinosum*: from almost similar shifts at low pH, their values diverge with increasing pH ($\Delta\delta_{max}$ = 15 ppm) and then exhibit comparable shifts again at high pH. In this case, we have also observed a marked difference in the δ values of these signals between H_2O and D_2O solutions: in D_2O, the pH titration shows an even higher divergence of their chemical shifts at intermediate pH ($\Delta\delta_{max}$ = 22 ppm). Curiously, these are the only signals whose shifts display such a large isotopic effect.

In the case of *C. vinosum*, the latest study pointed out that a better fitting of the pH dependence at low pH could be obtained by using three pK_a values (Banci et al., 1992), and this seems to be the case also for the cytochrome from *R. gelatinosus*. For the cytochromes from *R. rubrum* (Emptage et al., 1981), *R. molischianum* (La Mar et al., 1990), and *R. palustris* (Jackson et al., 1983), the fittings were performed using only two pK_a values. In the present case, a fitting including three pK_as yielded the values of 4.81 ± 0.04, 6.70 ± 0.03, and 9.03 ± 0.04 (3σ). It should be recalled that in the case of the *C. vinosum* protein the three pK_as found were 5.28 ± 0.03, 6.72 ± 0.03, and 8.84 ± 0.03 (3σ) (Banci et al., 1992).

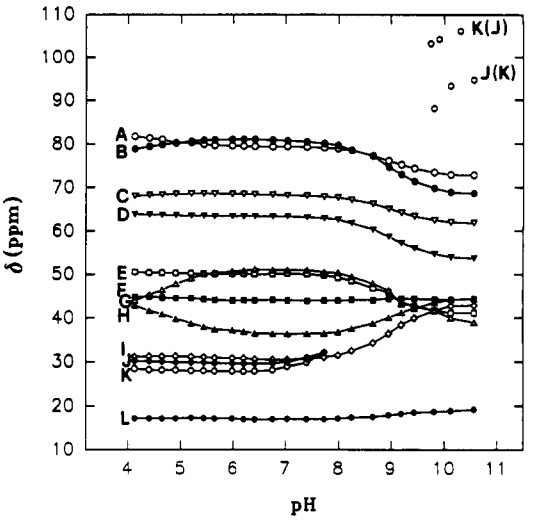

FIGURE 3: Plot of the chemical shift dependence against varying pH of the downfield ^1H NMR signals of *R. gelatinosus* ferricytochrome *c'*. The spectra were recorded at 600 MHz and 300 K in unbuffered H_2O solutions. The signals with chemical shifts larger than 105 ppm at high pH were detected in the 200-MHz spectra.

Table I: T_1 of Paramagnetically Shifted Signals in Reduced and Oxidized Forms of *R. gelatinosus* Cytochrome *c'*

signal	assignment	T_1 (ms)[a] oxidized[b]	T_1 (ms)[a] reduced[c]
A	1-CH_3 (3-CH_3)	5.0	35.0
B	8-CH_3	5.2	38.2
C	5-CH_3	5.8	36.4
D	3-CH_3 (1-CH_3)	4.8	45.1
E	6-CHα'	3.2	34.0[a]
F	4-CHα (2-Hα)	3.6	
G	7-CHα'	2.8	
H	7-CHα	3.6	17.2
I	6-CHα	3.3	55.7
J	Hisβ	2.1[d]	13.2 (22.3)
K	Hisβ'	2.5[d]	22.3[d] (13.2)
L	2-CHα (4-Hα)	3.0	

[a] Measured with an error of ±10%, except when indicated. [b] At 200 MHz, pH 7.2, and 300 K. [c] At 600 MHz, pH 5.2, and 300 K. [d] Measured with an error of ±20%.

The temperature dependence of the chemical shifts was followed, and the signals followed a Curie behavior. As previously noted in other cytochromes *c'* (Emptage et al., 1981; Jackson et al., 1983; Bertini et al., 1990), some signals have zero intercepts outside the diamagnetic region. The T_1 values for the paramagnetically shifted signals determined at 200 MHz at pH 7.6 are reported in Table I and fall in the same range as observed for the other cytochromes *c'* (Bertini et al., 1990).

The 600-MHz 295 K NOESY spectrum performed at pH 7.2 in D_2O with a mixing time of 7 ms is shown in Figure 4. NOESY experiments provide dipolar connectivities, and the cross-peaks are due to cross-relaxation (Gordon & Wüthrich, 1978). Hence, maximum intensity of NOESY cross-peaks is expected for mixing times of the order of T_1. Intense cross-peaks are observed between the pairs of signals G,H (cross-peak 3) and E,I (cross-peak 4). Signal I also shows a connectivity with signal C (cross-peak 2), and H shows a cross-peak (1) with the methyl signal B. When a similar NOESY

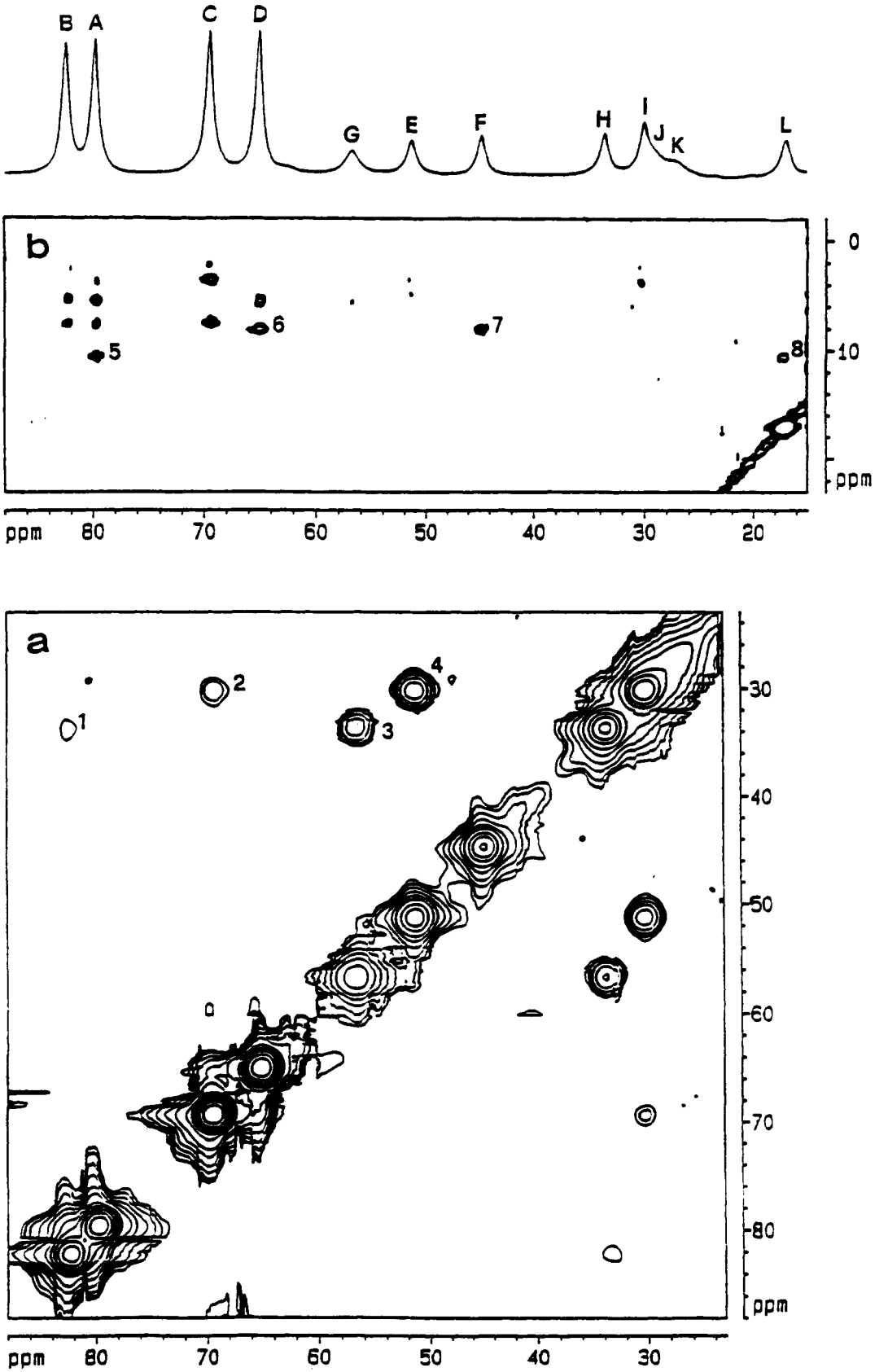

FIGURE 4: 600-MHz 295 K NOESY spectrum obtained at pH 7.2 in D_2O with a mixing time of 7 ms and a relaxation delay of 20 ms. (a) NOESY map in the 88/25 ppm region. (b) NOESY connectivities of the heme methyl signals A–D with signals in the diamagnetic envelope. Conditions: TPPI mode, 256 data points in f_2, 256 data points in f_1, zero-filled to 1K × 1K. A squared sine-bell weighting function was used in both cases. Region b was plotted using a lower threshold. Cross-peak assignments are as follows: (1) 8-CH_3, 7-propionate Hα; (2) 5-CH_3, 6-propionate Hα; (3) 7-propionate Hα, Hα'; (4) 6-propionate Hα, Hα'; (5) 1-CH_3, 2-β CH_3; (6) 3-CH_3, 4-β CH_3; (7) 4-αCH, 4-β CH_3; (8) 2-α CH, 2-β CH_3.

experiment is performed at pH 5.0, in which signals J and K are sufficiently separated, an intense NOESY connectivity is also detected between them (data not shown). Owing to their pH dependence, signals J and K may be assigned to the β-CH_2

protons of the proximal histidine in analogy with other cytochromes *c'* previously studied.

On the other hand, the pairs G,H and E,I are attributable to the α-CH_2 protons of the heme propionate side chains at positions 6 and 7, which display NOESY connectivities with methyl group 5 and 8 (signals B and C). The remaining signals F and L should correspond to the α-CH protons of the thioether bridge, which give intense cross-peaks with signals at 8.0 and 10.8 ppm, respectively (cross-peaks 7 and 8, Figure 4b). The latter signals which lie in the diamagnetic envelope also show dipolar connectivities with signals D and A (cross-peaks 6 and 5, respectively), which correspond to the heme methyl groups in positions 1 and 3. The two resonances at 8.0 and 10.8 ppm are thus attributable to the β-methyl groups in positions 2 and 4. This assignment is consistent with the X-ray structure of *R. molischianum* which shows that these methyl groups are oriented toward the 1- and 3-CH_3 groups, respectively. This is not the case for the α-CH protons of the thioether moiety, explaining the absence of dipolar connectivities between signals F,L and signals B,C. All these results are fully consistent with those previously reported for the *C. vinosum* cytochrome *c'* (Banci et al., 1992).

The heme methyl signals A–D display further cross-peaks with the diamagnetic envelope (see Figure 4b). Despite our attempts, we were not able to recognize any residue connected with the isotropically shifted signals.

Fe(II) Cytochrome c'. The reduced cytochrome *c'* displayed a monodimensional NMR spectrum (Figure 5) similar to those reported previously for *C. vinosum* and *R. rubrum*, i.e., with three methyl resonances (A–C) falling in the upfield region and the fourth one (D) displaying a downfield shift. The chemical shifts of the reduced cytochromes *c'* span from 30 to −15 ppm. Five additional signals of intensity 1 can be observed in the downfield region. The T_1 values measured at 600 MHz for each signal are reported in Table I. For samples in which both the oxidized and reduced cytochromes were present, the resonances for the two species could be observed. From this, it can be inferred that the electron exchange rate is slow on the NMR time scale.

Figure 5 shows the NOESY spectrum at 600 MHz of the reduced cytochrome *c'* at pH 5.0 with a mixing time of 80 ms. This spectrum allows us to identify three pairs of signals in the downfield region displaying strong cross-peaks which we assign to the geminal couples of the two α-CH_2 of the propionates (cross-peaks 2 and 4) and the β-CH_2 of the proximal histidine (cross-peak 3, from a NOESY experiment with a mixing time of 50 ms). Furthermore, two of these protons also show dipolar connectivities with two methyl groups (cross-peaks 1 and 6), and this enables the propionate signals and the neighboring methyl groups 5 and 8 to be identified. These assignments were verified by means of saturation-transfer experiments of the methyl groups in a sample containing both the oxidized and the reduced species (data not shown). Hence, the same lettering has been given to the signals corresponding to the same protons in the reduced and in the oxidized forms. We should note at this point that owing to the instability of the reduced form it was very difficult to perform long experiments and that repeated oxidation and reductions of the sample induced a considerable denaturation of the protein.

The heme methyls exhibit some dipolar connectivities in the diamagnetic region, which can be seen in the NOESY spectra, and these have been verified by means of 1D NOE experiments. We can point out that in the reduced form only signal C (methyl 5 or 8, up to now) displays a cross-peak (numbered 5) in the aromatic region, located at 6.78 ppm. According to the NOESY experiment performed with a mixing time of 80 ms and a recycle time of 250 ms, this signal corresponds to a phenylalanine residue. The inset in the upper left hand of Figure 5 shows the phenylalanine cross-peak pattern in the diamagnetic region which allowed us to identify the residue.

DISCUSSION

The first comment to be made concerns the pH dependence of the NMR spectra of the cytochromes *c'*. This point is of relevance, since it has been suggested that the ground-state wavefunction can be altered by pH changes (Maltempo, 1974, 1976; Maltempo et al., 1974). All the cytochromes *c'* investigated to date clearly show a strong pH dependence at high pH. The shift pattern in the 7–10 pH range is very similar for all of them (Emptage et al., 1981; Jackson et al., 1983; La Mar et al., 1990; Banci et al., 1992). This ionization has been previously attributed to the deprotonation of the Nδ1 of the proximal histidine ligand. The dramatic pH dependence of signals J and K is the best confirmation for this assignment. This is the only major ionization in cytochromes *c'* from *R. molischianum* (Jackson et al., 1983) and *R. palustris* (La Mar et al., 1990). Actually, the pH dependencies of the latter systems were fitted using two pK_as (6.0 and 8.2, and 6.2 and 7.5, respectively), but the low-pH ionization caused a very minor perturbation of the shifts.

On the other hand, the behavior of the *C. vinosum* (Bertini et al., 1990; La Mar et al., 1990; Banci et al., 1992) and the *R. gelatinosus* proteins shows, beyond any doubt, that another major perturbation occurs with a pK_a below 7. In this case, a minimum of two pK_a values is required to obtain an acceptable fit. Nevertheless, only the introduction of a third pK_a brings the calculated curves within the experimental error limits for the shifts over the whole pH range. We interpret this situation as arising from the presence of an ionizable group in the vicinity of the heme in the *C. vinosum* and the *R. gelatinosus* proteins and from its absence in the *R. molischianum* and *R. palustris* cytochromes.

In previous work from this lab (Banci et al., 1992), it was noted that the two most reasonable candidates for this group in the *C. vinosum* cytochrome *c'* were two Glu residues, namely, Glu-10 and Glu-17. In the *R. gelatinosus* protein, the Glu-17 is lacking (Ambler et al., 1979, 1981). Therefore, we assign the low-pH ionizable group in these two proteins as Glu-10. It should be noted that Glu-10 is lacking in the *R. molischianum* and *R. palustris* cytochromes *c'*. According to the X-ray structure of the *R. molischianum* protein (Weber et al., 1981), Glu-10 should be located in the terminus of an α-helix pointing toward the propionate moiety of carbon 7.

Glu-10 is also present in the *R. rubrum* cytochrome *c'*. Unfortunately, the published pH dependence for this system (Emptage et al., 1981) is limited to the four heme signals. Judging from these limited data, the low pK_a is also apparent.

A further comment can be made on the intermediate pK_a in the *C. vinosum* and the *R. gelatinosus* cases; its effects are far more marked in the former than in the latter. Actually, in the present system, the signals are almost pH-independent around the second pK_a values. It seems, therefore, that the presence of a third ionizable group is only demonstrated in the case of the *C. vinosum* cytochrome *c'*. It is tempting to assign this group as Glu-17, which is lacking in all the other cytochromes *c'*. Finally, the group responsible for the minor perturbations in the shifts (intermediate pK_a in the *R. gelatinosus* case and "low pH" in the *R. molischianum* and *R. palustris* cytochromes *c'*) could be the carboxylate group

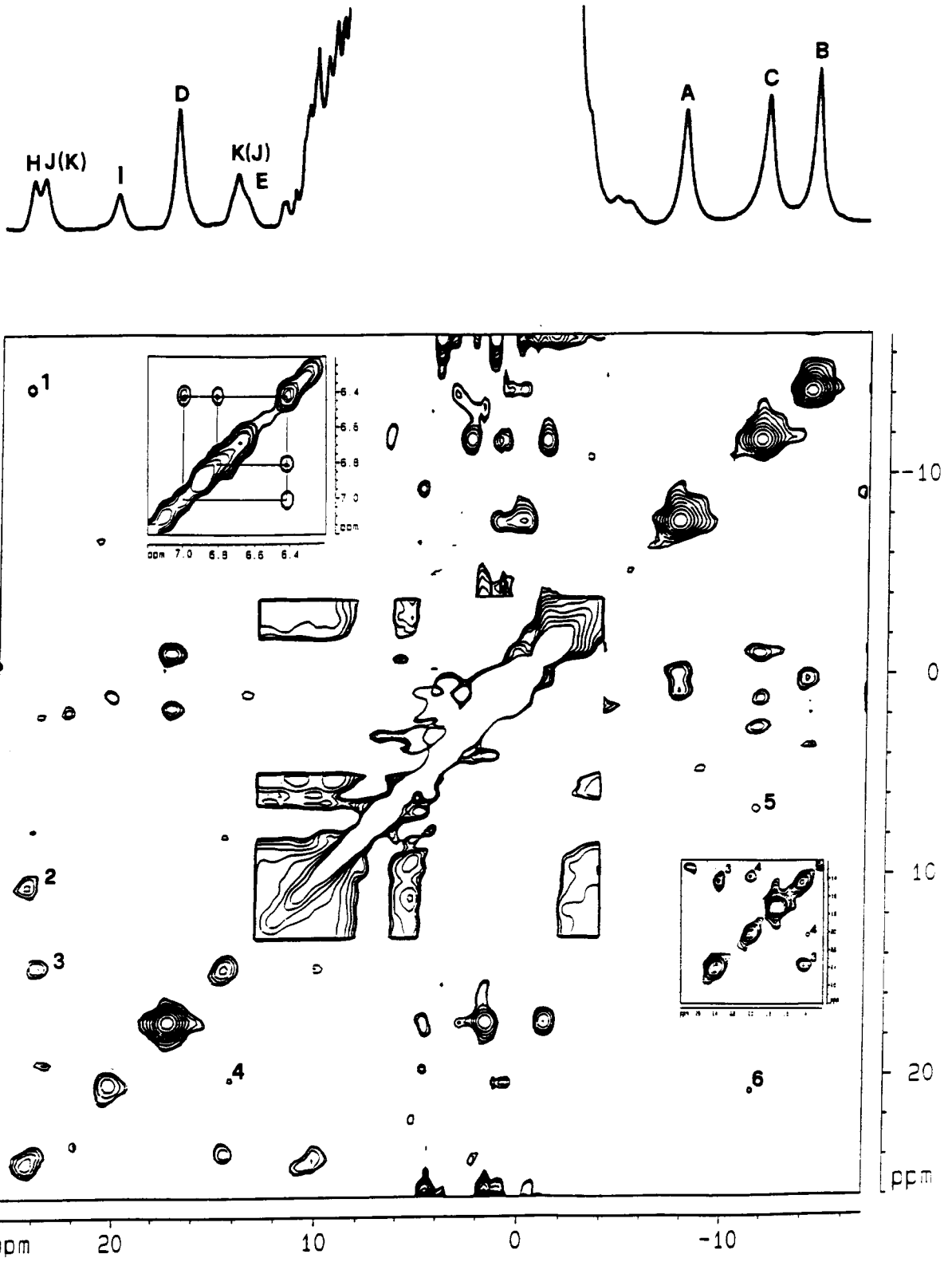

FIGURE 5: 600-MHz 295 K NOESY spectrum of the ferrocytochrome *c'* from *R. gelatinosus* in D_2O at pH 5.0. Cross-peak assignments are as follows: (1) 8-CH_3, 7-propionate Hα; (2) 7-propionate Hα, Hα'; (3) proximal histidine Hβ, Hβ'; (4) 6-propionate Hα, Hα'; (5) 5-CH_3, proton of a Phe residue; (6) 5-CH_3, 6-propionate Hα. Conditions: mixing time t_m = 80 ms, TPPI mode, 128 data points in f_2, 128 data points in f_1, zero-filled to 256 × 256 data points. A squared sine-bell weighting function was used in both cases. The inset to the lower right hand of the 28/12 ppm region corresponds to another experiment acquired with t_m = 50 ms, which allowed a more clear detection of cross-peak 4 between signals I and E. Processing conditions were the same. The inset to the upper left hand shows the aromatic region of the t_m = 80-ms experiment processed with the following conditions: TPPI mode, 1K data points in f_2, 512 data points in f_1, zero-filled to 2K × 1K data points.

of the propionate group at the 7 position, which is always present in cytochromes *c'*. Its effects may not be apparent in the protein from *C. vinosum*, being masked by other major effects.

The observed NOESY connectivities of signal C in the reduced form give a relevant clue to perform the assignment of the heme signals. Owing to the longer T_1 with respect to the oxidized form, we were able to observe an NOESY cross-

peak between C and a phenylalanine residue. This fact is of relevance since it has been previously pointed out that, in spite of the differences in the primary sequences of the different cytochromes *c'*, a region near the heme rich in aromatic residues is preserved (Weber et al., 1981; Weber, 1982; Ambler et al., 1979, 1981). Then, the tertiary structure in this region is very likely to be similar, and we can again refer to the X-ray structure of *R. molischianum* (Weber et al., 1981). In this case, Trp-58, Phe-82, and Phe-125 are in the vicinity of methyl 5. If these residues are not conserved in other cytochromes *c'*, they are always replaced by aromatic amino acid residues. In the *R. gelatinosus* case, both Phe residues remain, and Trp-58 is substituted by a Phe. Hence, it is reasonable to suppose that methyl 5 will be close to a Phe. On the other hand, the 8-methyl group has no aromatic residues nearby. These facts confirm that signal C corresponds to methyl 5.

So, having assigned signal C, the assignment of half of the heme signals should be straightforward by means of the dipolar connectivities found in the paramagnetic NOESY spectra. Considering that methyl C presents a dipolar connectivity with signal I, the couple E,I is assigned to the propionate group at position 6. Hence, signal B should correspond to methyl 8. On the other hand, proton H presents a dipolar connectivity with signal B, and on this basis, the geminal G,H pair is assigned to the 7-propionate.

In this way, we have performed a further step in the assignment of the heme signals. By examining the pH dependence (Figure 3) in light of these assignments, the proposal of Glu-10 as the major low-pH ionizing group is strongly confirmed. The pair of signals G,H (corresponding to propionate 7) experiences a dramatic pH dependence at low pH, while the shifts of the other geminal pair (signals E,I) remain almost unaffected in the pH range 4–7.5. Hence, the pair G,H corresponds to the α-CH_2 propionate of carbon 7, which is the one that should be mostly influenced by the ionizing Glu-10. It may be added that the assignment is also consistent with the pH dependence at low pH values of signal B (8-methyl), which is nearer to the ionizable residue than signal C (5-methyl), whose shift is unaffected in this pH range. It is possible that the ionization causes a small conformational change.

Regarding the rest of the heme resonances (A, D, F, and L), up to now we have only been able to assign them in pairs in the oxidized form. However, a useful clue to perform a tentative individual assignment of the remaining signals can be found in the spectrum of the reduced species.

Fe(II) may be found as a paramagnetic ion in the high-spin form ($S = 2$) or in an intermediate spin state ($S = 1$) (Scheidt & Reed, 1981). NMR studies on model porphyrin–Fe(II) complexes show a substantially different methyl pattern for the $S = 1$ and the $S = 2$ cases (Goff & La Mar, 1977; Goff et al., 1977). In the intermediate spin situation, all the methyl signals are found downfield, and their shifts experience an upfield bias when the substituents in the 2,4 position of the heme are more electron-withdrawing. On the other hand, when the signals split, the methyl resonances at positions 5 and 8 are expected to appear as a pair widely separated from the 1-CH_3, 3-CH_3 pair (Goff et al., 1977).

Meanwhile, a clearly different situation is encountered for the high-spin ferrous complexes ($S = 2$) (Goff & La Mar, 1977). Although the methyl signal spreading also increases with the electron-withdrawing power of the 2,4 substituents, no pairwise distribution is found in this case. While methyl groups 1,5 and 8 display similar shifts, the 3-CH_3 resonance is upfield-biased and even changes sign when acetyl groups are found in the 2,4 position.

For this unusual situation, a tentative explanation has been suggested for the porphyrin complexes. The preferred orientation of the fifth ligand, 2-methyl imidazole in this case, could be the cause of this asymmetric shielding (La Mar & Walker, 1978). In the protein, the π-plane of the proximal histidine should determine the methyl shifts. Moreover, the asymmetry of the shift pattern is expected to be enhanced by the protein environment, giving rise to a clearly distinct shift for the 3-methyl group (La Mar et al., 1978). In our case, the reverse is observed: only one methyl group (signal D) is found downfield while the remaining three show upfield shifts.

The present 2D and saturation-transfer experiments have demonstrated that signal D corresponds to a methyl group either at position 1 or at position 3. According to the experiments on model complexes, it would be tempting to assign signal D as corresponding to the 3-methyl group. If this assignment were correct, it would follow that signal A is 1-CH_3, signal D is 3-CH_3, signal F is 4-αCH, and signal L is 2α-CH.

An interesting point to note is that in the X-ray structure of *R. molischianum* it can be seen that pyrroles I, III, and IV are solvent-exposed, (Weber et al., 1981; Weber, 1982) while pyrrole II (the one bearing the 3-methyl group) is rather hidden. In the case of the low-spin cytochromes *c*, such a situation is reflected in a different shift pattern for the heme methyls, and it could also be correlated to how the electron transfer from the heme takes place (Keller & Wüthrich, 1978).

In conclusion, assignments for all the paramagnetically shifted signals either in the oxidized or in the reduced form of cytochrome *c'* are proposed for the first time. Additionally, the previous hypothesis regarding the ionizable group in the *C. vinosum* cytochrome *c'* has been confirmed, allowing a more precise identification of the residue responsible of the ionization at low pH values. Finally, NOESY 2D spectra of a ferrous high-spin ($S = 2$) protein are reported for the first time, yielding results consistent with the spectra of the oxidized form and verified by saturation transfer between the two species.

ACKNOWLEDGMENT

We thank Dr. Margarita Vicens Oliver for helping with the growth and purification of the protein and Mr. Stefano Rocchi for technical assistance.

REFERENCES

Ambler, R. P., Meyer, T. E., & Kamen, M. D. (1979) *Nature 278*, 661–662.

Ambler, R. P., Bartsch, R. G., Daniel, M., Kamen, M. D., McLellan, L., Meyer, T. E., & van Beeumen, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6854–6857.

Banci, L., Bertini, I., Luchinat, C., Scozzafava, A., Piccioli, M., & Turano, P. (1989) *Inorg. Chem. 28*, 4650–4656.

Banci, L., Bertini, I., & Luchinat, C. (1991a) *Nuclear and Electron Relaxation*, VCH Publishers, New York.

Banci, L., Bertini, I., Luchinat, C., & Piccioli, M. (1991b) in *NMR and Biomolecular Structure* (Bertini, I., Molinari, H., & Niccolai, N., Eds.) pp 31–55, VCH Publishers, Weinheim, FRG.

Banci, L., Bertini, I., Turano, P., Ferrer, J. C., & Mauk, A. G. (1991c) *Inorg. Chem. 30*, 4510–4516.

Banci, L., Bertini, I., Turano, P., Tien, M., & Kirk, T. K. (1991d) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6956–6960.

Banci, L., Bertini, I., Turano, P., & Vicens Oliver, M. (1992) *Eur. J. Biochem. 204*, 107–112.

Bartsch, R. G. (1971) *Methods Enzymol. 23*, 344–363.

Bartsch, R. G. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 249–279, Plenum Press, New York.
Bartsch, R. G. (1991) *Biochim. Biophys. Acta 1058*, 28–30.
Bertini, I., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin Cummings, Boston.
Bertini, I., Briganti, F., Monnanni, R., Scozzafava, A., Carlozzi, P., & Materassi, R. (1990) *Arch. Biochem. Biophys. 282*, 84–90.
Budd, D. L., La Mar, G. N., Langry, K. C., Smith, K. M., & Nayyar-Mazhir, R. J. (1979) *J. Am. Chem. Soc. 101*, 6091–6096.
Cusanovich, M. A. (1971) *Biochim. Biophys. Acta 679*, 238–241.
Cusanovich, M. A., & Gibson, Q. H. (1973) *J. Biol. Chem. 248*, 822–834.
Cusanovich, M. A., Meyer, T. E., & Tollin, G. (1988) *Adv. Inorg. Biochem. 7*, 37–91.
de Ropp, J. S., & La Mar, G. N. (1991) *J. Am. Chem. Soc. 113*, 4348–4350.
Dus, K., Sletten, K., & Kamen, M. D. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 367–370.
Ehrenberg, A., & Kamen, M. D. (1965) *Biochim. Biophys. Acta 102*, 333–340.
Emerson, S. D., & La Mar, G. N. (1990) *Biochemistry 29*, 1545–1556.
Emptage, M. H., Zimmerman, R., Que, L., Jr., Münck, E., Hamilton, W. D., & Orme-Johnson, W. H. (1977) *Biochim. Biophys. Acta 495*, 12–23.
Emptage, M. H., Xavier, A. V., Wood, J. M., Alsaadi, B. M., Moore, G. R., Pitt, R. G., Williams, R. P. J., Ambler, R. P., & Bartsch, R. G. (1981) *Biochemistry 20*, 58–64.
Gibson, Q. H., & Kamen, M. D. (1966) *J. Biol. Chem. 241*, 1969–1973.
Goff, H., & La Mar, G. N. (1977) *J. Am. Chem. Soc. 99*, 6599–6606.
Goff, H., La Mar, G. N., & Reed, C. A. (1977) *J. Am. Chem. Soc. 99*, 3641–3646.
Gordon, S. L., & Wüthrich, K. (1978) *J. Am. Chem. Soc. 100*, 7094–7096.
Hore, P. J. (1983) *J. Magn. Reson. 54*, 539–542.
Inubushi, T., & Becker, E. D. (1983) *J. Magn. Reson. 51*, 128–133.
Jackson, J. T., La Mar, G. N., & Bartsch, R. G. (1983) *J. Biol. Chem. 258*, 1799–1805.
Kassner, R. J. (1991) *Biochim. Biophys. Acta 1058*, 28–30.
Kassner, R. J., Kykta, M. G., & Cusanovich, M. A. (1985) *Biochim. Biophys. Acta 831*, 155–158.
Keller, R. M., & Wüthrich, K. (1978) *Biochim. Biophys. Acta 533*, 195–208.
Kennel, S. J., Meyer, T. E., Kamen, M. D., & Bartsch, R. G. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3432–3435.
Korszun, Z. R., Bunker, G., Khalid, S., Scheidt, W. R., Cusanovich, M. A. & Meyer, T. E. (1989) *Biochemistry 28*, 1513–1517.
La Mar, G. N., & Walker, F. A. (1978) in *The Porphyrins* (Dolphin D., Ed.) Vol. 4, pp 61–157, Academic Press, New York.
La Mar, G. N., Viscio, D. B., Smith, K. N., Caughney, W. S., & Smith, M. S. (1978) *J. Am. Chem. Soc. 100*, 8085–8091.
La Mar, G. N., Jackson, J. T., & Bartsch, R. G. (1981) *J. Am. Chem. Soc. 103*, 4405–4410.
La Mar, G. N., Jackson, J. T., Dugad, L. B., Cusanovich, M. A., & Bartsch, R. G. (1990) *J. Biol. Chem. 265*, 16173–16180.
Lecomte, J. T. J., Unger, S. W., & La Mar, G. N. (1991) *J. Magn. Reson. 94*, 112–120.
Maltempo, M. M. (1974) *J. Chem. Phys. 61*, 2540–2547.
Maltempo, M. M. (1976) *Q. Rev. Biophys. 9*, 181–215.
Maltempo, M. M., Moss, T. H., & Cusanovich, M. A. (1974) *Biochim. Biophys. Acta 342*, 290–305.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 962–974.
McRee, D. E., Redford, S. M., Meyer, T. E., & Cusanovich, M. A. (1990) *J. Biol. Chem. 265*, 5363–5365.
Meyer, T. E., & Kamen, M. D. (1982) *Adv. Protein Chem. 35*, 105–212.
Rajarathnam, K., La Mar, G. N., Chiu, M. L., Sligar, S. G., Singh, J. P., & Smith, K. M. (1991) *J. Am. Chem. Soc. 113*, 7886–7893.
Rawlings, J., Stephens, P. J., Nafie, L. A., & Kamen, M. D. (1977) *Biochemistry 16*, 1725–1729.
Satterlee, J. D., & Erman, J. E. (1991) *Biochemistry 30*, 4398–4405.
Scheidt, W. D., & Reed, C. A. (1981) *Chem. Rev. 81*, 543–572.
Spiro, T. G., Strong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.
Strekas, T. C., & Spiro, T. G. (1974) *Biochim. Biophys. Acta 351*, 237–245.
Taniguchi, S. & Kamen, M. D. (1963) *Biochim. Biophys. Acta 74*, 438–455.
Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831–3832.
Weber, P. C. (1982) *Biochemistry 21*, 5116–5119.
Weber, P. C., Bartsch, R. G., Cusanovich, M. A., Hamlin, R. C., Howard, A., Jordan, S. R., Kamen, M. D., Meyer, T. E., Weatherford, D. W., Xuong, Ng. H., & Salemme, F. R. (1980) *Nature 286*, 302–304.
Weber, P. C., Howard, A., Xuong, Ng. H., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399–424.
Wu. J.-Z., La Mar, G. N., Yu, L. P., Lee, K.-B., Walker, A., Chiu, M. L., & Sligar, S. G. (1991) *Biochemistry 30*, 2156–2165.
Yamamoto, Y., Osawa, A., Inoue, Y., Chujo, R., & Suzuki, T. (1989) *FEBS Lett. 247*, 263–267.