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EVOLUTIONARY CHANGE OF THE HEME C ELECTRONIC STRUCTURE: FERRICYTOCHROME C-551 FROM PSEUDOMONAS AERUGINOSA AND HORSE HEART FERRICYTOCHROME C

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SUMMARY: Individual assignments of the ^{1}H n.m.r. lines of heme c in reduced and oxidized cytochrome c-551 from Pseudomonas aeruginosa were obtained by nuclear Overhauser enhancement and saturation transfer experiments. Comparison with the corresponding data on horse heart cytochrome c showed that the locations of high spin density on the heme c periphery as well as the in-plane principal axes x and y of the electronic g-tensor are rotated by approximately 90° in ferricytochrome c-551 relative to horse ferricytochrome c. High spin density in ferricytochrome c-551 is thus localized on the pyrrole ring III. While this pyrrole ring is well shielded in the interior of mammalian-type cytochromes c, it is more easily accessible in cytochrome c-551. It is suggested that this evolutionary change of the heme c electronic structure would be compatible with the hypothesis that the electron transfer in both species is via solvent exposed peripheral ring carbon atoms.

The elucidation of the pathways through which electrons are transferred in and out of the protein have long been a central theme for investigations of structure-function relationships in cytochromes c (1,2). According to one of the mechanisms which have been proposed, the electron transfer is via the solvent exposed edge of heme c (3). It has also been suggested that the pronounced asymmetry of the electron spin distribution on the peripheral ring carbon atoms of heme c, which was observed in earlier proton nuclear magnetic resonance (n.m.r.) studies of ferricytochromes c, provides support for this hypothesis (4,5). A more detailed description of the electron spin density distribution in horse heart ferricytochrome c was recently obtained from individual assignments of the heme c proton n.m.r. lines (6). This data showed that high spin density is indeed localized on the solvent exposed pyrrole ring II of the heme c group (Fig. 1). Since extensive homologies among different cytochromes of the c-type were demonstrated on the level of the primary structure (7) as well as the spatial folding of the polypeptide chain in single crystals (8) and in solution (9-14), it seems of interest to complement these studies with a detailed comparison of the heme c electronic structures. The present paper describes individual assignments for numerous heme c ^{1}H n.m.r. lines in cytochrome c-551 from Pseudomonas aeruginosa and

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compares the resulting information on the electronic structure of the heme group with the corresponding data in horse cytochrome c.

The techniques used here are very similar to those described previously for horse cytochrome c (6). First, using nuclear Overhauser enhancement (n.o.e.) difference spectra (15), individual assignments were obtained for the heme c ^{1}H n.m.r. lines in ferrocytochrome c-551. The corresponding lines in the oxidized protein were then identified by saturation transfer studies in partially oxidised solutions (5) and n.o.e. experiments with ferricytochrome c-551.

MATERIALS AND METHODS: Most of the experiments described in this paper were done with a sample of cytochrome c-551 provided to us by Dr. P. Debrunner, University of Illinois, Urbana, USA. Some measurements were also done with material obtained from Dr. I. Pecht and M. Goldberg of the Weizmann Institute of Science, Rehovot, Israel. The heme c resonances in the ^{1}H n.m.r. spectra of the proteins from the two sources were essentially identical. For the n.m.r. studies, solutions in D_2O or in 0.05 M deuterated phosphate buffer, pD = 7.0, were prepared. Partial or complete reduction of the protein was obtained by addition of solid disodium dithionite.

High resolution proton n.m.r. spectra were recorded in the Fourier mode on a Bruker HX-360 spectrometer. Chemical shifts are in parts per million (ppm) from internal sodium 3-trimethyl-silyl-[2,2,3,3-$^2H_4$]-propionate. N.o.e. difference spectra were recorded as described previously (15). Similarly, difference spectra were obtained for studies of saturation transfer in solutions of the partially oxidized protein.

RESULTS: Previously, the identification of the singlet resonances of the four meso-protons and the four ring methyl groups of heme c (Fig. 1) in ferrocytochrome c-551 had been described (13). In addition, two A_3X spin systems (16) with chemical shifts corresponding approximately to those expected for the thioether bridges (17) were identified by spin decoupling (Table 1). In this group of resonances, those corresponding to neighboring atoms were now identified by n.o.e. measurements.

Fig. 2A shows the ^{1}H n.m.r. spectrum of ferrocytochrome c-551. The n.o.e. difference spectra used for the resonance assignments in the reduced protein (Fig. 2, B-F) were obtained by subtracting spectra with n.o.e. from reference spectra (15). For the spectra with n.o.e., a 2s low power saturating pulse was applied at the chemical shifts indicated by the arrows, followed immediately by a 90° observation pulse. Fig. 2B shows that two resonances with chemical shifts corresponding to heme c ring methyl lines (13) appeared in the n.o.e. difference spectrum obtained with irradiation of the highest field meso-proton line. Inspection of the heme c structure (Fig. 1) then shows that the three lines in Fig. 2B must come from the ring methyls 1 and 8, and the

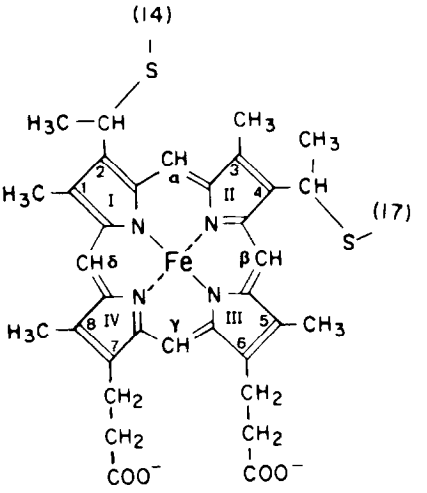

Fig. 1 Structure of heme c. The four pyrrole rings are numbered I to IV, the peripheral β-positions 1 to 8, and the four meso-positions α to δ.

meso-proton δ. Similarly, an unambiguous assignment of meso-proton γ (Table 1) was obtained from the observation that none of the ring methyls appeared in the n.o.e. difference spectrum. The traces C and D of Fig. 2 show that upon irradiation of either one of the remaining two meso-proton resonances, one heme ring methyl resonance and one of the thioether bridge A_3X spin systems appears, as one would expect for the meso-protons α and β (Fig. 1). The α and β meso-proton resonances were then distinguished with the experiments E and F in Fig. 2. In trace E the preirradiation was on the resonance position of one of the thioether methyl groups, which caused one of the heme ring methyl resonances 3 or 5 to appear in the n.o.e. difference spectrum. Trace F shows that irradiation of the other thioether methyl resonance caused the appearence of one of the two ring methyl lines attributed in trace B to positions 1 and 8. The individual resonance assignments in Fig. 2 and Table 1 are then the only ones compatible with all the data in Fig. 2.

Two general comments on the analysis of the experiments in Fig. 2 are called for. One is that the experimental conditions used yield "steady state" n.o.e. spectra (18). As a consequence mainly of spin diffusion (18,19), some of these spectra contain numerous lines of peptide protons in addition to the heme c resonances. Prior knowledge of the chemical shifts of the heme c lines (13) was therefore needed for the analysis of the data. That the present interpretation of the steady state n.o.e.'s of heme c protons is a valid approach

TABLE 1

Assignments of the heme c ^{1}H n.m.r. lines in cytochrome c-551 and hyperfine shifts of corresponding resonances in ferricytochrome c-551 and ferricytochrome c from horse heart.

Resonance Assignment	Cytochrome c-551		Horse heart cytochrome c
	Chemical shift in the reduced protein (ppm), T = 27°C	Hyperfine shift [a] in the oxidized protein (ppm), T = 27°C	Hyperfine shift [a] in the oxidized protein (ppm), T = 35°C [c]
Mesoproton α	9.87	- 1.2 [b]	
Mesoproton β	9.36	-10.1 [b]	
Mesoproton γ	9.42	- 2.7 [b]	
Mesoproton δ	9.24	-11.0 [b]	
Ringmethyl 1	3.69	21.1	3.9
Ringmethyl 3	3.76	9.6	27.3
Ringmethyl 5	3.32	28.8	6.9
Ringmethyl 8	3.42	14.4	31.7
Thioether Bridge 2: methine	5.97		
methyl	1.87	0.8	- 3.6
Thioether Bridge 4: methine	6.18		
methyl	2.44	- 2.5	0.5

a The hyperfine shift is the chemical shift difference between corresponding lines in the paramagnetic oxidized and the diamagnetic reduced protein (16). Positive numbers indicate shifts to lower field.

b from ref. (13), T = 42°C

c from ref. (6)

was confirmed with transient n.o.e. experiments with horse ferrocytochrome c (20). Secondly, it was previously suggested that the A_3X spin systems of the thioether bridges (Table 1) correspond to protons of amino acid residues located near the heme group (21). From the large steady state n.o.e.'s of approximately -60 % for the proton X (Fig. 2, C and D) and in the case of horse ferrocytochrome c also from the time course of the spin polarization in the transient n.o.e. studies (20), the assignment to the thioether groups seems unambiguous. Furthermore, the n.o.e. data imply that the methine protons of the thioether bridges are directed towards the nearest-by meso-proton (20).

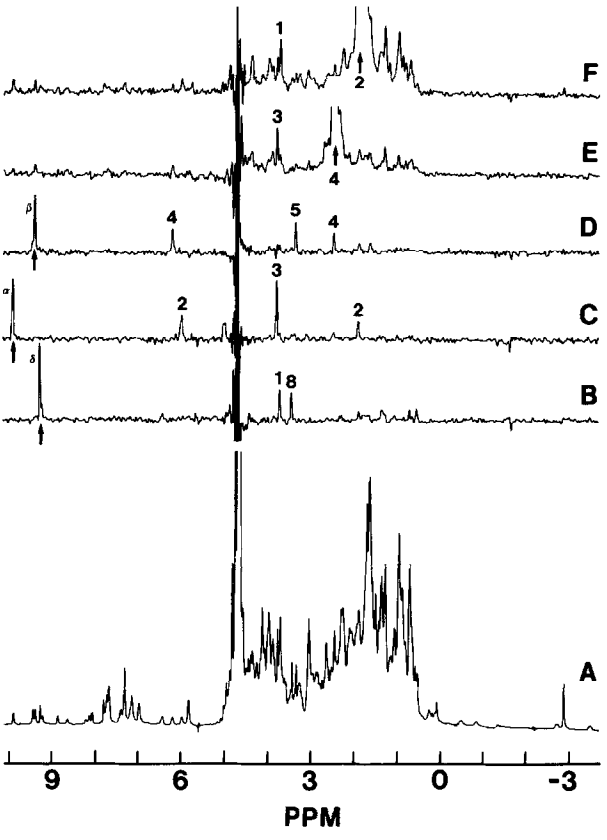

Fig. 2 A. ^{1}H n.m.r. spectrum at 360 MHz of a 0.002 M solution of ferrocytochrome c-551 in 0.05 M deuterated phosphate buffer, pD 6.8, T = 35° C. B to F. N.o.e. difference spectra obtained upon irradiation at the positions indicated by the arrows. The individual assignments of the heme c resonances obtained from these experiments (see text) are also given, where the four heme ring methyls and the two thioether bridges are indicated by the number of the β-carbon atoms to which they are attached (Fig. 1).

For the heme c ring methyl resonances individual assignments in the oxidized protein were obtained from the saturation transfer experiments in Fig. 3. Since the electron exchange in partially oxidized solutions of cytochrome c-551 is considerably faster than for mammalian type cytochromes c (13), the condition of rapid exchange (16) prevails for the other heme c resonances in Table 1, which experience much smaller hyperfine shifts than the ring methyls. Instead of the saturation transfer technique, the dependence of the chemical shifts on the concentration of oxidized protein was therefore used to obtain approximate resonance positions in ferricytochrome c-551. Corresponding meso-proton chemical shifts in ferro- and ferricytochrome c-551 (Table 1)

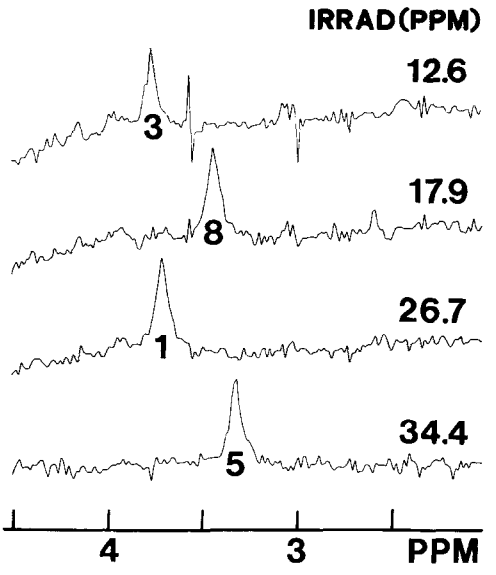

Fig. 3 Saturation transfer experiments in a mixed solution of 98 % reduced and 2 % oxidised cytochrome c-551, T = 4° C, total protein concentration 0.002 M. The traces correspond to the differences between spectra obtained with and without double resonance irradiation at the positions indicated, which correspond to the chemical shifts of the heme ring methyl groups (Fig. 1) in ferricytochrome c-551.

were thus determined previously (13), and for the thioether methyls the hyperfine shifts given in Table 1 were obtained by repeating the experiments of Fig. 2, C and D, in slightly oxidized cytochrome c-551 solutions. These assignments of the thioether methyl lines in ferricytochrome c-551 were then confirmed by the n.o.e. experiments in Fig. 4.

DISCUSSION: The individual assignments of the heme c ^{1}H n.m.r. lines in cytochrome c-551 and horse heart cytochrome c (6), which appears to be representative for mammalian type cytochromes c (8-11), show that the electronic heme structure in the oxidized form of the two proteins is markedly different. It was previously noted that the anisotropy of the spin density distribution on the peripheral heme ring carbon atoms is less pronounced in ferricytochrome c-551 than in horse ferricytochrome c (13). The hyperfine shifts for the heme ring methyls, which arise primarily from contact interactions with the unpaired electron (16), now show that while high spin density is localized on the pyrrole rings II and IV in horse ferricytochrome c, spin delocalization in ferricytochrome c-551 is primarily on the pyrrole rings III and I, in this order (Table 1).

In contrast to the heme ring methyls, the resonance positions of the two thio-

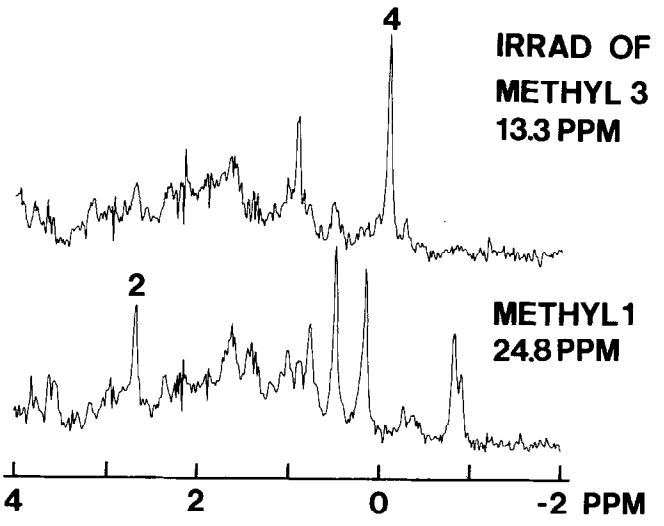

Fig. 4 N.o.e. difference spectra in a 0.01 M solution of ferricytochrome c-551 in D_2O, pD = 7.0, T = 27° C, obtained upon presaturation of the heme ring methyl resonances 1 and 3 in the low field part of the spectrum. The traces contain lines with the chemical shifts of the neighboring thioether methyls 2 and 4, respectively (Table 1). The appearence of numerous additional lines in these "steady state" n.o.e. spectra is mainly a consequence of spin diffusion (17,18).

ether methyls are only little affected by contact coupling. These resonances thus provide a basis for the determination of the axes of the electronic g-tensor. The data in Table 1 then indicate that the g-tensor is rotated by approximately 90° about an axis perpendicular to the heme plane, so that the in-plane x- and y-axes are essentially interchanged when going from horse ferricytochrome c to ferricytochrome c-551. The simultaneous changes in the spin density distribution and the directions of the g-tensor axes are in agreement with theoretical considerations of the electronic state of the heme in low spin ferric hemoproteins (22,23).

With regard to the electron transfer mechanism it is interesting that the presently available low resolution X-ray structure of cytochrome c-551 (8) indicates that as a consequence of the deletion of the peptide fragment 39 to 56, the pyrrole ring III of heme c should be accessible to the solvent on the surface of the protein. Accordingly, the previous arguments relating the electronic heme structure with electron transfer through the heme edge (3-6) appear to be applicable for both proteins, in spite of the markedly different spin density distributions in the heme groups of horse cytochrome c and cytochrome c-551.

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