INVESTIGATION OF PHOTOSYNTHETIC CYTOCHROMES C BY HIGH RESOLUTION NMR SPECTROSCOPY*

by

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The high-field NMR spectra of the reduced c-type cytochromes of four photosynthetic organisms are reported. All exhibit a resonance at -3.0 to -3.4 ppm which closely corresponds to the familiar methionine S-methyl peak of mammalian cytochromes c. Both bacterial cytochromes c_2, however, show an additional resonance in the range of -1.9 to -2.4 ppm which is tentatively assigned to the alkyl side chains of a particular leucyl and valyl residue, respectively. The most probable positions of these residues within the linear sequences of these cytochromes are discussed.

C-type cytochromes of photosynthetic bacteria are distinguished from the cytochromes c of aerobic tissues in that they do not react appreciably with mammalian cytochrome oxidase. Moreover, they have a significantly more positive redox potential and usually also contain a longer polypeptide chain. Nevertheless, the primary structures of cytochromes c_2 of Rhodospirillum rubrum (Dus et al., 1968) and Rhodopseudomonas capsulatus (Turner et al., 1971) reveal extensive sequence homology to eukaryotic cytochromes c. Further support for this homology is derived from a comparison of low resolution X-ray diffraction data on cytochrome c_2 of R. rubrum (Kraut et al., 1968; Singh, 1968) and on oxidized horse heart muscle cytochrome c (Dickerson et al., 1967; Dickerson et al., 1968). In this context it is of great interest to probe into the conformational details of the heme environment in cytochrome c in order to correlate differences of redox potentials and biological activities with small variations in the steric arrangement of otherwise

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homologous portions of the polypeptide chains surrounding their heme groups.

From recent work of several groups (McDonald and Phillips, 1967; Wüthrich, 1968; Wüthrich et al., 1968; McDonald and Phillips, 1969), it has become increasingly apparent that application of 220 MHz proton magnetic resonance spectroscopy offers a unique approach to the study of these subtle structural features in the vicinity of the heme group in heme proteins. For instance, McDonald and Phillips (1967) have shown that methionine is indeed coordinated to the heme iron of cytochrome c in position 6 as was previously inferred on the basis of chemical experiments (Harbury et al., 1965; Ando et al., 1965; Tsai and Williams, 1965) and preliminary results from X-ray crystallography (Dickerson et al., 1967). Based on estimations of the ring current effect of the porphyrin π electron system of the reduced protein, the protons of the methionine S-methyl group were shifted to -2.0 to -4.0 ppm above TMS.

We wish to report in this communication our preliminary observations on the high-field NMR spectra of several photosynthetic cytochromes and to compare them to the corresponding spectra of horse heart cytochrome c.

The photosynthetic cytochromes were purified as previously reported (Dus et al., 1968; Turner et al., 1970; Dus et al., 1970; Yamanaka et al., 1967). Horse heart cytochrome c, type III, was purchased from Sigma Chemical Company. All cytochromes were reduced by adding a small amount of solid ascorbic acid to the protein solution in the NMR tube. The spectra were determined in D_2O of pD approximately 7, using protein concentrations in the range of 30-50 mg per ml.

Chemical shifts were measured relative to tetramethylsilane with the HDO peak at 4.75 ppm as the internal standard. The spectra were recorded on a Varian HA-220 NMR spectrometer, and peak amplification was obtained with a Varian time averaging computer C-1024.

Fig. 1 shows the high-field NMR spectra of three ferrocytochromes c. Two cytochromes c_2, obtained from the photosynthetic organisms R. rubrum and Rps. capsulatus, respectively, are compared to cytochrome c of horse heart muscle. Both bacterial cytochromes exhibit a proton resonance at -3.1 ppm above TMS which corresponds very closely to the resonance at -3.4 ppm of mammalian cytochromes c. Based on estimates of the ring current effect of the porphyrin ring McDonald and Phillips (1967) assigned this peak to the protons

of the S-methyl group of methionine in position 80 which functions as the 6th ligand to the heme iron in cytochrome c. High-field shifts of the protons on the β and γ-carbons of this methionine are believed to give rise to the three smaller peaks at -2.0, -2.5, and -3.9 ppm. Corresponding peaks can be seen in the spectra of Rps. capsulatus cytochrome c_2, and also, but less clearly because of an unfavorable signal to noise ratio, in the spectra of R. rubrum cytochrome c_2. Moreover, the singlets observed at -0.9 ppm for both cytochromes c_2 roughly match with spectral peaks of mammalian cytochromes. It seems reasonable, therefore, to assume that the sulfur of a methionine resi-

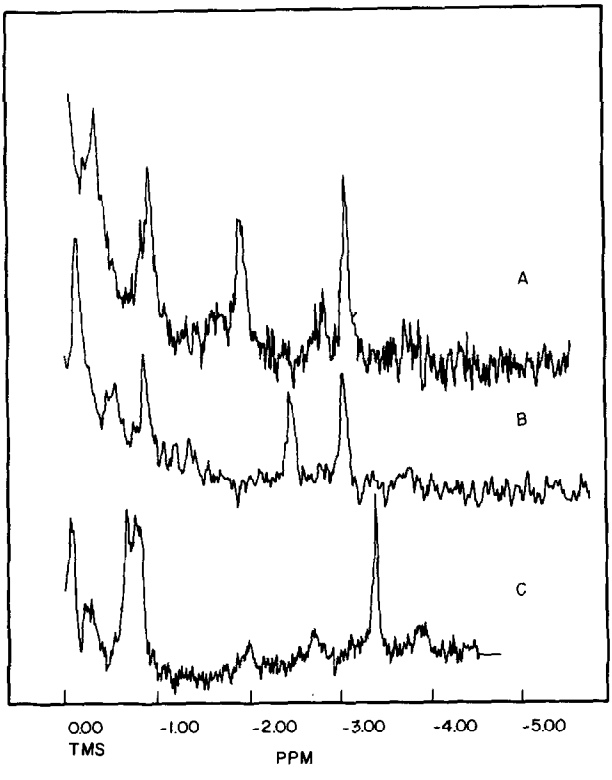

Fig. 1 High-Field NMR Spectra of Reduced Cytochromes.

A. Rhodopseudomonas capsulatus cytochrome c_2,
B. Rhodospirillum rubrum cytochrome c_2,
C. Horse Heart Cytochrome c.

All spectra were recorded in D_2O at pD ~ 7 on a Varian 220 MHz spectrometer with HDO as internal standard. Peak amplification was obtained with a Varian time averaging computer C-1024.

due, corresponding to position 80 on the basis of sequence homology (Dus et al., 1968), is indeed bound at the 6th ligand position to the heme iron in both ferrocytochromes c_2. In contrast, the observed similarity of spectra between 0 and -1.00 ppm (Fig. 1A, B, and C) might well be fortuitous because resonances in this area could arise from ring current of either heme or aromatic side chains. A decision between these alternatives could be attempted with the help of the X-ray diffraction data obtained by Dickerson et al. (1970 a) at 2.8 Å resolution. On first sight, it appears, however, that no alkyl side chain of ferricytochrome c would be close enough to the heme group to be sufficiently influenced by its ring current.

The most obvious difference between the spectra of these bacterial cytochromes and mammalian cytochromes c, however, is the proton resonance at -2.4 ppm for R. rubrum and at -1.9 ppm for Rps. capsulatus. While resonances in this range are likely to arise from alkyl side chains of leucyl, isoleucyl, or valyl residues which are close to the porphyrin ring but not chelating to the heme iron, it would seem very difficult to make a specific assignment of the observed resonances to a particular residue within the two sequences since no high-solution X-ray analysis is currently available for cytochrome c_2. On the other hand, several pieces of indirect evidence have become available recently which support tentative assignment of a specific residue in each sequence.

The X-ray structure of ferricytochromes c of horse and bonita heart muscle at 2.8 Å resolution (Dickerson et al., 1970 a) as well as chemical modifications of side chains of cytochromes c (summarized by Dickerson et al., 1970 b) suggest that the "left side" of this protein molecule, comprising approximately residues 48-94, may be somewhat flexible.

This flexibility may be due, in part at least, to a "left channel" which can be seen in the X-ray model of cytochrome c (Dickerson et al., 1970 a). The photosynthetic cytochromes c_2 of R. rubrum and Rps. capsulatus, on the other hand, while extensively homologous to the eukaryotic cytochromes c, each con-

TABLE I

	72					77	77A	B	C	D	E	F	G	H	78		80	
Horse Heart Cytochrome c	. . . Lys	Lys	Tyr	Ile	Pro	Gly-	D -	D -	D -	D -	D -	D -	D -	D -	-Thr	Lys	Met	. . .
R. rubrum Cytochrome c_2	. . . Lys	Ala	Phe	Val	Leu	Glu	Lys	Ser	Gly	Asp	Pro	Lys	Ala	Lys	Ser	Lys	Met	. . .
Rps. capsulatus Cytochrome c_2	. . . Lys	Glu	Phe-	D -	D -	-Asp	Lys	Val	Gly	Asp	Pro	Lys	Ala	Lys	Ser	Gly	Met	. . .

Table I Sequence Comparison.

The comparison is limited to the segment of the sequence which contains the "additional" eight amino acid residues in bacterial cytochromes c_2. D indicates sequence gap.

tain additional sequences of eight residues which are located between positions 77 and 78 of the eukaryotic cytochromes (Table 1), perhaps in order to fill this left channel, or to curtail or block the access of O_2 or CN^- to the heme iron. The photosynthetic cytochromes $\underline{c}_2$ indeed exhibit a substantially higher midpoint potential (300-330 mV) than do the cytochromes $\underline{c}$ of aerobic tissues (230-250 mV), and they do not react with KCN. This structural difference constitutes the most pronounced deviation of the cytochrome $\underline{c}_2$ sequences from the sequences of cytochrome $\underline{c}$, and it is all the more remarkable as it occurs in a region which is absolutely constant for all eukaryotic cytochromes $\underline{c}$. As a consequence of replacing Pro in cytochromes $\underline{c}$ at position 76 by Leu in cytochrome $\underline{c}_2$ of R. rubrum, the polypeptide chain is thought to come up closer to the heme group. Thus, if the side chain of this leucyl residue could reach close enough to the porphyrin ring for its methyl protons to be exposed to the ring current of the porphyrin, one should expect to see a high-field shift of these protons by NMR spectroscopy similar to those of the methyl protons of methionine at position 80. The same argument would apply to the valyl side chain (at position 77B) in cytochrome $\underline{c}_2$ of Rps. capsulatus. This side chain is one CH_2-group shorter but the position of the residue relative to the porphyrin ring may be somewhat different. In fact, the resonance believed to result from shielding of the valyl side chain is not shifted as much upfield as the protein resonance corresponding to the leucyl side chain in R. rubrum cytochrome $\underline{c}_2$. Thus, despite similar interactions the actual positions of the shifted resonances reflect both distance and attitude of the alkyl group relative to the heme. This comparison, however, does not take into account the possibility of a drastic change in conformation due to changes in the redox state of the heme iron.

A small cytochrome $\underline{c}$ isolated from the obligate photoanaerobe R. molischianum (Dus et al., 1970) shows a high-field peak at -3.2 ppm (Fig. 2) which is strikingly similar to the proton resonance of the S-methyl group of methionine 80 in horse heart cytochrome $\underline{c}$ at -3.4 ppm. From this, we assume that despite its smaller size this cytochrome $\underline{c}$ nevertheless does contain a

methionine residue in a position suitable for coordination of its sulfur to the heme iron analogous to mammalian cytochrome c. The case of R. molischianum cytochrome c is reminiscent of the small cytochrome c of Pseudomonas fluorescens for which coordination of methionine sulfur to the heme iron was indicated from chemical experiments (Harbury et al., 1965). A close similarity of these two small cytochromes was implied by studies of sequence homology (Dus, 1970) which suggested that relative to mammalian cytochromes c similar portions of the sequence are missing in these two bacterial cytochromes. Except for an unfavorable signal to noise ratio which may not permit us to see the single protons on

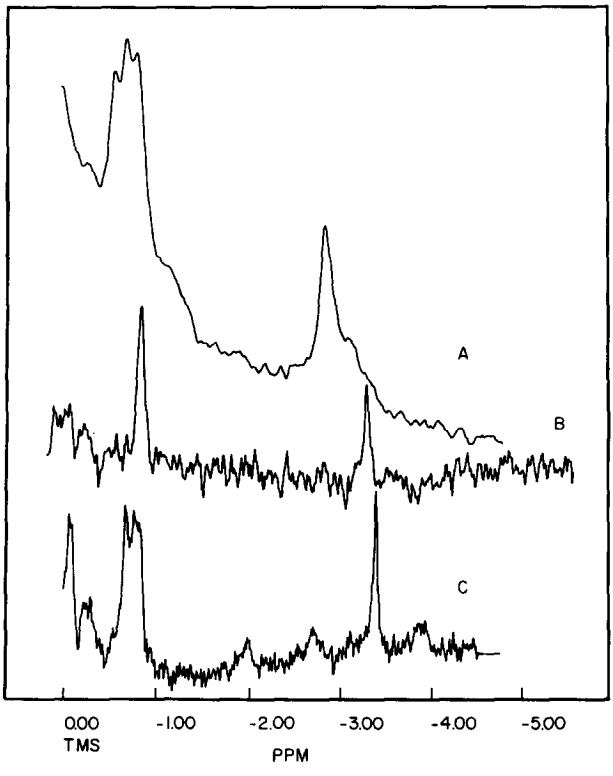

Fig. 2 High-Field NMR Spectra of Reduced Cytochromes.

A. Navicula pelliculosa cytochrome c,
B. Rhodospirillum molischianum cytochrome c (small),
C. Horse Heart Cytochrome c.

All spectra were recorded in D_2O at pD $\sim$ 7 on a Varian 220 MHz spectrometer with HDO as internal standard. Peak amplification was obtained with a Varian time averaging computer C-1024.

the β and γ-carbons of the methionine, the high-field NMR spectra of the two proteins appear to be very similar.

We have included in Fig. 2 the high-field NMR spectra of a ferrocytochrome c of the diatom Navicula pelliculosa because it adds further support for a similar tertiary structure of c-type cytochromes from photosynthetic organisms. In this case, a resonance corresponding in position to the S-methyl protons of methionine appears as rather broad peak centered at -2.8 ppm with a shoulder at about -3.1 ppm.

We expect that the significance of the reported observations will be more apparent once a high-solution X-ray structure of cytochrome c_2 becomes available for direct comparison to the three dimensional structure of horse heart cytochrome c. It is, therefore, our hope that the pursuit of these investigations by NMR spectroscopy, using both diamagnetic and paramagnetic spectra, will implement this information and improve our understanding of the structural details of the heme environment in cytochrome c.

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