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STRUCTURAL STUDIES OF MODIFIED CYTOCHROMES *c* BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY*

K. WÜTHRICH[a], I. AVIRAM[b] AND A. SCHEJTER[b]

[a]*Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, Zürich (Switzerland) and* [b]*Department of Biochemistry, Tel-Aviv University, Tel-Aviv (Israel)*

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SUMMARY

The binding of methionine to one of the axial positions of the heme iron in various modified mammalian-type cytochromes *c* has been studied by nuclear magnetic resonance spectroscopy. The modifications of the protein included formylation of tryptophan, alkylation of methionine, and polymerization by treatment with ethanol. Comparison of the data on methionine coordination with data on the ability of the modified cytochromes *c* to restore the respiration of cytochrome *c*-depleted rat liver mitochondria, indicates that the redox carrier properties of cytochrome *c* are maintained only if methionine is bound to the heme iron in the oxidized and the reduced form.

INTRODUCTION

Cytochrome *c*, a hemoprotein which functions as an oxidation–reduction carrier in the respiratory chain, is presently among the most widely investigated protein molecules. The amino acid sequences are known for the cytochromes *c* of over 30 species[1], and the properties of a number of chemically modified cytochromes *c* have been reported[2–10]. More recently, the molecular structure in single crystals of horse ferricytochrome *c* was determined by X-ray methods[11]. Yet relatively little is known about specific correlations between molecular structure and physical, chemical, or physiological properties. On the one hand, it has been observed that the electron-transferring properties are essentially identical in all mammalian-type cytochromes *c* even though there are quite extensive differences in primary structure[12,13]; similarly there are chemical modifications of cytochrome *c* which appear not to influence the redox carrier properties[2]. On the other hand, a number of rather limited structural modifications are known which seem to destroy this physiological property of cytochrome *c*[2–10]. This paper presents an attempt to correlate structural properties observed by nuclear magnetic resonance spectroscopy with the electron-transferring properties of several modified cytochromes *c*.

High resolution nuclear magnetic resonance spectroscopy has been applied

Abbreviation: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

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to structural studies of various hemoproteins[14]. Because of its low molecular weight cytochrome *c* is particularly suited for the application of this technique. In recent experiments it was possible to show that the axial ligands of the heme iron in reduced and oxidized cytochrome *c* are histidine and methionine[15] and the typical proton resonances of the iron-bound methionine were identified[15,16]. In the experiments described in this paper the NMR technique was employed to study the axial ligands of the heme iron in various biologically active and inactive modified cytochromes *c*. The modifications include carboxymethylation of the methionyl residues[7], formylation of tryptophan-59[10], and polymerization by treatment with 60% ethanol[17].

EXPERIMENTAL PROCEDURE

Horse heart cytochrome *c*, Type II (Sigma Chemical Co.) was purified according to MARGOLIASH AND WALASEK[18]. The modified cytochromes *c* were prepared as described in the appropriate references[7,10,17].

For the NMR studies the lyophilized ferricytochromes *c* were dissolved in 0.1 M deuterated phosphate buffer, p^{2}H 7.0. The concentration was between $4 \cdot 10^{-3}$ and $15 \cdot 10^{-3}$ M, depending on the amount of protein available. The cyanide complexes of the ferricytochromes *c* were obtained by addition of KCN to the solutions. Ferrocytochromes *c* were obtained by reduction with dithionite or ascorbic acid.

High resolution proton NMR spectra were recorded on a Varian HR-220 spectrometer equipped with a standard Varian variable temperature control unit. The signal:noise ratio was in some experiments improved by data accumulation in a Varian 1024 computer of average transients. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference. Chemical shifts are expressed in parts per million (ppm) from DSS, where shifts to low field at constant radio frequency are assigned negative values.

Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E-3 spectrometer equipped with a standard Varian temperature control unit. The samples contained $5 \cdot 10^{-4}$ M cytochrome *c* in deuterated phosphate buffer, p^{2}H 7.0.

RESULTS

The proton NMR spectrum of the diamagnetic reduced cytochrome *c* (Fe^{2+}, $S = 0$) contains in the spectral region from DSS to -10 ppm the strongly overlapping resonances of about 650 protons of the polypeptide chain. In addition a number of well-resolved resonances with intensities corresponding to one, two or three protons are in rather unusual positions between DSS and +4 ppm[15,16]. The resonances at 3.7, 3.3, 2.7, and 1.9 ppm (Fig. 1) correspond to protons of the iron-bound methionyl residue in position 80 of the polypeptide chain[11,15], which are shifted upfield by the local ring current field of the heme group[14]. In particular the resonance at 3.3 ppm corresponds to the methyl group of methionine. It was also observed that displacement of methionine from the coordination site on the heme iron, *e.g.* by cyanide ion, causes all the four resonances between 1.9 and 3.7 ppm to be shifted to positions downfield from DSS[15].

In Fig. 1 the spectral region from DSS to +6 ppm is shown for native horse ferrocytochrome *c*, ferrocytochrome *c* carboxymethylated at methionine-65, dicarboxymethylated at the methionyl residues 65 and 80, and formylated at tryptophan-59. In addition the spectrum of reduced cytochrome *c* polymers is also shown. With the exception of the dicarboxymethylated ferrocytochrome *c* all these compounds contain the typical high field resonances of the iron-bound methionine. As one would expect from the increase of the particle size upon polymerization the resonances in cytochrome *c* polymers appear to be somewhat broader than in the other compounds. In the dicarboxymethylated cytochrome *c* there are two lines of equal intensity at +2.5 and +4.0 ppm. These resonances might correspond to an amino acid residue located near the heme in place of methionine-80.

The NMR spectrum of the paramagnetic ferricytochrome *c* (Fe^{3+}, $S = 1/2$) contains in addition to the polypeptide resonances between DSS and −10 ppm a number of well resolved lines in the spectral regions from −35 to −10, and from 0 to +25 ppm[15]. These unusual resonance positions arise mostly from hyperfine interactions between the protons of heme *c* and the axial ligands of the iron and the unpaired electron of the heme group. Particularly prominent are two resonances at −35.0 and −32.3 ppm at 27°, which correspond to two of the ring methyl groups of heme *c*, and a resonance at +24.2 ppm, which corresponds to the methyl group of

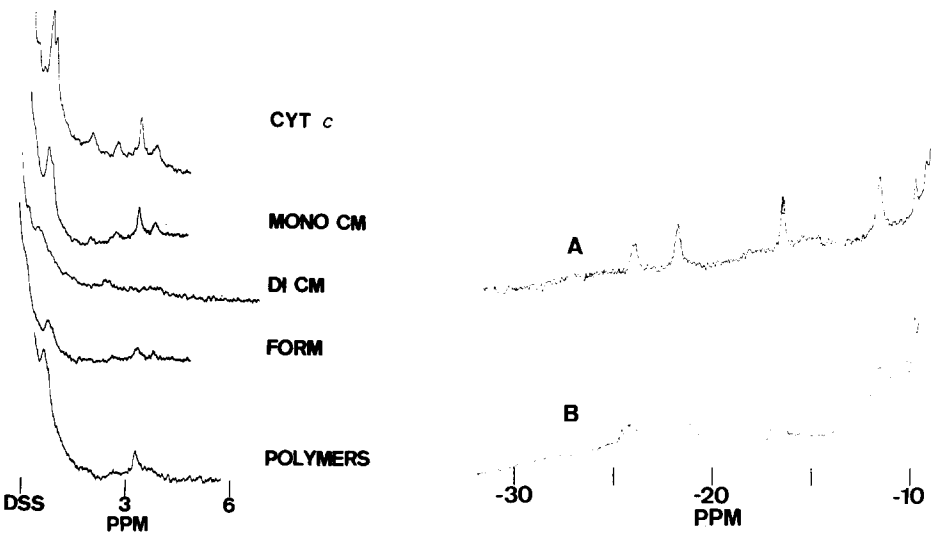

Fig. 1. Proton NMR spectra at 220 MHz in the spectral region from the internal reference DSS to +6 ppm of native horse ferrocytochrome *c* (CYT *c*), ferrocytochrome *c* carboxymethylated at methionyl-65 (MONO CM), ferrocytochrome *c* dicarboxymethylated at the methionyl residues 65 and 80 (DI CM), ferrocytochrome *c* formylated at tryptophanyl-59 (FORM), and reduced cytochrome *c* polymers. Solutions in 0.1 M deuterated phosphate buffer, p^2H 7.0, were studied at 27°. The protein concentration varied from about $4 \cdot 10^{-3}$ (FORM) to about $15 \cdot 10^{-3}$ (CYT *c*).

Fig. 2. Proton NMR spectra at 220 MHz in the spectral region from −10 to −25 ppm of cyanoferricytochrome *c* dicarboxymethylated at the methionyl residues in positions 65 and 80. The compounds were studied in 0.1 M deuterated phosphate buffer, p^2H 7.0, at 27°. The signal:noise ratio was improved by data accumulation in a computer of average transients for several hours. The carboxymethylated protein was obtained by reacting ferricytochrome *c* with 0.18 M bromoacetic acid at pH 7.0 in the presence of 0.1 M KCN. The reaction was terminated after: A, 10 h at 25°; B, 24 h at 25°.

the iron-bound methionine[15]. The positions of these resonances are intimately related to the environment of the heme iron. For example, they vary greatly when the axial methionine is replaced by cyanide ion in cyanoferricytochrome *c*[15]. When the oxidized forms of the modified cytochromes *c* of Fig. 1 were studied, only the monocarboxymethylated ferricytochrome *c* displayed the typical resonances of the native protein. In formylated ferricytochrome *c* several apparently hyperfine-shifted lines were observed between —9 and —11 ppm. Our experiments so far do not allow unequivocal assignments of hyperfine-shifted resonances in oxidized cytochrome *c* polymers and in dicarboxymethylated ferricytochrome *c*.

The NMR spectrum of cyanoferricytochrome *c* contains at 27° four hyperfine-shifted resonances at −23.8, −21.7, −16.5, and −11.4 ppm[15]. When KCN is added to the solutions of the modified cytochromes *c* very similar spectra are observed. Fig. 2 shows the spectrum of dicarboxymethylated cyanoferricytochrome *c*. It is seen that Spectrum A corresponds exactly to the resonances observed in the cyanide complex of native ferricytochrome *c*. Depending on the specific conditions for the carboxymethylation more than one set of hyperfine-shifted resonances may be present (Fig. 2B). The most likely explanation for Spectrum B appears to be that there are two magnetically non-equivalent species, one being identical to the derivative observed in Spectrum A. This is further supported by the observation that the relative intensities of the two sets of resonances are different in different preparations. Investigation of the ferricyanide forms of monocarboxymethylated and formylated cytochrome *c*, and of cytochrome *c* polymers, yielded similar results.

The absorption spectra of the ferric cytochrome *c* derivatives at pH 7.0 indicate that all of them are in the low-spin (S + 1/2) state. In order to ascertain this fact the electron paramagnetic resonance (EPR) spectra of these compounds were recorded in the spectral region from $g = 1.5$ to $g = 6.5$. The typical resonance signal of high spin ferric heme compounds at g values around 6.0[19] could be detected in all the cytochrome *c* derivatives. From comparison with ferrimyoglobin (S = 5/2) and cyanoferrimyoglobin (S = 1/2) it was found that at −140° all the modified cytochromes *c* contained less than 1% high spin heme iron.

DISCUSSION

The above NMR observations indicate that methionine coordination to the heme iron is preserved in ferricytochrome *c* carboxymethylated at methionine-65, whereas other ligands seem to have displaced methionine-80 from the heme group in the ferric forms of dicarboxymethylated cytochrome *c*, cytochrome *c* formylated at tryptophan-59, and cytochrome *c* polymers. On the other hand methionine binding to the heme iron seems to be maintained in the reduced form of all these modified cytochromes *c*, with the exception of the protein which had been dicarboxymethylated at the methionyl residues in positions 65 and 80. This exceptional behavior of the dicarboxymethylated compound is not surprising since the modification of methionine-80 should prevent methionine coordination to the heme iron in both oxidation states[7].

These findings on the coordination of the heme iron were compared to the

data on the ability of the modified cytochromes *c* in restoring the respiration of cytochrome *c*-depleted rat liver mitochondria. This biological role of native cytochrome *c* is preserved in the protein which had been carboxymethylated at methionine-65, but it is lost in the other compounds of Fig. 1. Within the group of species studied it would then appear that the redox carrier properties of cytochrome *c* are maintained only if one of the axial ligands of the heme iron is methionine in both oxidation states. Methionine coordination in the reduced state only is apparently not a sufficient structural feature for cytochrome *c* activity.

Studies of reactions with ligands have shown that the native cytochrome *c* crevice is a stronger structure in the reduced than in the oxidized form of the molecule[2]. This may be due to the stronger affinity of the methionine sulfur for the ferrous than for the ferric heme iron, as shown by studies with heme peptides[20], although conformational differences between the oxidized and the reduced protein may also contribute to the different strength of their respective crevices. Our results with modified cytochromes *c* indicate that alterations of the polypeptide chain that do not chemically affect the methionyl-80 residue, yet prevent methionine coordination in the ferric state, do not necessarily have this same effect upon the structure of the ferrous state.

When used in a similar way as in studies of chemically modified cyanoferrimyoglobins[21], the NMR method is a sensitive tool for the investigation of the homogeneity of preparations of chemically modified cytochromes *c*. For example, the disappearance of the hyperfine-shifted ferricytochrome *c* resonances indicates the completion of the carboxymethylation of methionine-80. A magnetically homogeneous species of modified cyanoferricytochrome *c* is then observed (Fig. 2A), in which the hyperfine-shifted resonances are essentially identical with those in the cyanide complex of native ferricytochrome *c*[15] was observed when the carbox methylation reaction had been continued for 10 h under the conditions given in Fig. 2. If the protein is reacted for a longer period of time, "side-reactions" with other amino acid residues appear to produce sizeable quantities of different magnetically non-equivalent species (Fig. 2B). These results agree with the different rates at which residues 65 and 80 react with the alkylating agent in the presence of cyanide[7].

The near identity of the NMR spectra in the cyanide complexes of native and of dicarboxymethylated ferricytochrome *c* may be of interest in view of the intions of native ferricytochrome *c* and imidazole (I. AVIRAM, A. SCHEJTER AND K. WÜTHRICH, unpublished data) or azide ion[22] only partial displacement of methionine is observed even at extremely high concentrations of these ligands. This result is in keeping with the low affinities of these ligands for ferric cytochrome *c*[23,24]. In the dicarboxymethylated cytochrome *c* the tendency to form complexes with these ligands should be increased[7]. Therefore a detailed study of the hyperfine-shifted resonances in the complexes with a variety of low molecular weight ligands should be possible. In analogy to the cyanide complexes it is to be expected that suitable conditions will be found under which the heme resonances of the dicarboxymethylated cytochrome *c* complexes correspond closely to those of the corresponding complexes of native cytochrome *c*.

The EPR measurements indicate that in all the compounds of Fig. 1 the ferric heme iron is in a low spin state ($S = 1/2$). This suggests that in the place of methionine-80 there are other amino acid residues bound to the heme iron in the oxidized form

of the modified proteins. Further studies of the NMR spectra of these low spin ferric species are in progress.

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Recent additional studies of dicarboxymethylated cytochrome *c* in the reduced state revealed that this species is in the paramagnetic high spin ferrous state (Fe^{2+}, $S = 0$). It is therefore possible that the unusual positions of the proton resonances at +2.5 and +4 ppm (Fig. 1) are due also to interactions with the unpaired electrons of the heme iron (R. M. KELLER, I. AVIRAM, A. SCHEJTER AND K. WÜTHRICH, *FEBS Lett.*, submitted for publication).

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REFERENCES

1 M. O. DAYHOFF, *Atlas of Protein Sequence and Structure*, Vol. 4, Natl. Biomed. Res. Found., Silver Spring, Md., 1969.
2 E. MARGOLIASH AND A. SCHEJTER, *Adv, Protein Chem.*, 21 (1966) 113.
3 K. ANDO, H. MATSUBARA AND K. OKUNUKI, *Biochim. Biophys. Acta*, 118 (1966) 240.
4 K. ANDO, H. MATSUBARA AND K. OKUNUKI, *Biochim. Biophys. Acta*, 118 (1966) 256.
5 M. W. FANGER, T. P. HETTINGER AND H. A. HARBURY, *Biochemistry*, 6 (1967) 713.
6 E. STELLWAGEN, *Biochemistry*, 7 (1968) 2496.
7 A. SCHEJTER AND I. AVIRAM, *J. Biol. Chem.*, 245 (1970) 1552.
8 M. SOKOLOVSKY, I. AVIRAM AND A. SCHEJTER, *Biochemistry*, 9 (1970) 5113.
9 A. SCHEJTER, I. AVIRAM AND M. SOKOLOVSKY, *Biochemistry*, 9 (1970) 5118.
10 I. AVIRAM AND A. SCHEJTER, *Biochim. Biophys. Acta*, 229 (1971) 113.
11 R. E. DICKERSON, T. TAKANO, D. E. EISENBERG, O. B. KALLAI, L. SAMSON, A. COOPER AND E. MARGOLIASH, *J. Biol. Chem.*, 246 (1971) 1511.
12 R. MARGALIT AND A. SCHEJTER, *FEBS Lett.*, 6 (1970) 278.
13 V. BYERS, D. LAMBETH, H. A. LARDY AND E. MARGOLIASH, *Fed. Proc.*, 30 (1971) 1286.
14 K. WÜTHRICH, *Struct. Bonding*, 8 (1970) 53.
15 K. WÜTHRICH, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 1071.
16 C. C. MCDONALD, W. D. PHILLIPS AND S. N. VINOGRADOV, *Biochem. Biophys. Res. Commun.*, 36 (1969) 442.
17 E. MARGOLIASH AND J. LUSTGARTEN, *J. Biol. Chem.*, 237 (1962) 3397.
18 E. MARGOLIASH AND O. F. WALASEK, in R. W. Estabrook and M. E. Pullman, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 339.
19 J. F. GIBSON, D. J. E. INGRAM AND D. SCHONLAND, *Disc. Faraday Soc.*, 26 (1958) 76.
20 H. A. HARBURY, J. R. CRONIN, M. W. FANGER, T. P. HETTINGER, A. J. MURPHY, Y. P. MYER AND S. N. VINOGRADOV, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1658.
21 K. WÜTHRICH, R. G. SHULMAN, T. YAMANE, B. J. WYLUDA, T. E. HUGLI AND F. R. N. GURD, *J. Biol. Chem.*, 245 (1970) 1947.
22 K. WÜTHRICH, *Probes of Structure and Function of Macromolecules and Membranes, Vol. 2: Probes of Enzymes and Hemoproteins*, Academic Press, New York, 1971, p. 465.
23 A. SCHEJTER AND I. AVIRAM, *Biochemistry*, 8 (1969) 149.
24 P. GEORGE, S. C. GLAUSER AND A. SCHEJTER, *J. Biol. Chem.*, 242 (1967) 1690.