EVIDENCE FOR PENTACOORDINATED IRON (II) IN CARBOXYMETHYLATED CYTOCHROME *C*

Regula M. KELLER*, Irit AVIRAM**, Abel SCHEJTER** and Kurt WÜTHRICH*

**Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, 8049 Zürich, Switzerland*

and

***Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel*

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1. Introduction

In biological systems the occurrence of the paramagnetic high spin form of iron (II) has been demonstrated in deoxymyoglobin and deoxyhemoglobin [1], where the heme iron is pentacoordinated and located ca. 0.8 Å out of the plane formed by the 4 nitrogen atoms of protoheme IX [2,3]. In the present paper we report the observation by nuclear magnetic resonance (NMR) spectroscopy of high spin iron (II)(Fe^{2+},S=2) in chemically modified horse cytochrome *c* (CM-Cyt *c*), where the methionyl residues had been carboxymethylated with 2-bromoacetate in the presence of KCN [4]. The paramagnetism of the reduced form of CM-Cyt *c* is manifested in the NMR spectra by the appearance of largely shifted resonances outside of the spectral region for diamagnetic molecules [5]. Since theoretical considerations [6] and observations in "inorganic" compounds [7] seem to indicate that systems with the electronic configuration nd^6 tend to be in the diamagnetic low spin form even in relatively weak ligand fields, it seems probable that the paramagnetism in reduced CM-Cyt *c* is related to pentacoordination of the heme iron. It was pointed out previously that the reduced form of CM-Cyt *c* has many other properties in common with deoxymyoglobin, e.g. it binds molecular oxygen and carbon monoxide at neutral pH [4, 8].

2. Materials and methods

Horse heart ferricytochrome *c* (Sigma, Type II) was purified by column chromatography on Amberlite IRC-50 [9]. The methionyl residues in positions 65 and 80 were then carboxymethylated in a reaction mixture containing 0.18 M 2-bromoacetate, 0.1 M KCN and 0.1 M Tris-HCl buffer, pH 7.0 [4]. The reaction was carried on for 10 hr at 25°. Particular care was taken to remove CN^- by dialysis after the reaction. Ferrous CM-Cyt *c* was obtained by reduction with dithionite under an N_2 atmosphere.

The composition of the reaction product was determined from the NMR spectra. There was less than 20% of cytochrome *c* unreacted at methionine-80, which agrees with the rate observed for the reaction of methionine-80 with bromoacetate [4], and less than 10% of the total cytochrome *c* was complexed with CN^-. A single set of hyperfine-shifted resonances was observed in ferric CM-Cyt *c* and its complex with cyanide ion. This indicates that possible "side-reactions" of bromoacetate with other amino acid residues which would affect the heme proton resonances had occurred at most to a very limited extent [10].

For the NMR studies lyophilized CM-Cyt *c* was redissolved in 0.1 M deuterated phosphate buffer, pD = 7.0. The concentration was ca. 8×10^{-3} M. High resolution proton NMR spectra were recorded on a Varian HR-220 spectrometer equipped with a standard Varian variable temperature control unit. The signal:

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noise ratio was in some experiments improved by data accumulation in a Varian 1024 computer of average transients. Chemical shifts are expressed in parts per million (ppm) from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), where shifts to low field at constant radio frequency are assigned negative values.

3. Results and discussion

In the proton NMR spectrum of reduced CM-Cyt *c* (fig. 1) the resonances between 0 and −9 ppm correspond to the bulk of the 650 protons of the polypeptide chain. In addition several weak resonances are observed in the spectral regions from +2 to +4 ppm and from −10 to −20 ppm. The downfield shifts of these latter resonances have to come mainly from hyperfine interactions with unpaired electrons; they could not conceivably be caused by any mechanism [11]. The hyperfine-shifted lines correspond probably mainly to protons of heme *c*, which can interact with unpaired electrons of the heme iron through Fermi-type contact coupling and through pseudo-contact coupling [5]. Overall the spectrum in fig. 1 is qualitatively very similar to the spectra observed in the deoxyforms of myoglobin [5] and hemoglobin [12].

The dependence on temperature of the hyperfine-shifted resonances in fig. 1 deviates markedly from the Curie-type behaviour generally observed for systems in which the electronic ground state is well separated in energy from the other states [7]. This indicates that at least 2 different states are in thermal equilibrium in CM-Cyt *c*. There could either be a rapid interchange between different molecular species containing respectively high spin and low spin iron (II), or a thermal equilibrium between two or more energetically close-lying electronic states in one molecular species. It appears that a higher concentration of paramagnetic ferrous CM-Cyt *c* is present at lower pH-values. This would agree with earlier observations in the optical spectra, which indicated the presence of high spin iron (II) in CM-Cyt *c* at pH-values around 4.0 [8]. Further investigations of these equilibria, including NMR and optical spectroscopy and susceptibility measurements, are in progress.

Oxidized CM-Cyt *c* and various complexes of CM-Cyt *c* with low molecular weight ligands have also been examined. The NMR spectrum of oxidized CM-Cyt *c* is typical for a low spin ferric hemoprotein [5]. This agrees with earlier observations by optical and electron paramagnetic resonance spectroscopy [4, 10]. The positions of the hyperfine-shifted resonances in CM-Cyt *c* and its cyanide complex [10] are quite similar, yet they differ sufficiently for the complex formation to be observed by NMR [13]. The complexes of reduced CM-Cyt *c* with CO and CN^- are, in contrast to the corresponding ferrocytochrome *c* compounds [14], stable at neutral pH [4]. No hyperfine-shifted resonances were observed in these complexes, and overall their NMR spectra are typical for diamagnetic proteins [5]. In analogy to the corresponding complexes of myoglobin and hemoglobin [1] one would have anticipated that these compounds are diamagnetic.

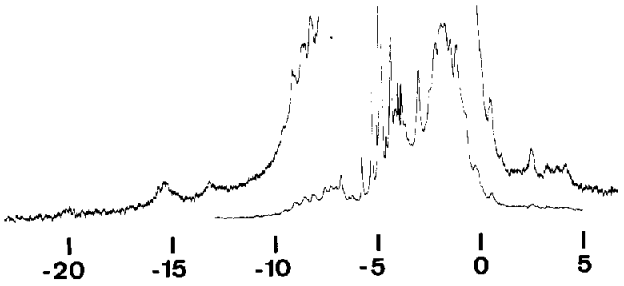

Fig. 1. Proton NMR spectrum at 220 MHz of horse ferrocytochrome *c* carboxymethylated at the methionyl residues in positions 65 and 80 (CM-Cyt *c*). A solution of ca. 8×10^{-3} M deuterated phosphate buffer, pD = 7.0, was studied at 26°. The intense lines between −3.8 and −6 ppm correspond to the resonance of the residual protons of the solvent and its spinning side bands. The resonances outside the spectral region from 0 to −9 ppm are shown with two different scales.

In conclusion we have on the one hand that oxidized CM-Cyt *c* contains low spin ferric heme iron. This indicates that another amino acid residue is bound to the heme iron in the place of methionine-80, which is one of the axial ligands in native cytochrome *c* [15]. From the X-ray structure of ferricytochrome *c* [16] and from chemical considerations the most likely axial ligand in oxidized CM-Cyt *c* would seem to be lysine-79. On the other hand we have that an appreciable portion of the heme iron in reduced CM-Cyt *c* is in the paramagnetic high spin ferrous form at neutral pH. Since the occurrence of the high spin ferrous state in hemoproteins appears to be quite generally

linked with pentacoordination of the heme iron [1–3] it seems reasonable to relate also the paramagnetism in reduced CM-Cyt *c* with this type of coordination, i.e. the heme iron would then be bound to the 4 nitrogen atoms of heme *c* and one nitrogen atom of imidazole of the axial histidine-18 [16]. This explanation of the paramagnetism in reduced CM-Cyt *c* is further supported by theoretical considerations [6] and by observations in "inorganic complexes" [7] which show that systems with the electronic configuration nd^6 tend to take on the low spin state even in relatively weak ligand fields. Also the closely related properties of the heme iron in CM-Cyt *c* and deoxymyoglobin, in particular the ability to bind O_2, CO, CN^- and other small molecules at neutral pH, would then be understandable. It will be of interest in the future to investigate whether the transition from hexacoordinated to pentacoordinated heme iron during reduction of CM-Cyt *c* is due mainly to the lesser affinity of the sixth ligand, possibly lysine-79, for ferrous iron, or to major changes of the molecular conformation which would tend to open the heme crevice.

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