STRUCTURAL STUDIES OF CYTOCHROME *c*-551 BY ^{1}H NMR SPECTROSCOPY AT 360 MHz

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1. Introduction

High resolution ^{1}H NMR studies have in the past resulted in significant contributions to the elucidation of the molecular conformations of cytochromes of the *c*-type [1–6]. In addition, NMR provided also insights into the relations between electronic structure of the heme group and the biological role of cytochromes [3,6–9], and on electron exchange reactions between reduced and oxidized cytochromes *c* [10–12]. In the present study high resolution ^{1}H NMR was used to investigate a new species, i.e. cytochrome *c*-551 of *Pseudomonas aeruginosa* [13–14].

Cytochrome *c*-551 consist of one polypeptide chain with 82 amino acid residues and one heme *c* group. The amino acid sequence includes the typical heme binding fragment -Cys-X-Y-Cys-His-[14]. A preliminary description of a low resolution X-ray study of cytochrome *c*-551 at 4 Å has been presented [15], and extensive studies have been described of the electron transfer reaction between cytochrome *c*-551 and azurin of *Pseudomonas aeruginosa* [16–18]. In view of these earlier kinetic investigations, the present paper includes also studies of the NMR manifestations of electron exchange in solutions of half reduced cytochrome *c*-551. As will be seen in the following text, observation of these dynamic phenomena was of considerable importance for the NMR characterization of the fully oxidized and fully reduced states of the protein.

2. Materials and methods

Cytochrome *c*-551 from *Pseudomonas aeruginosa* was extracted and purified according to the procedure of Ambler [13]. The protein was stored in ammonium acetate buffer, at pH 3.9, which was then replaced with 0.05 M deuterated phosphate buffer by ultrafiltration. Some of the protein was subsequently lyophilized and redissolved in D_2O. NMR spectra taken before and after lyophilization showed the same features. The protein concentrations in the NMR samples were between 0.002 M and 0.005 M. The pH values reported are those measured with a combination glass electrode without correction for deuterium isotope effects [19]. Ferrocytochrome *c*-551 was obtained by adding solid disodium dithionite to the solution of ferricytochrome *c*-551. To obtain a fully oxidized solution of the protein it was necessary to add a slight excess of $K_3Fe(CN)_6$.

High resolution proton NMR spectra were recorded in the Fourier mode on a Bruker HX-360 spectrometer equipped with a standard Bruker variable temperature control unit. 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 are assigned positive values. Spectrum simulations were carried out with a modified version of the multisite exchange program by M. Saunders [20].

3. Results

The ^1H NMR spectra of reduced and oxidized cytochrome *c*-551 are shown in fig.1. Table 1 contains chemical shifts of a number of resonance lines of the heme group and the axial methionyl residue. As is shown in the following individual resonances could be assigned to specific protons on the basis of comparison with other cytochromes *c*, consideration of the amino acid sequence of cytochrome *c*-551 and observations on electron exchange phenomena in mixed solutions of reduced and oxidized cytochrome *c*-551 (fig.2).

Ferrocytochrome *c*-551 (fig.1A) contains the spectral features known to arise from methionyl coordination to the heme iron [1–3], i.e. a methyl resonance at –2.9 ppm and four one-proton resonances between –0.5 and –3.5 ppm. Additional high field resonances are several rather broad lines between –0.2 and 0.3 ppm, which correspond in intensity to 5 to 6 protons. It appears likely that these lines correspond to ring current shifted methylene-proton resonances [21] of Pro-60, Pro-62 and Pro-63 which are next to the axial Met-61 in the amino acid sequence [14]. Since the amide protons had been exchanged with deuterium of D_2O, the low field region of spectrum 1A contains only the resonances of the aromatic amino acids and the meso-protons of heme *c*. The four heme meso-protons are well resolved between 9.2 and 9.9 ppm. In the aromatic resonances some fine structure arising from spin-spin coupling can be seen even without application of digital resolution enhancement techniques [22] (fig.2A). The analysis of this spectral region so far revealed the presence of only two singlet resonances at 7.3 and 7.8 ppm. Since Cyt *c*-551 contains two Trp and one His

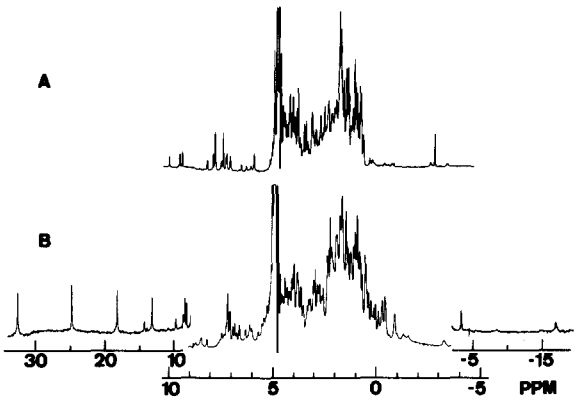

Fig.1. ^1H NMR spectra at 360 MHz. A. 0.002 M solution of ferrocytochrome *c*-551 in 0.05 M deuterated phosphate buffer, p*D* 7.0, T = 40°C. B. 0.005 M solution of ferricytochrome *c*-551 in 0.05 M deuterated phosphate buffer, p*D* 7.5, T = 24°C. The solution contained 0.002 M $K_3Fe(CN)_6$. Note that the vertical and horizontal scales are different for the central part and for the outer parts of the spectrum. The strong lines between 4 and 5 ppm correspond to the HDO resonance and its spinning sidebands.

Table 1
^1H NMR chemical shifts in ppm from DSS of heme *c* and the iron-bound methionyl residue in cytochrome *c*-551 at 42°C

Resonance assignments	Reduced cyt *c*-551	Oxidized cyt *c*-551
Heme meso-protons	9.86 ppm	8.7[a] ppm
	9.39	6.7[a]
	9.36	–0.7[a]
	9.24	–1.8[a]
Heme ring methyl groups	3.74	30.5
	3.68	23.2
	3.40	17.9
	3.30	13.7
Met-61 ε-methyl group	–2.90	–15.7
β,γ methylene-protons	–0.52	>130[a]
	–0.87	8[a]
	–2.72	0[a]
	–3.52	–8.8

[a]Obtained from the spectral simulation in fig.2B (see text)

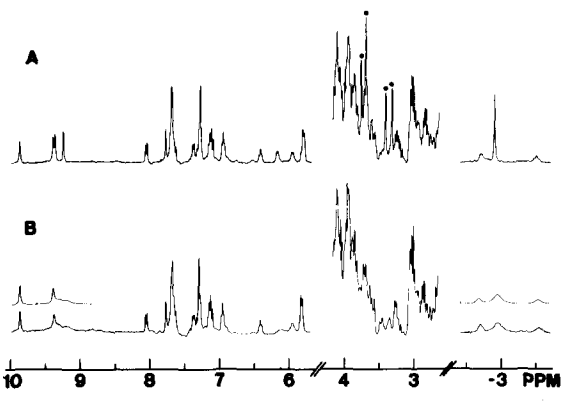

Fig.2. Three spectral regions of the ^{1}H NMR spectra at 360 MHz of 0.002 M solutions of cytochrome *c*-551 in 0.05 M deuterated phosphate buffer, p*D* 7.0, T = 42°C. A. Reduced cytochrome *c*-551. B. Mixture of 99% reduced and 1% oxidized cytochrome *c*-551. Compared to spectrum A some resonances are sizeably broadened by the intermolecular electron exchange. In particular, four sharp resonances between 3 and 4 ppm (indentified by an asterix in spectrum A) could thus be assigned to the 4 heme methyl groups (see text). The inserts show simulations of the regions 9 to 10 ppm and −2 to −4 ppm of spectrum B obtained with a value of 1.2×10^7 M^{-1} sec^{-1} for the bimolecular rate constant of the electron exchange.

[14], this is what one would expect if the only His residue were bound to the heme iron. Cyt *c*-551 contains also one Tyr and two Phe [14]; at ambient temperature it was found that one Phe ring is rotating rapidly about the C^{β}–C^{γ} bond [22] whereas the spin systems of the other two rings have not yet been assigned with certainty.

Ferricytochrome *c*-551 contains the methyl resonance in an extreme high field position, i.e. −16.9 ppm, which is characteristic for methionine coordination to the heme iron [1,7,10]. Additional resonances assigned in analogy to other cytochromes *c* [5] are those of the thioether bridges, i.e. two one-proton lines which 24°C are superimposed at −3.2 ppm and two methyl resonances at −0.84 ppm and −0.89 ppm. At low fields there are four resonances of intensity 3 protons at 32.6, 24.8, 18.2 and 13.2 ppm, which were from comparison with heme model complexes assigned to the four heme methyls [3].

In fig.2 three regions of the ^{1}H NMR spectrum of reduced cytochrome *c*-551 are compared with the corresponding spectral features of a solution of cytochrome *c*-551 in which approximately 1% of the protein had been autoxidized. Intermolecular electron exchange leads to line broadening and displacement of the resonances corresponding to protons which have sizeably different chemical shifts in the two oxidation states of the protein. For example the methyl resonance of the iron bound Met-61 at around −2.9 ppm is markedly broadened and slightly shifted to higher field by the presence of the oxidized protein (fig.2B). The data on the methyl resonance of Met-61 in figs.1 and 2 were used as the starting point for a mathematical analysis of the electron exchange effects on the ^{1}H NMR spectra of cytochrome *c*-551. It became then readily apparent that the one-proton resonances at −3.5 ppm in the reduced protein and at −8.8 ppm in the oxidized protein correspond to the same proton, which is probably a methylene-proton of Met-61 [3,10]. By simulation of these two lines we evaluated the rate of the intermolecular electron exchange and the fraction of oxidized protein. Good agreement between observed and simulated resonance positions and lines shapes (fig.2B) was achieved for $k = 1.2 \times 10^7$ M^{-1} sec^{-1} and 1% oxidized protein.

With the electron exchange rate and the degree of autoxidation thus established, manifestation of exchange phenomena in other spectral regions (fig.2B) could be used to obtain chemical shifts for the heme ring methyl- and meso-protons in both oxidation states of the protein. From earlier studies of diamagnetic hemes [3] and hemoproteins [10], the ring methyl resonances of ferrocytochrome *c*-551 were expected to be at around 4 ppm. Since these resonances experience large hyperfine shifts (fig.1B), they should be very broad in the spectrum of fig.2B. Indeed there are four sharp lines of intensity three protons in the reduced protein (marked with an asterix in fig.2A) which disappeared in spectrum B of fig.2 and were therefore assigned to the four ring methyls. For the meso-protons, only the chemical shifts in ferrocytochrome *c*-551 (fig.1A) and the exchange effects in fig.2B could be observed. The chemical shifts for the oxidized species (table 1) were then obtained from a computer fit of spectrum B of fig.2. Similarly, the chemical shifts were obtained for methylene-protons of Met-61; for the fourth methylene-proton of Met-61, only a lower limit for the absolute value of the hyperfine shift could be derived (table 1).

4. Discussion

The ^{1}H NMR data presented in this paper revealed both structural similarities and differences between cytochrome *c*-551 and other cytochromes *c* [1,2,4–6]. Methionine coordination to the heme iron is clearly manifested in both oxidized and reduced cytochrome *c*-551, and the spectrum of the reduced protein contained also evidence for coordination of the single histidyl residue [14] to the second axial position.

The assignments of resonance lines of heme *c* given in table 1 provide a basis for future quantitative investigations of the electronic structure. Inspection of the NMR spectrum in fig.1B shows that ferrocytochrome *c*-551 does not contain the typical distribution of heme ring methyl resonances found for other cytochromes *c* [1,5–7]. An apparent C_2- symmetry is on the other hand manifested in the meso-proton hyperfine shifts (table 1). Overall it appears that the principal features of the local magnetic fields of the heme in cytochrome *c*-551 are similar to those generally found for low spin ferric hemoproteins [22,23]. In particular, the pseudocontact shifts and the ring current shifts have opposite sign, as indicated by the chemical shifts of the resonances assigned to the thioether bridges in the oxidized protein.

As usual with hemoproteins [22,23], information on the structure of the heme crevice is contained in the high field region of the ^{1}H NMR spectra of cytochrome *c*-551. In the reduced protein the complete absence of methyl resonances other than that of Met-61 at higher field than 0.6 ppm shows that no aliphatic methyl group is located near the plane of the heme. The presence of ten well resolved methyl lines between 0.5 and −0.5 ppm in ferricytochrome *c*-551 shows on the other hand that numerous lipophilic aliphatic side chains are located near the edges of the heme.

Compared to horse cytochrome *c* [10,11] the electron exchange between reduced and oxidized cytochrome *c*-551 is two to three orders of magnitude faster. At 42°C the exchange phenomena in the ^{1}H NMR spectra could be accounted for by the presence of one oxidized and one reduced species. Preliminary data from studies of the temperature dependence, which are currently being continued, indicate that equilibria between different oxidized species might also affect the NMR spectra at lower temperatures. The NMR observations on the electron exchange, in particular the relatively high rate of the exchange between reduced and oxidized cytochrome *c*-551, is also of special interest for the analysis of the electron transfer kinetics with azurin [16–18].

Acknowledgements

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