LOCATION OF Cr(III) IN THE Cr(III)–CYTOCHROME C COMPLEX AS OBSERVED BY NMR SPECTROSCOPY

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1. Introduction

The site of chromium binding in the Cr(III)–cytochrome *c* (cyt. *c*) complex formed by the Cr(II)-reduction of ferricytochrome *c* is of interest for understanding the mechanism of electron transfer [2]. The stability of the Cr(III)–cyt. *c* complex allowed the hydrolyzation of this protein with trypsin and attempt to locate the exchange-inert Cr^{3+} by analysis of the resulting fragments with chromatographic procedures [3]. They concluded that the most likely position for Cr(III) was in a crosslinking configuration with the metal ligated to both Tyr_{67} and Asp_{52} residues. This conclusion implicated the participation of Tyr_{67} in electron transfer during the reduction of ferricyt. *c* [3].

We sought to utilize the paramagnetic effects of Cr(III) on ^1H nuclear relaxation rates of well-assigned proton resonances of cyt. *c* to obtain distances from these protons to the Cr(III) label.

2. Materials and methods

Horse-heart cyt. *c* (type VI) was obtained from Sigma and was further purified by chromatography on Sephadex G-25 in ammonium carbonate buffer (pH 10). The resulting cyt. *c* solution was neutralized with carbonic acid and lyophilized. A deoxygenated solution of chromic perchlorate (100 mM in 0.1 mM $HClO_4$) was reduced to Cr(II) with zinc amalgam. A 10 mM solution of purified cyt. *c* was adjusted to pH 4.1 with acetate buffer and carefully treated with equimolar Cr^{2+} under an atmosphere of nitrogen at 0°C with slight agitation [2]. The solution was then passed through a short Dowex-50 column to remove any chromium ions not bound to cyt. *c*. When required the oxidation of ferrocyt. *c* was carried out with ferricyanide without loss of the Cr(III) label. A 1:1 complex of Cr(III) with cyt. *c* was confirmed by chromium analysis using the diphenylcarbazide method [4] and by double integration of the first derivative ESR signal from Cr(III). The Cr(III)-labeled cyt. *c* proved to be unstable to lyophilization. Therefore, deuteration of the protein to avoid the strong H_2O signal in NMR was accomplished by extensive dialysis against 100 mM phosphate buffer in D_2O (pH 6.7, uncorrected pH meter reading) in an Englander dialysis cell.

The longitudinal nuclear relaxation rates ($1/T_1$) of water protons were measured at 24.3, 100 and 360 MHz using a home-built spin echo spectrometer, a Varian XL-100-FT spectrometer and a Bruker WH-360 spectrometer, respectively. Longitudinal relaxation rates $1/T_1$ for well resolved and previously assigned [5–7] methyl group protons of cyt. *c* and the Cr(III)–cyt. *c* complex were measured on the Varian XL-100 system at 100 MHz using the inversion–recovery technique. All measurements were made at 21 ± 1°C, unless otherwise specified. The paramagnetic effects

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of Cr(III) on the ^1H longitudinal relaxation rates ($1/T_{1p}$) were evaluated by subtracting the relaxation rates of native cyt. *c* from those obtained in the Cr(III)–cyt. *c* complex. When dipolar interaction predominates, the distance-dependence of T_{1p} is described by the following eq. [8]:

$$\frac{1}{fqT_{1p}} = \frac{2\mu_S^2\gamma_I^2}{15r^6}\left\{\frac{3\tau_S}{1+\omega_I^2\tau_S^2} + \frac{7\tau_S}{1+\omega_S^2\tau_S^2}\right\} \quad (1)$$

where symbols characterizing the electron and nuclear moments, and their interactions, have their usual meaning. For the Cr(III)-cyt. *c* complex, the electron spin relaxation time τ_S will be the correlation time for the Cr(III)–nuclear dipolar interaction [9]. The distance-dependence of $1/T_{1p}$ described by eq. (1) may be used to calculate distances from Cr(III) to the various ^1H atoms of the cyt. *c* molecule. However, implicit in the derivation of eq. (1) is the assumption that the contact hyperfine contributions to paramagnetic effects of $1/T_{1p}$ are negligible. This is true in our case since $\omega_S\tau_S \gg 1$ [10]. When used to describe the relaxation behaviour of water protons, an additional assumption implicit in eq. (1) is that $1/T_{1p}$ is not exchange-limited [10]. We consider the validity of this assumption later.

3. Results

The presence of Cr(III) in the 1:1 Cr(III)–cyt. *c* complex causes small but significant enhancements in the $1/T_1$ of the well-resolved methyl resonances of cyt. *c* in either oxidation state, presumably due to the paramagnetic effects of Cr(III) (table 1). However, the positions of the hyperfine-shifted resonances in Cr(III)–ferricyt. *c* and the diamagnetically upfield-shifted resonances in Cr(III)-ferrocyt. *c* are unaffected by the presence of Cr(III) in the molecule. This indicates that Cr(III) does not significantly perturb the heme or the protein conformation. The magnitudes of the paramagnetic effects of Cr(III) on the ^1H relaxation rates $1/T_1$ in the Cr(III)–cyt. *c* complex are also presented in table 1. Assignments of the resonances to individual methyl groups are well-established [5–7] and are indicated in fig.1.

The paramagnetic effects of Cr(III) on the ^1H relaxation rates may be used to obtain distances between Cr(III) and the relaxing protons by use of eq. (1) provided the correlation time for metal–nuclear dipolar interaction is known. This correlation time was determined from the magnetic field-dependence of the paramagnetic effects of Cr(III)–cyt. *c* complex on the relaxation rate of water protons in the same complex. This approach is valid since the correlation time for both the Cr(III)-^1H(cyt. *c*) and the Cr(III)-$^1H(H_2O)$ dipolar interactions is dominated by τ_S, the electron spin relaxation time of Cr^{3+}. The magnitude of the longitudinal component of molar paramagnetic relaxivity of the Cr(III)–cyt. *c* complex for water protons decreased by ∼1.5-fold at 360 MHz from its value at 24.3 MHz (table 2), presumably due to the dispersion of the ω_I term in eq. (1), the ω_S term being negligibly small at all frequencies used. The

Table 1
Paramagnetic effects of chromium on the hyperfine-shifted or diamagnetically shifted methyl proton resonances in the chromium(III)–cytochrome *c* complexes at 100 MHz

Methyl group	δ_{ppm}	T_1 (ms)		$1/T_{1M}$ (s^{-1})	Cr(III)–^1H distance[c] (Å)
		Native	Cr(III) complex		
Methionine 80[a]	+ 3.3	233 ± 14	118 ± 4	4.2	17
Heme-ring (b_1)[b]	−35.0	26 ± 1	22 ± 2	∼ 7	∼16
Heme-ring (b_2)[b]	−32.0	37 ± 3	27 ± 2	∼10	∼15
Thioether bridge (c)[b]	+ 2.5	123 ± 7	94 ± 3	2.5	19
Heme-ring (b)[b]	−10.5	116 ± 8	89 ± 4	2.6	19

[a] In the Cr(III)–ferrocyt. *c* complex
[b] In the Cr(III)–ferricyt. *c* complex
[c] Calculated using eq. (1) and a $\tau_S = 3.2 \times 10^{-10}$ s

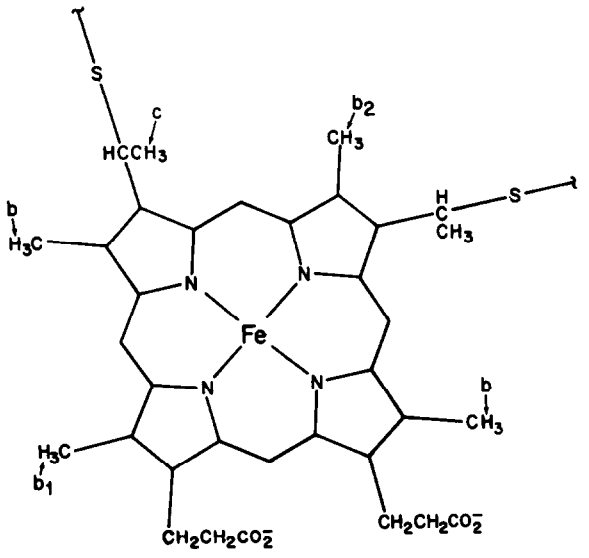

Fig.1. The heme ring of cytochrome *c*. According to the X-ray crystallographic structure of cyt. *c* [14], the ring methyl labeled b_1 is located on the buried edge of the heme near Trp_{59} and Tyr_{67} while ring methyl b_2 is located on the exposed edge of the heme.

observed paramagnetic effect of Cr(III)–cyt. *c* on $1/T_1$ of water protons was in the fast-exchange region on the NMR time scale, since the magnitude of the effect at 24.3 MHz decreased by ∼20% with a 40°C increase in the sample temperature (table 3). Exchange-limited paramagnetic relaxation would be expected to show a more pronounced temperature dependence in the opposite direction [10]. An analysis of the magnetic field-dependence of water proton relaxation data yielded a value of

Table 2
Magnetic field-dependence of the paramagnetic effect of Cr(III)–cytochrome *c* on water proton relaxation[a]

^{1}H resonance frequency (MHz)	360	100	24.3
Molar paramagnetic relaxivity (s^{-1})	4750	6525	6835

[a] A τ_S value of 3.2×10^{-10} s was obtained from an analysis of the magnetic field-dependence of the relaxation data using eq. (1). Cr(III)–ferricyt. *c* and Cr(III)–ferrocyt. *c* had similar effects on water relaxation

Table 3
Temperature dependence of paramagnetic water proton relaxivity of Cr(III)–cytochrome *c* at 24.3 MHz[a]

T (°C)	1	11	21	30	41
Molar paramagnetic relaxivity (s^{-1})	7560	6970	6835	6645	6250

[a] Decreasing relaxivity with increasing temperature indicates fast exchange of protons out of the paramagnetic environment

∼3.2×10^{-10} s for the τ_S of Cr(III) in the Cr(III)–cyt. *c* complex at 100 MHz.

The observed paramagnetic effects on the hyperfine or diamagnetically shifted resonances, which arise from the heme group and its axial ligands, are small indicating that the Cr^{3+} is located far away from the heme-iron. From the magnitudes of the paramagnetic effects (table 1), using the dipolar correlation time τ_S derived from the magnetic field-dependence of water proton relaxation, metal–nuclear distances were obtained from Cr(III) to the ligand methionine methyl (17 Å), 3 heme-ring methyls (∼15–19 Å) and a thioether bridge methyl (19 Å) via eq. (1) (table 1).

4. Discussion

The distances between the chromium label and various protons on or near the heme group of cyt. *c*, calculated from the paramagnetic effects of Cr(III) on the longitudinal nuclear relaxation rates of the protons, indicate that the Cr(III) is located on or near the surface of the cyt. *c* molecule ∼14 Å away from the iron. The results are incompatible with Cr(III) coordination to the oxygen of Tyr_{67} as suggested [3]. Cr(III)-crosslinking Tyr_{67} and Asp_{52} would position the metal ion ∼6.1 Å from the sulfur atom of Met_{80}. In comparison we find a distance of 17 Å from Cr(III) to the methyl protons of Met_{80}.

Although we may not yet be able to pin point the exact position of Cr(III) on the cyt. *c* molecule, the distances obtained here place the metal near His_{26}. It is possible that electron transfer occurs via spatial overlap of the electronic orbitals of Cr(II) with the delocalized d-orbitals of sulfur in the thioether bridge

to Cys_{17}, although other pathways can not be ruled out at present. It is, however, clear that the position of Cr(III) is incompatible with the direct transfer of electron from chromium to the heme group. The reductive electron-transfer must therefore occur via the protein moeity of cytochrome *c*. Similar conclusions implicating the protein moeity in oxidative electron transfer from the heme of cyt. *c* to that of cyt. *c* peroxidase were obtained from the large separation (~25 Å) between the hemes in the complex of cyt. *c* with cyt. *c* peroxidase [12]. Differences in the rates of self redox reaction for different species of cyt. *c*, with identical hemes, are also consistent with the involvement of the polypeptide chain in the electron transfer process. [13].

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