The reaction of cytochrome *c* with $[Fe(EDTA)(H_2O)]^-$

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The interaction of horse ferricytochrome *c* with the reagents $[Fe(EDTA)(H_2O)]^-$ and $[Cr(CN)_6]^{3-}$ were studied at pH 7 and 25°C by ^{1}H-NMR spectroscopy. Two binding regions near to the heme crevice of cytochrome *c* were identified. Both regions bound both reagents but they exhibited different selectivities. The relevance of this finding to the electron-transfer function of cytochrome *c* is discussed.

NMR *Cytochrome c* *$[Fe(CN)_6]^{3-}$* *$[Fe(EDTA)(H_2O)]^-$* *Binding site*

1. INTRODUCTION

The reactions of electron-transfer proteins with small redox reagents such as $[Fe(CN)_6]^{3-/4-}$ and $[Fe(EDTA)(H_2O)]^-$ have been extensively studied [1,2] because they are simpler reaction systems than the physiological ones. The aims of such studies are to relate observed kinetic and thermodynamic parameters to specific theories of electron transfer. In this respect the Marcus theory [3] has been widely discussed [1,2].

A complete description of the electron-transfer process requires that the reactive species be adequately described. The simplest reaction scheme is the following:

Association:
$$A^o + B^r \rightleftharpoons (A^o \text{ ---- } B^r) \quad (1)$$

Equalisation of energy levels:
$$(A^o \text{ ---- } B^r) \rightleftharpoons (A^o \text{ ---- } B^r)^* \quad (2)$$

Electron transfer:
$$(A^o \text{ ---- } B^r)^* \rightleftharpoons (A^r \text{ ---- } B^o)^* \quad (3)$$

Relaxation:
$$(A^r \text{ ---- } B^o)^* \rightleftharpoons (A^r \text{ ---- } B^o) \quad (4)$$

Dissociation:
$$(A^r \text{ ---- } B^o) \rightleftharpoons A^r + B^o \quad (5)$$

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where A^o and B^o are the oxidised counterparts of A^r and B^r. These 4 species are well characterised for most reactions of cytochrome *c* with small inorganic reagents. Steps 1, 2, 4 and 5 are generally very rapid and thus they do not appear in the rate law for the reaction. In such cases the rate of reaction describes:

$$A^o + B^r \rightleftharpoons A^r + B^o \quad (6)$$

Nevertheless, electron transfer does occur through the agency of a reactive complex, although it is a transient one, and it is particularly important that the geometry of the complex be defined because one of the factors that the rate of electron transfer is dependent upon is the distance between the donor and acceptor sites [1–3].

Until recently there has not been a rigorous method of determining the geometry of the precursor complexes. However, with the increasing definition of the NMR spectra of electron-transfer proteins, cytochromes in particular [4,5], a method of locating and defining interaction sites has been developed. This method makes use of the paramagnetic effects of the redox reagent, or its structural analogues, to induce perturbations in NMR spectra of the protein that are related to the distance between the paramagnetic centre and the perturbed nucleus [4,6].

An NMR investigation of the interaction of eukaryotic cytochrome *c* with $[Fe(CN)_6]^{3-}$ was

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recently reported [7] and the suggestion made that there was more than one interaction site near to the heme group. In this paper are reported analogous studies with $[Fe(EDTA)(H_2O)]^-$, together with a further discussion of the previously reported $[Fe(CN)_6]^{3-}$ data. The improved definition of the interaction regions reported here results from an increase in the number of assigned resonances in the spectrum of cytochrome *c*. The reagents $[Fe(CN)_6]^{3-}$ and $[Fe(EDTA)(H_2O)]^-$ were chosen because their reactions with cytochrome *c* have been particularly well characterised [1,2,8–10].

2. MATERIALS AND METHODS

Horse cytochrome *c* (type VI) was obtained from the Sigma Chemical Co. and samples prepared for NMR as in [7]. $[Fe(EDTA)(H_2O)]^-$ was prepared from Analar grade reagents immediately prior to use by the admixture of $Fe(NO_3)_3$ and Na_2H_2EDTA (10% excess) in ^2H_2O. The pH of the resulting solution was adjusted to 7.0 with NaOD.

^1H NMR spectra were obtained using a Bruker WH-300 spectrometer and the 470 MHz spectrometer developed by the Oxford Enzyme Group. Resolution enhancement was carried out by Gaussian multiplication [11]. The paramagnetic difference spectra were obtained as in [7].

3. RESULTS

3.1. *The spectrum of ferricytochrome* c

The resolution enhanced 470 MHz ^1H NMR spectrum of horse ferricytochrome *c* at pH 7 and 25°C is given in fig. 1 along with some of the resonance assignments. The assignment procedure for many of these resonances has either been published [5,12,13] or will be reported elsewhere. About 40% of the –CH ^1H-NMR resonances have been assigned. Previous NMR studies of the interaction of cytochrome *c* with paramagnetic molecules suffered from the lack of resonance assignments which led to an inadequate description of the interaction sites [4,7].

3.2. *Effect of $[Fe(EDTA)(H_2O)]^-$ on the spectrum of ferricytochrome* c

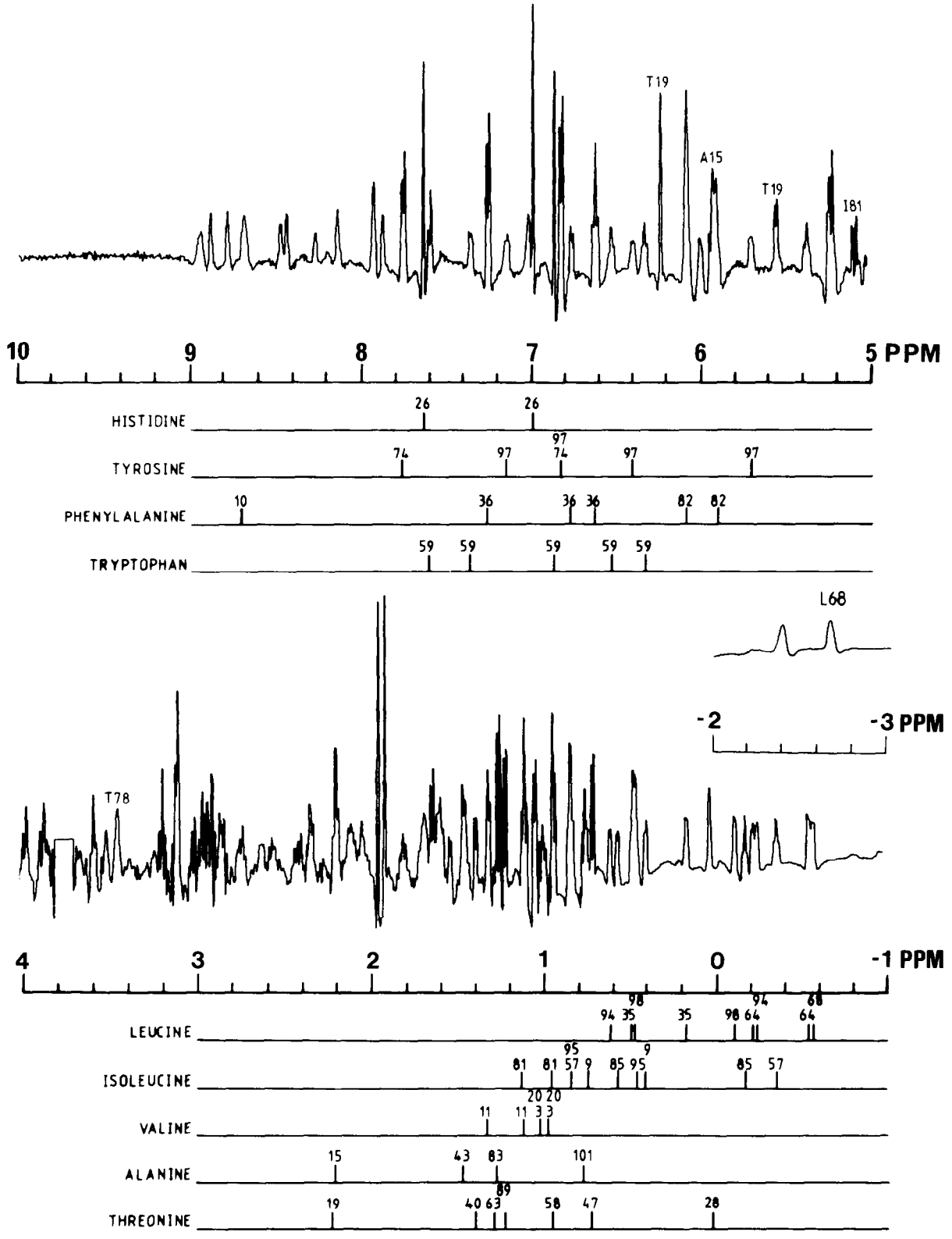
Increasing concentrations of $[Fe(EDTA)(H_2O)]^-$ at constant cytochrome concentration resulted in some resonances of ferricytochrome *c* being markedly broadened. The perturbed resonances appear in different spectra obtained by subtraction of the spectra of cytochrome *c* in the presence of $[Fe(EDTA)(H_2O)]^-$ from the spectrum of cytochrome *c* in the absence of $[Fe(EDTA)(H_2O)]^-$ (fig. 2). At 32 mol% $[Fe(EDTA)(H_2O)]^-$ resonances of the following groups were perturbed in the order of decreasing effect: Thr19 $\geqslant$ Ala15 > Thr28 > Tyr97 $\geqslant$ Phe10 > Phe82 $\geqslant$ Ala83 = Thr89 = (*Ile81γ/Val11) > Val11 = Ile81δ = Ile85γ > His26 (*Ile81 and Val11 methyl resonances overlap at 1.1 ppm; more than one methyl resonance is affected at this position but it is not certain in what order). Resonances of the following groups were unaffected at 32 mol% $[Fe(EDTA)(H_2O)]^-$: *N*-acetyl, Val3, Ile9, Leu32, Leu35, Phe36, Thr40, Ala43, Thr47, Ala51, Ile57, Trp59, Leu64, Met65, Leu68, Tyr74, Leu94, Tyr97 and Leu98. At 32 mol% $[Fe(EDTA)(H_2O)]^-$ the α and γ resonances of Thr19 are so broad that they are no longer observable. However, even at 160 mol% $[Fe(EDTA)(H_2O)]^-$ resonances of Phe82 and Ile85 are observable in the normal spectrum; the increase in their line-widths caused by $[Fe(EDTA)(H_2O)]^-$ is 20 ± 8 Hz.

The $[Fe(EDTA)(H_2O)]^-$ is the fast exchange between bound and unbound states. This deduction is based on the observations that resonance linewidths increase rather than intensities decrease, and that while some resonances are strongly affected at <10 mol% of the reagent, others are weakly affected at >100 mol% of the reagent (fig. 2).

In a separate experiment at 300 MHz the effect of increasing concentrations of $[Fe(EDTA)(H_2O)]^-$ upon resonances outside the spectral range 10 ppm to –4 ppm was investigated. These resonances come from the heme group and axial ligands [14,15]. None of them were specifically affected, even at 300 mol% $[Fe(EDTA)(H_2O)]^-$.

3.3. *Geometry of the complex*

The X-ray structures of both $[Fe(EDTA)H_2O]^-$ [16] and cytochrome *c* are known [17]. Cytochrome *c* is a prolate sphaeroid of dimensions 30 Å × 34 Å × 34 Å [18]. There is good evidence for believing that its solid state and solution structures are similar [17–19]. In the solid state $[Fe(EDTA)H_2O]^-$ is 7 coordinate with 5 oxygen and 2 nitrogen ligand atoms. In solution, 7-fold coor-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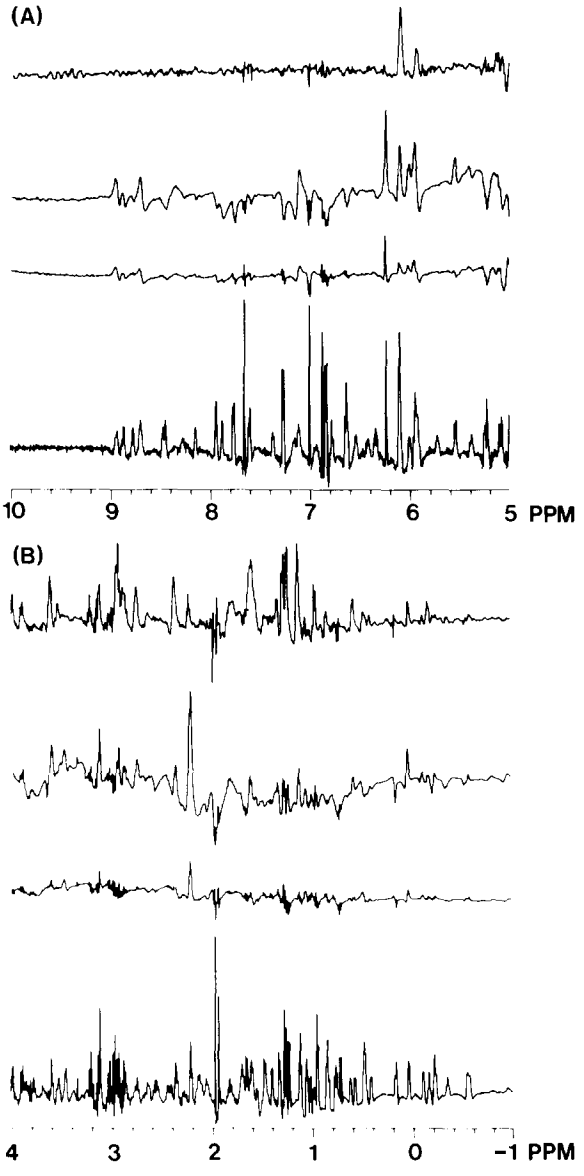

Fig. 2. Aromatic (A) and aliphatic (B) regions of the 470 MHz spectra of 4 mM horse ferricytochrome *c* at pH 7 and 25°C (vertical scale of difference spectra in A is ~2 × that of difference spectra in B): (i) resolution enhanced spectrum; (ii) difference spectrum obtained on addition of 4 mol% $[Fe(EDTA)(H_2O)]^-$; (iii) difference spectrum obtained on addition of 32 mol% $[Fe(EDTA)(H_2O)]^-$; (iv) difference spectrum obtained on addition of 25 mol% $[Cr(CN)_6]^{3-}$.

dination of the iron is maintained by 5 oxygen and 2 nitrogen ligands but it is not clear if the complex contains 1 or 2 coordinated H_2O molecules [20]. For our purposes we will assume the complex is spherical with an effective radius of 4.5 ± 0.5 Å. Despite this uncertainty there are no clefts in cytochrome *c* large enough to accommodate an $[Fe(EDTA)H_2O]^-$ molecule.

$[Fe(EDTA)(H_2O)]^-$ is a relaxation probe, as expected for a high-spin ferric complex which has a relatively long electron-spin lattice relaxation time [6]. Accordingly, the relative broadening of 2 resonances *i* and *j* is related to the ratio of their distances from the ferric ion thus:

$$\frac{(\pi\Delta\nu)_i}{(\pi\Delta\nu)_j} = \frac{r_j^6}{r_i^6}$$

where

r = distance between nuclei and Fe^{3+};
$\Delta\nu$ = increase in line-width caused by the Fe^{3+}.

Inspection of the pattern of perturbed resonances, together with the X-ray structure, reveals that $[Fe(EDTA)(H_2O)]^-$ binds close to the exposed edge of the heme in 2 different regions of the protein. One of the positions is on the front left face of cytochrome *c* and the other is on the front right face; the terminology front, left side and right side, refers to the conventional view of cytochrome *c* [18] shown in fig. 3. Resonances of groups in the right-hand region, which consists of Phe10, Ala15, Thr19, Thr28 and Tyr97, are more affected than resonances of groups in the left-hand region, which contains Ile81, Phe82 and Ile85. The Fe–Fe distances in the 2 regions are: for the right-hand region 13 ± 2 Å, and for the left-hand region 12 ± 1.5 Å.

4. DISCUSSION

Both $[Fe(EDTA)(H_2O)]^-$ and $[Cr(CN)_6]^{3-}$ bind at the heme crevice of ferricytochrome *c*. $[Cr(CN)_6]^{3-}$ also binds weakly at the back of the protein close to Phe36 [7], but there is no evidence

Fig. 1. 470 MHz resolution enhanced ^{1}H-NMR spectrum of horse ferricytochrome *c* at pH 7 and 25°C with the positions of assigned resonances indicated. With the exception of Ala15 (α), Thr17 (α, β) and Ile81 (α), only assigned resonances of aromatic groups and methyl groups are indicated. At the resolution of the labelling scheme methyl resonances of Ile57 and Ile95 overlap at 0.83 ppm and methyl resonances of Val3 and Val20 overlap at 1.04 ppm and 0.99 ppm.

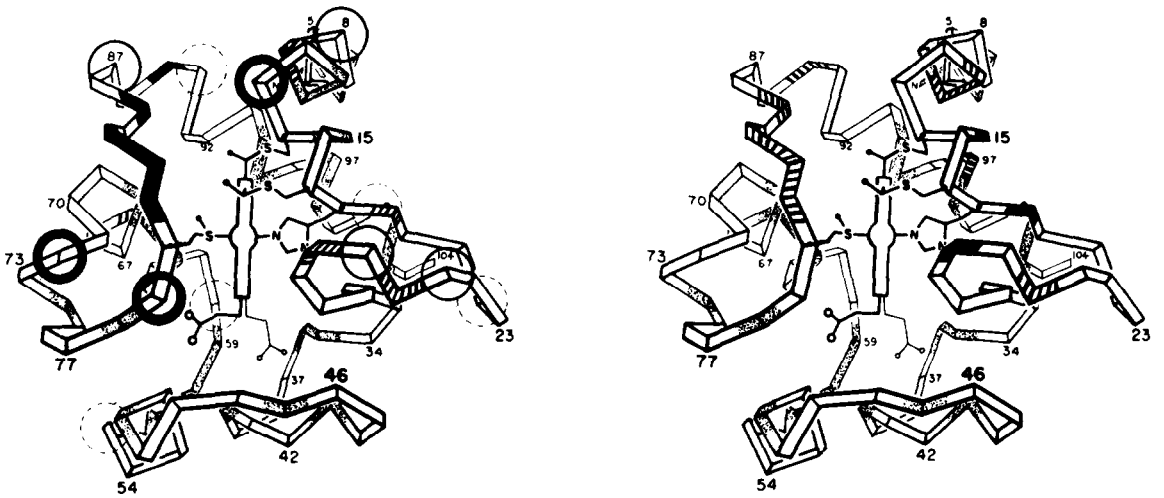

Fig. 3. Ribbon diagrams of the polypeptide fold of horse cytochrome *c*. Residues for which NMR assignments are known for all or some of its CH protons are indicated by ▬, ▭ and ▩, the different markings signifying, respectively, those resonances strongly affected, those that are weakly affected and those that are unaffected. The diagram on the left is for the interaction of ferricytochrome *c* with $[Cr(CN)_6]^{3-}$ and the diagram on the right is for the interaction of ferricytochrome *c* with $[Fe(EDTA)(H_2O)]^-$. The selectivity of the two binding regions is clearly indicated by the distribution of affected residues. The circles on the left diagram illustrate the approximate positions of some of the lysine residues that have been shown by studies of chemically modified cytochrome *c* [21,22] to have either a large effect (●), a weak effect (○) or no effect (◌) upon rates of reaction of cytochrome *c* with $[Fe(CN)_6]^{3-/4-}$.

that $[Fe(EDTA)(H_2O)]^-$ binds in this region. The heme crevice binding can be described in terms of 2 separate interaction regions [7]. This scheme is the minimal scheme that qualitatively fits the data. In [7], which analysed the relaxation effects of $[Cr(CN)_6]^{3-}$ and $[Cr(oxalate)_3]^{3-}$ on NMR spectra of cytochrome *c*, the 2 heme crevice interaction regions (fig. 3), the left region (region-L) and the right region (region-R), were not well defined because there were fewer resonance assignments known at that time. In particular, only resonances of Phe82, Ile85 and heme methyl-3 in region-L were known to be perturbed. This work shows that the newly-assigned resonances of Ile81, Ala83 and Thr89, all in region-L, are also strongly affected by $[Cr(oxalate)_3]^{3-}$ (see fig. 3 of [7]). The unexpected result that $[Cr(CN)_6]^{3-}$ and $[Cr(oxalate)_3]^{3-}$ produce larger perturbations to resonances of groups in region-L compared to those in region-R, while with $[Fe(EDTA)(H_2O)]^-$ the order is reversed (fig. 2), not only confirms the presence of the two binding regions but also shows them to have different selectivities.

The differences between the magnitudes of perturbations caused by $[Fe(EDTA)(H_2O)]^-$ and $[Cr(oxalate)_3]^{3-}$ must be due in part to the different binding strengths of region-L and region-R for the 2 reagents, and in part to the different physical properties of the 2 reagents. Assuming that the electron-spin lattice relaxation time for both reagents is about the same, then $[Fe(EDTA)(H_2O)]^-$ will be a better relaxation agent than $[Cr(oxalate)_3]^{3-}$ because it has 5 unpaired electrons compared to 3. The relaxation enhancement is proportional to $S(S+1)$ [6] which makes high-spin Fe^{3+} 2.3-times more effective than Cr^{3+}. After accounting for this factor, the markedly larger perturbations in region-L for $[Cr(oxalate)_3]^{3-}$ compared to $[Fe(EDTA)(H_2O)]^-$ must result from a higher affinity of region-L for $[Cr(oxalate)_3]^{3-}$. The differences in perturbations of resonances of groups in region-R probably result from a higher affinity of region-R for $[Fe(EDTA)(H_2O)]^-$, but in part they may also be due to the different physical properties of $[Fe(EDTA)(H_2O)]^-$ and $[Cr(oxalate)_3]^{3-}$. The difference in binding affinities is probably a reflection of the difference in polarity between the reagents.

The structures defined here are relevant to

previous studies of the electron transfer function of cytochrome *c*: [21,22] were concerned with defining the regions of the protein that interact with $[Fe(CN)_6]^{3-/4-}$ by measuring the rates of reaction of a variety of derivatives of horse cytochrome *c*, all singly modified at particular lysine residues. A total of 12 out of the 19 lysine residues were modified. Modification of lysines 13, 72 and 79 had the greatest effect on the rate, modification of lysines 8, 25, 27 and 87 had a weak effect, and modification of lysines 22, 55, 60, 99 and 100 had no effect. Fig. 3 shows that this pattern fits in reasonably well with the NMR-defined binding regions; lysines 13 and 72 bind $[Fe(CN)_6]^{3-/4-}$ in region-L, and lysines 25 and 27 bind $[Fe(CN)_6]^{3-/4-}$ in region-R. The importance of other lysines to either of these regions is not clear.

The Fe–Fe distances for region-L and region-R are 12 ± 1.5 Å and 13 ± 2 Å, respectively. These are the first such distance measurements made using a structural technique. The electron-transfer distance for the ferricytochrome *c*–$[Fe(CN)_6]^{4-}$ complex has been estimated from kinetic data to be 10 ± 0.8 Å [10]. Although this estimate was obtained on the assumption of one binding site at the heme crevice, the agreement between the distance measured by NMR and the distance estimated from the kinetic data is encouraging. The only way that the kinetic analysis could be improved is to measure the rate of electron-transfer at each individual site.

The result that both $[Fe(EDTA)(H_2O)]^-$ and $[Fe(CN)_6]^{3-}$ bind to cytochrome *c* in the region including the exposed edge of the heme, is in excellent agreement with the correlation of their rates of reaction with relative Marcus theory [1–3,23], which suggests that both sets of reactions occur by outer-sphere electron transfer at a common site.

Finally, we note that the discovery of 2 different anion-binding regions at the heme crevice of cytochrome *c* may be important for the definition of interaction regions on cytochrome *c* for its biological redox partners. These all interact at the heme crevice [24], though the nature of the complexes has still to be fully defined. The paramagnetic reagents we have used are NMR probes of structure that will allow a detailed analysis of the surface of cytochrome *c*. Competition for cytochrome *c* between its different redox partners and the different paramagnetic reagents should then enable the interaction sites on cytochrome *c* to be better defined.

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