Preferred Sites for Electron Exchange between Cytochrome c and $[Fe(edta)]^{2-}$ and $[Co(sep)]^{2+}$ Complexes†

Graeme D. Armstrong, Jill A. Chambers, and A. Geoffrey Sykes*
Department of Inorganic Chemistry, The University, Newcastle upon Tyne NE1 7RU

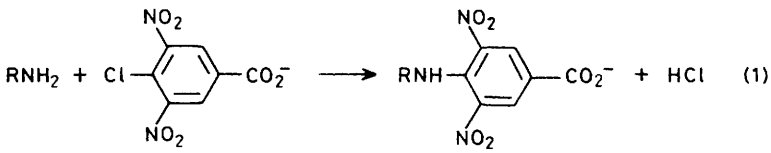
Rate constants (25 °C) for the reduction of eight different singly substituted 4-carboxy-2,6-dinitrophenyl (cdnp) horse heart cytochrome c derivatives, modified respectively at lysine 7,13,25,27,60,72,86, and 87, and of one 2,4,6-trinitrophenyl (tnp) derivative modified at lysine 13, by the 2− and 2+ complexes $[Fe(edta)]^{2-}$ (edta = ethylenediaminetetra-acetate) and $[Co(sep)]^{2+}$ (sep = 1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]icosane) have been determined at pH 7.5 (Tris–HCl) [Tris = tris(hydroxymethyl)methylamine], I = 0.10 M (NaCl). The influence of the modified residues on second-order rate constants for these reactions has been used to define the regions on the protein surface involved. In both cases the solvent accessible edge of the heme prosthetic group on the 'front' surface of the molecule is relevant. The reaction with $[Fe(edta)]^{2-}$ is most strongly influenced by the modification at lysine 72 followed by 27, the latter representing a shift in reactivity as compared to $[Fe(CN)_6]^{3-}$. With $[Co(sep)]^{2+}$ the region around lysine 27 to the right of the heme edge is the most influential as in the case of $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline). The preferred sites with $[Fe(edta)]^{2-}$ and $[Fe(CN)_6]^{3-}$ are the same as those identified using n.m.r.

Lysine modified cytochrome c derivatives, prepared and characterised by Margoliash and co-workers,[1–3] are invaluable in the identification of binding sites on the protein surface for different redox partners. The method provides an opportunity to explore a wide range of different redox partners including inorganic reagents,[4–8] as well as natural (mitochondrial) and non-physiological protein partners,[1,9–23] in order better to understand factors which influence protein reactivity. So far, a recurring theme is the relevance of the solvent accessible exposed heme edge of cytochrome c. However with different reagents different localities around this edge are involved. Previously the preferred sites for two inorganic oxidants $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) have been identified.[4] Whereas $[Fe(CN)_6]^{3-}$ is most influenced by the modification at the lysine 72 site to the left of the exposed heme edge, with $[Co(phen)_3]^{3+}$ it is the region around lysine 27 to the right of the heme edge that is most important. In this work the reactions of two reductants, the ethylenediaminetetra-acetate complex of Fe^{II}, here written as $[Fe(edta)]^{2-}$, and the sepulchrate cage Co^{II} complex, $[Co(sep)]^{2+}$, with cytochrome c in the Fe^{III} state are studied. The effect which different ligands on the inorganic reagent have is of interest.

Use has been made of n.m.r. spectroscopy to investigate the interaction of $[Fe(CN)_6]^{3-}$ and $[Fe(edta)(H_2O)]^{-}$ with cytochrome c also in the Fe^{III} state, cyt c(III).[24–27] Since this is a situation in which no electron transfer is observed, it is important to compare the two approaches. The n.m.r. work was with tuna cytochrome c at pH 6.0, and some initial work was required therefore to ensure that the different protein source and conditions were not important.

Experimental

Protein.—Native horse-heart and tuna cytochrome c (Sigma) were purified by the procedure already described.[28] The singly modified 4-carboxy-2,6-dinitrophenyl (cdnp) lysine derivatives of horse-heart cytochrome c and the 2,4,6-trinitrophenyl (tnp) derivative were obtained and generously supplied by Professor Margoliash (Northwestern) by modification as in equation (1),[1–3] where RNH_2 represents the lysine residue undergoing modification. The cdnp modification replaces the 1+ charge of RNH_3^+ at pH 7.5 by a 1− charge. The cytochrome c derivatives were recycled after use. To convert to the cyt c(III) form a few crystals of $K_3[Fe(CN)_6]$ (BDH, Analar) were added and the protein then purified on a CM32 (Whatman) ion-exchange column (1 cm diameter by 3 cm long) equilibrated in 10 mM phosphate buffer at pH 7.0. The cytochrome c was eluted with 65 mM phosphate. Protein solutions were dialysed (Sigma 250-9U tubing) against the required buffer with three changes prior to use.



(1)

Complexes.—The aqua(ethylenediaminetetra-acetato)ferrate(III) complex, $Na[Fe(edta)(H_2O)]$ (Sigma Chemicals), was used without further purification. The sepulchrate complex $[Co(sep)]Cl_3 \cdot H_2O$, where sep is the cage ligand 1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]icosane, was prepared by the method described,[29] u.v.–visible peak positions: λ/nm (ε/M^{-1} cm^{-1}) 472 (109), 340 (116). Solutions of $[Fe(edta)]^{2-}$ generated in the presence of 20% excess $Na_2(edta)$ (BDH, Analar) (E^0 + 0.12 V),[30] and $[Co(sep)]^{2+}$ (E^0 − 0.26 V),[31] were prepared by controlled potentiometry using a Chemical Electronics Ltd. (Washington, Tyne & Wear) potentiostat model DD505U.

† edta = Ethylenediaminetetra-acetate, sep = sepulchrate (1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]icosane).

Non-S.I. unit employed: M = mol dm^{-3}.

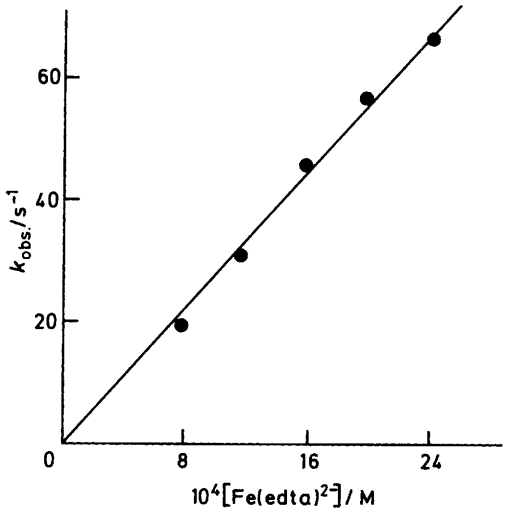


Figure 1. Dependence of first-order rate constants, $k_{obs.}$ (25 °C) for the reduction of horse heart cytochrome c(III) on $[Fe(edta)^{2-}]$ at pH 6.0 (cacodylate), I = 0.10 M (NaCl)

Concentrations of reductant were determined by titration against $[Fe(phen)_3]^{3+}$, prepared as $[Fe(phen)_3][ClO_4]_3 \cdot H_2O$,[32] using the strong absorbance due to $[Fe(phen)_3]^{2+}$ ($\Delta\varepsilon$ at 540 nm of 5 900 M^{-1} cm^{-1}), or by titration against $[Fe(CN)_6]^{3-}$ ($\Delta\varepsilon$ at 420 nm of 1 010 M^{-1} cm^{-1}).

Kinetics.—All reactions were studied at 25 °C. Sodium cacodylate, $Na[AsMe_2CO_2]$ (BDH, GPR grade) (0.050 M) in HCl at pH 6.0, and tris(hydroxymethyl)methylamine (Tris, Sigma Chemicals, 0.020 M) in HCl at pH 7.5, were used as buffers. The ionic strength was adjusted to I = 0.10 M with NaCl (BDH, Analar). The reductants were in > 10-fold excess of the cyt c(III). Reactions were monitored at 416 nm at which wavelength molar absorption coefficients for reduced and oxidised cytochrome c are 1.29×10^5 and 0.89×10^5 M^{-1} cm^{-1} respectively. In all cases the absorbance changes were consistent with 1:1 reduction of the cyt c(III) to cyt c(II) (E^0 0.26 V), equations (2) and (3).

$$[Fe(edta)]^{2-} + \text{cyt c(III)} \longrightarrow [Fe(edta)(H_2O)]^{-} + \text{cyt c(II)} \quad (2)$$

$$[Co(sep)]^{2+} + \text{cyt c(III)} \longrightarrow [Co(sep)]^{3+} + \text{cyt c(II)} \quad (3)$$

Kinetic changes were monitored on a Dionex D-110 stopped-flow spectrophotometer equipped with a logarithmic amplifier and the output stored digitally in a Datalab DL901 transient recorder. Reaction traces were displayed on an oscilloscope using the digital analog facility of the transient recorder. A Commodore PET 2001-16K computer was interfaced to the recorder, and absorbance changes ln $(A_\infty - A_t)$ against time were displayed. Such plots giving $k_{obs.}$ (slope) were linear to at least four half-lives.

Results

The reactivities of tuna and horse heart cytochrome c and effect of varying the pH (6.0—7.5) on the reactivity of horse-heart cytochrome c were first determined.* Five runs at 25 °C, pH 6.0 (cacodylate), in which the $[Fe(edta)]^{2-}$ concentration was varied in the range (5.5—17.2) $\times 10^{-4}$ M, and that of tuna cytochrome c(III) was varied in the range (1—2) $\times 10^{-6}$ M, gave a second-order rate constant (2.6 ± 0.3) $\times 10^4$ M^{-1} s^{-1}. Similarly, five runs in which the concentration of $[Fe(edta)]^{2-}$ was varied in the range (7.7—23.9) $\times 10^{-4}$ M with horse heart cytochrome c(III) (1—2) $\times 10^{-6}$ M, gave a rate constant (2.9 ± 0.2) $\times 10^4$ M^{-1} s^{-1} (Figure 1). At pH 7.5 (Tris) horse-heart cytochrome c gave a rate constant of (2.8 ± 0.2) $\times 10^4$ M^{-1} s^{-1} (Figure 2).

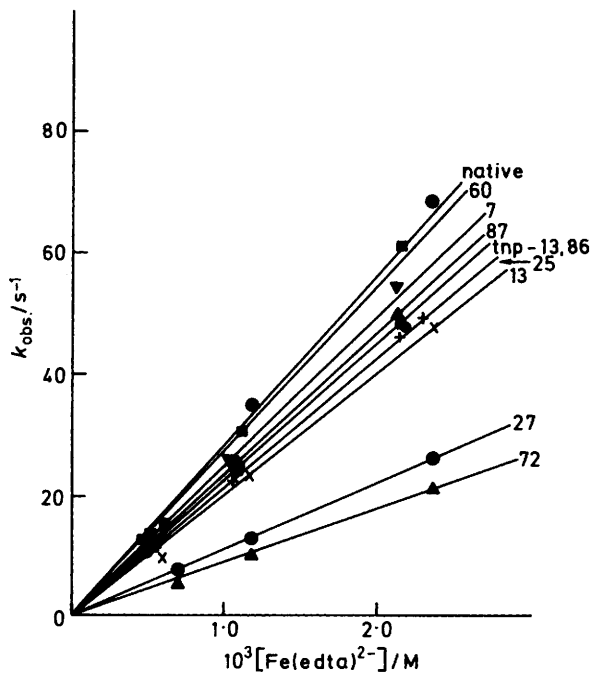


Figure 2. Dependence of first-order rate constants, $k_{obs.}$ (25 °C) on $[Fe(edta)^{2-}]$ for the reduction of cdnp- and tnp-modified horse heart cytochrome c(III) derivatives at pH 7.5 (Tris), I = 0.10 M (NaCl). The numbers refer to the sequence positions of the modified (cdnp, except where indicated) lysyl residues

First-order rate contants, $k_{obs.}$, for the reactions of native and modified horse heart cytochrome c derivatives also gave linear dependences on the reductant concentration, Figures 2 and 3. Second-order rate constants, k, defined in equation (4), are listed in the Table. Each rate constant was obtained from an average of *ca.* 20 kinetic traces using three different concentrations of inorganic complex. The effect of the cdnp modifications is to decrease rate constants with $[Fe(edta)]^{2-}$ as reductant, and to increase those with $[Co(sep)]^{2+}$. The effectiveness of each modification as compared to native cytochrome c is clearly illustrated in Figures 2 and 3. The tnp-13 modification, which replaces the 1+ charge of RNH_3^+ with a neutral charge, has an intermediate effect on rate constants in each case.

$$k_{obs.} = k[\text{reductant}] \quad (4)$$

Discussion

Reactivity contour maps illustrating the influence of the different modifications on electron transfer rate constants for horse heart cytochrome c are shown in Figure 4. These maps were obtained from the ratio R defined in equation (5) for $[Fe(edta)]^{2-}$, or the inverse for $[Co(sep)]^{2+}$. The logarithmic term is appropriate to give a measure of the difference in

* Tuna cytochrome c was selected for the n.m.r. studies because it does not have a histidine residue at position 33 with a pK_a of 6.5.[33] A pH of 6.0 was appropriate in experiments with $[Fe(edta)(H_2O)]^{-}$ to avoid effects stemming from the acid dissociation of the H_2O ligand (pK_a 7.6).[34] Cacodylate was used because it does not appear to bind to cytochrome c.[35]

Table. Second-order rate constants k (25 °C) at pH 7.5 (Tris) for the reduction of native and chemically modified cytochrome c by $[Fe(edta)]^{2-}$ (k_{Fe}) and $[Co(sep)]^{2+}$ (k_{Co}), I = 0.10 M (Tris)

Modified site	$10^{-4}k_{Fe}$/ M^{-1} s^{-1}	$\ln\left[\frac{k_{Fe}(native)}{k_{Fe}(derivative)}\right]$	$10^{-5}k_{Co}$/ M^{-1} s^{-1}	$\ln\left[\frac{k_{Co}(derivative)}{k_{Co}(native)}\right]$
Native	2.80		2.60	
cdnp-60	2.76	0.01	2.65	0.02
cdnp-7	2.38	0.16	3.47	0.29
cdnp-87	2.31	0.19	2.88	0.10
cdnp-86	2.29	0.20	3.06	0.16
cdnp-25	2.07	0.30	4.30	0.50
cdnp-27	1.07	0.96	7.19	1.02
cdnp-13	1.90	0.39	3.72	0.36
tnp-13	2.29	0.20	3.02	0.15
cdnp-72	0.85	1.19	3.53	0.31

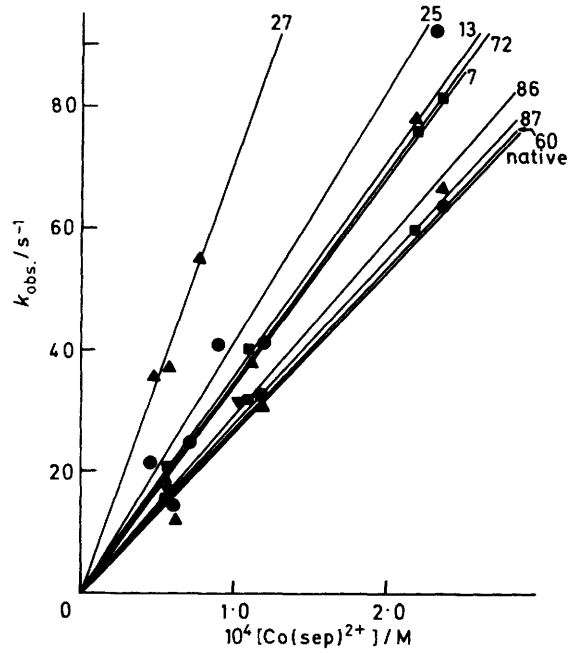


Figure 3. Dependence of first-order rate constants, $k_{obs.}$ (25 °C), on $[Co(sep)^{2+}]$ for the reduction of cdnp- and tnp-modified horse heart cytochrome c(III) derivatives at pH 7.5 (Tris), I = 0.10 M (NaCl). The numbers refer to the sequence positions of the (cdnp) modified lysyl residues

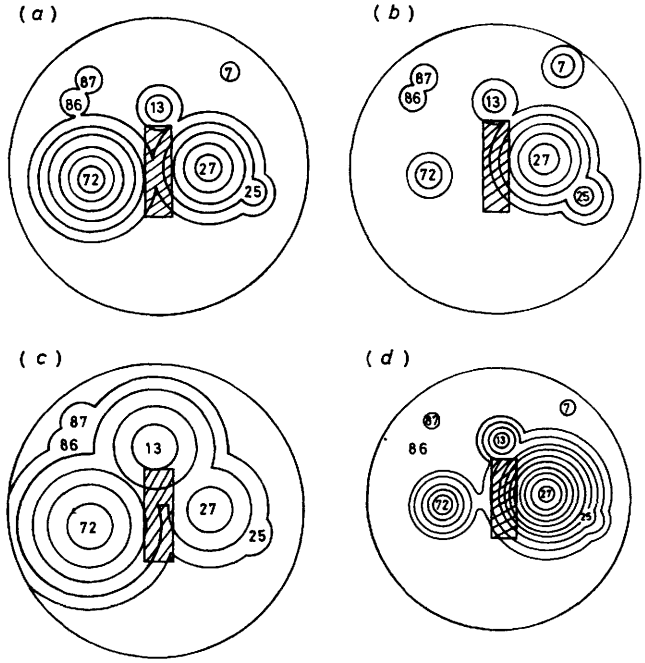


Figure 4. Reactivity contour maps representing a planar projection of the front hemisphere for (*a*) $[Fe(edta)]^{2-}$ + cyt c(III), (*b*) $[Co(sep)]^{2+}$ + cyt c(III), (*c*) $[Fe(CN)_6]^{3-}$ + cyt c(II), (*d*) $[Co(phen)_3]^{3+}$ + cyt c(II). The shaded area represents the exposed heme edge, and the numbers refer to the positions of the α-carbon atoms of the modified lysyl residues of the cytochrome c derivatives examined

activation free energy for the native and modified protein derivative. For a line joining two centres of modification each contour line represents a change of 0.2 units in R. The lysine residue which is surrounded by the largest number of concentric circles constitutes the position of greatest influence. In the case of $[Fe(edta)]^{2-}$ this represents a decrease in rate constant and for $[Co(sep)]^{2+}$ an increase which is according to the charge introduced by the modification. Reactivity contour maps for the two oxidants $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ are also included in Figure 4.[4]

$$R = \ln[k(\text{native})/k(\text{derivative})] \quad (5)$$

Comparison of the contour maps confirms the importance of the solvent accessible exposed heme edge as a centre for electron transfer. The modification at lysine 60 on the remote side of the protein has little or no effect. Reactions involving the negatively charged complexes are affected most by the modification at lysine 72. For the $[Fe(edta)]^{2-}$ reactant there is a shift to greater involvement of lysine 27 instead of lysine 13 for $[Fe(CN)_6]^{3-}$. The positively charged complexes $[Co(sep)]^{2+}$ and $[Co(phen)_3]^{3+}$ are most influenced by the modification at lysine 27. Interaction at these respective sites must constitute a lower coulombic barrier although the source of the effects (and precise residues involved) is not entirely clear. The results obtained with the 3+ and 2+ complexes establish that the aromatic and aliphatic ligands have little influence on site selectivity. Also the oxidation state of the protein has little or no effect on the site of electron transfer. Differences in rate constants for the reactions of tuna and horse heart cytochrome c(III) with $[Fe(edta)]^{2-}$ suggest that amino-acid variations for the two cytochromes do not produce significant changes in reactivity. Of the lysine

residues considered in this study only lysine 60 is not conserved in tuna cyt c(III). Modification of this residue in horse heart cyt c(III) has no effect on rate constants. Rate constants for the reduction of horse-heart cytochrome c with $[Fe(edta)]^{2-}$ at pH 6 in cacodylate (2.6×10^4 M^{-1} s^{-1}), at pH 7.0 in phosphate (2.6×10^4 M^{-1} s^{-1}),[36] and at pH 7.5 in Tris (2.8×10^4 M^{-1} s^{-1}), all at 25 °C with $I = 0.10$ M (NaCl), confirm that there are no significant effects of pH or buffer over this range.

The greater influence of the lysine 27 region in the case of the $[Fe(edta)]^{2-}$ reduction as compared to the $[Fe(CN)_6]^{3-}$ oxidation is in excellent agreement with the n.m.r. results in which the binding of $[Fe(edta)(H_2O)]^-$ and $[Fe(CN)_6]^{3-}$ to cytochrome c(III) were explored.[24–27] The more extensive work with $[Fe(CN)_6]^{3-}$ has implicated up to four sites for interaction.[27] These need not all be relevant to electron transfer and are not expected to be equally relevant in view of the greater distance separating some of the sites from the heme group. Previous work[4] singles out the lysine 72 site as contributing most strongly to electron transfer. In neither the $[Fe(edta)]^{2-}$ or $[Fe(CN)_6]^{3-}$ reactions[4] has any evidence for limiting kinetics been obtained indicative of a strong association of the reactants (K) prior to electron transfer. An upper limit of 200 M^{-1} was indicated in the $[Fe(CN)_6]^{4-}$ reduction of cytochrome c(III).[37] In the case of the $[Fe(edta)]^{2-}$ reduction a value of $K \leqslant 25$ M^{-1} is suggested by the results obtained for horse heart cytochrome c (Figure 1). It is not clear from the kinetic experiments whether for $[Fe(edta)]^{2-}$ a single reaction centre encompassing lysine 72 and 27 is relevant, or whether two separate reaction sites should be invoked. More detailed structuring within the lysine 27 site is required in order to account for the influence of this region on the reactions of both positively and negatively charged inorganic complexes.

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