Electron-transfer Reactions of Cytochrome b_5: Binding Site for Inorganic Complexes and Cytochrome c

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Redox inactive $[Cr(en)_3]^{3+}$ (en = ethylenediamine) has been shown to inhibit the oxidation of cytochrome b_5 with $[Co(NH_3)_6]^{3+}$, $[Pt(NH_3)_6]^{4+}$, and $[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$, and block association with cytochrome c.

Cytochrome b_5 is a negatively charged (pH 7) membrane bound mono-haem protein which is known to participate in a number of important oxidation–reduction processes involving stearyl-CoA desaturation,[1] cytochrome P450 reduction,[2] methemoglobin reduction,[3] sulphite oxidation,[4] and reduction of cytochrome c.[5] Although studies in which inorganic complexes are used as redox partners for such metalloproteins are of considerable interest and merit attention in their own right, some concern is often expressed as to whether they relate to natural processes involving protein–protein reactions. Here we describe experiments in which the reaction site on cytochrome b_5 used by the oxidants $[Co(NH_3)_6]^{3+}$, $[Pt(NH_3)_6]^{4+}$, and $[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$ is also implicated in the reaction with cytochrome c which is positively charged at pH 7.

The haem group of cytochrome b_5 is held in a hydrophobic pocket with Fe (oxidation states II and III) co-ordinated axially to two histidine side chains. The tryptic fragment (84 amino acids, M.Wt. *ca.* 9400) of the protein was isolated from fresh calf liver,[6] and purified to a u.v.–visible absorbance ratio $A_{413}/A_{280} = 5.8$. At 25 °C the reduction potential for the cytochrome b_5(III)/(II) couple is 5 mV (*vs.* normal hydrogen electrode) at pH 7.0 (phosphate), $I = 0.10$ M,[7] and has a similar value (20 mV) when bound to microsomes.[8] Cytochrome b_5 concentrations were determined from the absorbance at 413 nm (ϵ 1.17 × 10^5 M^{-1} cm^{-1}), and kinetic runs were monitored at 555 nm by the stopped-flow method.

First-order rate constants, k_{obs}, for the one-electron oxidation of cytochrome(cyt) b_5(II) with $[Co(NH_3)_6]^{3+}$, $[Pt(NH_3)_6]^{4+}$, and $[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$ (oxidant in ⩾10-fold excess) give a less than first-order dependence on the oxidant. This can be summarised by equation (1), which is consistent

$$k_{obs} = Kk_{et}[\text{oxidant}]/(1 + K[\text{oxidant}]) \quad (1)$$

with a mechanism involving association of the reactants (K) prior to electron transfer (k_{et}), equations, (2) and (3). Rate

$$\text{cyt } b_5(\text{II}) + \text{oxidant} \overset{K}{\rightleftharpoons} \text{cyt } b_5(\text{II}), \text{oxidant} \quad (2)$$

$$\text{cyt } b_5(\text{II}), \text{oxidant} \xrightarrow{k_{et}} \text{products} \quad (3)$$

constants were determined assuming the second stage of the conversion $Pt^{IV} \rightarrow Pt^{III} \rightarrow Pt^{II}$ to be rapid, and of the change $Co^{III}{}_2 \rightarrow Co^{III} + Co^{II} \rightarrow 2\ Co^{II}$ to be slow and negligible. Both assumptions are supported by earlier studies.[9] Values of K and k_{et} are listed in Table 1.

The observation that association (K) prior to electron transfer (k_{et}) can be detected for 3+, 4+, and 5+ oxidants introduces the possibility of adding redox inactive complexes

Table 1. Summary of data from kinetic studies on the oxidation of cytochrome b_5(II) with inorganic complexes at 25 °C, pH 7.4, ionic strength $I = 0.10$ M (NaCl).

	K /M^{-1}	k_{et} /s^{-1}
$[Co(NH_3)_6]^{3+}$ [a]	600	0.075
$[Pt(NH_3)_6]^{4+}$ [b]	14,800	0.080
$[(NH_3)_5Co{\cdot}NH_2{\cdot}Co(NH_3)_5]^{5+}$ [b]	16,600	3.8

[a] 2×10^{-2} M phosphate buffer. [b] 10^{-2} M Tris/HCl buffer. Phosphate gives a precipitate with the Pt^{IV} complex and is assumed to interact also with the 5+ complex.

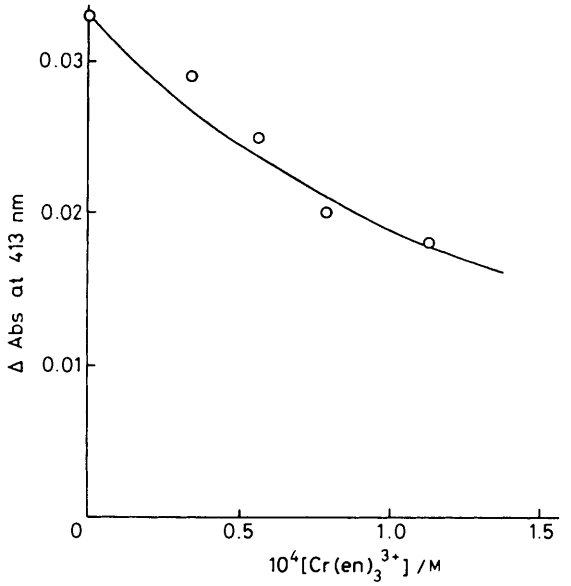

Figure 1. The effect of $[Cr(en)_3]^{3+}$ on the absorbance change (Δ Abs) at the cytochrome b_5(III) (3.56×10^{-6} M) peak at 413 nm on addition of cytochrome c(III) (3.60×10^{-6} M) at 25 °C, pH 7.0 (phosphate), ionic strength, I = 0.010 M (NaCl).

of similar charges as inhibitors. Competitive inhibition was observed in all three cases with redox inactive $[Cr(en)_3]^{3+}$ (K_{Cr} 309 M^{-1}; en = ethylenediamine). The excellent fit of data to equation (4), in the case of $[Co(NH_3)_6]^{3+}$ suggests complete

$$k_{obs} = \frac{Kk_{et}}{1 + K_{Cr}[Cr^{III}] + K[\text{oxidant}]} \quad (4)$$

blocking of the redox process, and that a specific as opposed to broad region of protein surface (such as is effective in reactions of plastocyanin[10]) is involved. The most likely explanation is that the exposed haem edge is the site of electron transfer since, from crystal structure information, this is known to be surrounded by negatively charged carboxylate residues.[11]

On mixing cytochrome b_5(III) with cytochrome c(III), a difference spectrum is obtained and the absorbance at 413 nm in the Soret region changes.[12] Other reports have provided supporting evidence for 1 : 1 adduct formation.[13,14] At 25 °C, pH 7.0 (phosphate), and an ionic strength I of 0.010 M (so that more extensive association is obtained), an association constant K of 8.3×10^4 M^{-1} has been obtained.[12] Using the same procedure we have demonstrated that $[Cr(en)_3]^{3+}$ impedes association with cytochrome c, as illustrated in Figure 1. Since cytochrome c(III) has charge 9+ this is believed to originate from $[Cr(en)_3]^{3+}$ associating with the cytochrome b_5(III), charge 9−. The specificity of $[Cr(en)_3]^{3+}$ and other positively charged complexes for a site on cytochrome b_5, and an overlap of this site with that used by cytochrome c is clearly indicated in these studies.

From crystallographic information Salemme[15] has proposed a detailed model for association of the two proteins in which positive (cytochrome c) and negative (cytochrome b_5) charges around the exposed haem edges are involved, and the two haems take up nearly coplanar positions. The present studies with inorganic complexes, in particular the magnitude of K values, indicate a dependence on electrostatics which adds support to this approach.

Received, 13th May 1983; Com. 612

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