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Reactivity of cyancytochrome *c*

Ferricytochrome *c* reacts slowly with cyanide, the absorption maximum in the visible region shifting from 530 to 535 mμ (ref. 1). The subsequent addition of dithionite causes the appearance of a reduced compound (555 mμ α-band), presumably ferrocytochrome *c*–cyanide[2], which slowly gives rise to normal ferrocytochrome *c* (550 mμ α-band). These complexes are of interest because their properties may help to define the ligands in the 5th and 6th coordination positions[3,4], and provide clues concerning the mechanism of electron transfer[5] to and from the heme group of cytochrome *c*.

GEORGE AND SCHEJTER[2] observed the spectrum of the cyanferrocytochrome *c* complex by rapid scanning at 2°. We have obtained it by adding dithionite to cyanferricytochrome at a series of single wavelengths and extrapolating the observed absorbances to zero time. Using horse-heart cytochrome *c* (Sigma type III), reduced with palladium and hydrogen, or oxidized by ferricyanide followed by dialysis, reactions were followed spectrophotometrically in pH 7.0 to 7.4 phosphate or Tris–maleate buffers (Tris–maleate is preferable for reactions involving copper), with a Zeiss PMQ II single-beam spectrophotometer. Ferrocytochrome *c*–cyanide was readily formed by adding a few mg $Na_2S_2O_4$ to 3 ml of 15 μM ferricytochrome *c* previously incubated in 0.03 M Tris–maleate, 9 mM KCN, final pH 7.4. Excess dithionite may be removed by rapid aeration.

Addition of dithionite to cyancytochrome *c* gives a rapid rise in absorbance at 550 mμ followed by a slow rise to the absorption characteristic of ferrocytochrome *c*. At 555 mμ, a rapid rise to a high absorption value is followed by a slow fall to the absorption level of reduced cytochrome *c*. These phenomena are consistent with the scheme of Eqn. 1 (*cf.* Eqns. 1–3 of GEORGE AND SCHEJTER[2]):

$$c\mathrm{Fe}^{3+}\,\mathrm{CN}^- + \mathrm{e}^- \xrightarrow{\text{fast}} c\mathrm{Fe}^{2+}\,\mathrm{CN}^- \xrightarrow{\text{slow}} c\mathrm{Fe}^{2+} \qquad (1)$$

Table I compares the rates of cyanferrocytochrome *c* disappearance obtained by GEORGE and in the present investigation with the rates of ferrocytochrome *c* formation. The two events are evidently synchronous, with no evidence for any intermediates. This was confirmed qualitatively by means of a series of rapid scans through

TABLE I

RATES OF DISSOCIATION OF CYANIDE FROM CYANFERROCYTOCHROME *c*

Ref.	*Medium*	*First-order velocity constants* (sec^{-1})	
		$cFe^{2+}\,CN^-$ *decay* §	cFe^{2+} *formation* §§
GEORGE AND SCHEJTER[2]	Phosphate*	$4.3 \cdot 10^{-3}$	—
This study	Phosphate**	$3.8 \cdot 10^{-3}$	$4.1 \cdot 10^{-3}$
This study	Tris–maleate***	$4.9 \cdot 10^{-3}$	$4.7 \cdot 10^{-3}$

* 0.10 M phosphate, pH 7.0, 0.05 M cyanide, 25°.
** 0.10 M phosphate, pH 7.4, 0.009 M cyanide, 20–25°.
*** 0.03 M Tris–maleate buffer, pH 7.4, 0.009 M cyanide, 20–25°.
§ Measured by decrease in absorbance at 555 mμ.
§§ Measured by slow increase in absorbance at 550 mμ.

the 560–540-mμ region during the reaction using a Beckman DB spectrophotometer.

Fig. 1 gives the spectrum of cyanferrocytochrome *c* obtained by the extrapolation method. The maximum absorption is at the same wavelength as previously reported[2] for 2°, but the intensity at 550 mμ is slightly less than that given by GEORGE AND SCHEJTER[2], *i.e.*, the peak appears sharper. The isosbestic point at 553 mμ could be used to detect the appearance of ferricytochrome *c* in mixtures of the ferrous derivatives.

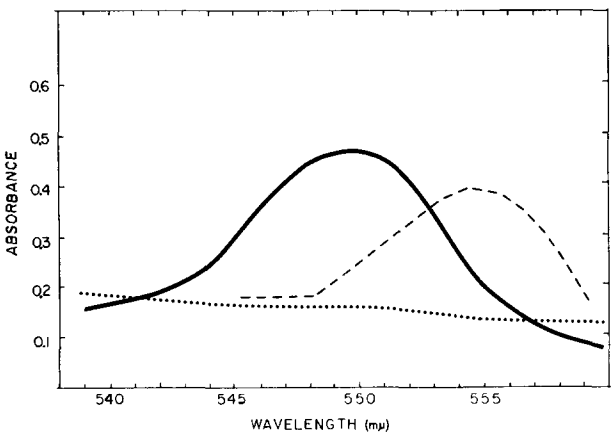

Fig. 1. Spectra of cytochrome *c* and of cyanferrocytochrome *c* in the region of the α-band, obtained by extrapolation to zero time at each wavelength (as described in text). Solutions contained 16.9 μM cytochrome *c* in 0.1 M Tris–maleate buffer, pH 7.0, at 25° in the presence and absence of 0.05 M CN^-. Reduction was achieved by the addition of a few mg dithionite. ———, ferrocytochrome *c*; – – – –, cyanferrocytochrome *c*; ·····, ferricytochrome *c* (indistinguishable from cyanferricytochrome *c* in this region).

Small amounts of $K_3Fe(CN)_6$, down to levels approximately stoichiometric with ferrocytochrome *c* present if dithionite has been completely removed, cause an immediate collapse of the absorption at 553 mμ, concomitant with the formation of cyanferricytochrome *c* (as demonstrated by its inability to be reduced by ascorbate). The cyanide complex thus reacts with ferricyanide without an initial or subsequent dissociation of the cyanide. Whether the rate of reaction is slower than that of free ferrocytochrome *c* ($1.6 \cdot 10^7$ M^{-1} sec^{-1})[6] is uncertain. The present observations can only give a lower bound of $\sim 10^5$ M^{-1} sec^{-1} for this rate. In contrast, the oxidation of the complex by $FeSO_4$ in air, or by $Fe_2(SO_4)_3$, was slow, compared with the effects of these ions on free ferrocytochrome *c*. The oxidation by $CuSO_4$ was also slow when followed at 553 mμ; measurements at 555 mμ and 550 mμ, however, showed an increase in the rate of cyanide dissociation induced at a copper concentration (1.5 mM) causing oxidation of normal ferrocytochrome *c* (Table II). Ferric ions may produce a similar effect, but the rate was too small for it to be distinguished from the spontaneous rate.

It is possible that cyanferrocytochrome *c* and copper form an unstable bridged intermediate which decays with transfer of the "pontal group" but without electron transfer (Eqn. 2). The fates of the reacting cyanide and copper moieties have, however,

$$c\mathrm{Fe}^{2+}\ \mathrm{CN}^- + \mathrm{Cu}^{2+} \xrightarrow{k_1} (c\mathrm{Fe}^{2+}\ \mathrm{CNCu}^+) \xrightarrow{k_2} c\mathrm{Fe}^{2+} + \mathrm{CuCN}^+ \qquad (2)$$

yet to be determined. The absence of spectroscopic evidence for the proposed intermediate indicates that $k_2 \gg k_1$ [Cu^{2+}] when [Cu^{2+}] < 2 mM.

TABLE II

REACTIONS OF CYTOCHROME *c* WITH INORGANIC COPPER IONS

Reaction *	*Pseudo-first-order velocity constants* (sec^{-1})	
	550 mμ **	*555 mμ* ***
(a) 1.5 mM Cu^{2+} *plus* cFe^{2+} CN^-	$2.8 \cdot 10^{-2}$	$3.8 \cdot 10^{-2}$
(b) 1.5 mM Cu^{2+} *plus* normal cFe^{2+}	$1.5 \cdot 10^{-2}$	—

* 0.03 M Tris–maleate buffer, ± cyanide, as in Table I.
** Formation of ferrocytochrome *c* (a), or ferricytochrome *c* (b).
*** Decay of cyanferrocytochrome *c*.

These reactions may provide models for both oxidative and energy conserving enzymic processes. Two intramolecular "routes" of cytochrome *c* reduction and oxidation may normally be available[5,7], the first being the path taken by the electron in reduction of endogenous *c* by the mitochondrion or in oxidation by reversed electron transfer, the second being the path used in oxidation by the oxidase or in the reduction of exogenous *c* by submitochondrial particles[7] mediated by cytochrome *a*. Evidently cyanide disrupts the "route" (or mechanism) involved in oxidation by copper, but not that involved in oxidation by ferricyanide. The absence of reduction by succinic–cytochrome *c* reductase and by ascorbate[1,2] may be due to the unfavourable redox potential of the cyanferro–cyanferricytochrome system, and not to any "mechanistic" fault. Whether cytochrome oxidase oxidizes cyanferrocytochrome *c* (*i.e.*, behaves like copper or like ferricyanide) is also unknown, as cyanide is a potent inhibitor of the oxidase; attempts to prepare cyanferrocytochrome *c* in cyanide-free media are being made to test this point.

The reduction of cyanferricytochrome *c* by dithionite can be regarded as an "energy-conserving" reaction when compared with the corresponding reduction of uncombined cytochrome *c*. The low redox potential of the cyancompound[1], the effect of pH on the equilibria with cyanide[8], and the failure of ferrocytochrome *c* to bind cyanide directly even at very high cyanide concentrations[1,8], all indicate the dissociation of cyanferrocytochrome *c* (Eqn. 1) to be a highly exergonic process. The sequence of Eqn. 3, given by CHANCE AND WILLIAMS[9], may be compared with that of Eqn. 4, proposed here to account for the reactions with cyanide and copper. It is not unreasonable to suppose that, although this particular speculative analogy may be no

$$c^{III} + I \rightleftharpoons c^{III}\,I \qquad c^{III} + CN^- \rightleftharpoons c^{III}\,CN^-$$

$$c^{III}\,I + b^{II} \rightleftharpoons b^{III} + c^{II} \sim I \quad (3) \qquad c^{III}\,CN^- + e^- \longrightarrow c^{II}\,CN^- \quad (4)$$

$$c^{II} \sim I + X \rightleftharpoons c^{II} + X \sim I \qquad c^{II}\,CN^- + Cu^{2+} \longrightarrow c^{II} + CuCN^+$$

more than that, the formation of "high energy" carrier compounds may be due to the oxidation or reduction of a metal group bound to a dissociable ligand with very different affinities for the two oxidation states.

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