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FERROCYANIDE AS ELECTRON DONOR TO CYTOCHROME aa_3

CYTOCHROME *c* REQUIREMENT FOR OXYGEN UPTAKE

K. KRAB * and E.C. SLATER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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Summary

1. In the absence of cytochrome *c*, ferrocyanide or ferrous sulphate reduces cytochrome *c* oxidase (EC 1.9.3.1), but no continuous oxygen uptake ensues, as it does with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or reduced phenazine methosulphate as reductants, unless a substoichiometric amount of cytochrome *c* or an excess of clupein is present. Cytochrome *c* cannot be replaced by porphyrin cytochrome *c*.

2. Cytochrome *c*, porphyrin cytochrome *c* and clupein all stimulate the reduction of cytochrome aa_3 by ferrocyanide.

3. A model is proposed to explain these findings in which a high-affinity site for cytochrome *c* on the oxidase regulates the access of hydrophilic electron donors to a low-affinity site, and reduction via the high-affinity site is required for continuous oxygen uptake.

4. Furthermore, it is shown that upon reaction of oxidase with ferrocyanide, cyano-oxidase is formed.

Introduction

The object of this paper is to shed some light on the nature of the reaction of cytochrome *c* oxidase with its substrate cytochrome *c*. At present it is

* Present address: Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, 00170 Helsinki 17, Finland.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Mops, morpholinopropane sulphonic acid.

suspected, but still not certain, that cytochrome *c* has a function in the enzyme-catalysed reduction of oxygen, in addition to that of being the source of electrons. As early as 1960, Sekuzu et al. [1] reported that isolated cytochrome *c* oxidase requires cytochrome *c* to be oxidizable by oxygen, but Minneart was unable to confirm this [2]. Indeed, oxidation of TMPD is catalysed by the oxidase in the absence of cytochrome *c* (see below). It is noteworthy that Jacobs and coworkers [3] have shown that tetrahalogenated *p*-benzoquinols can act as substrate only in the presence of polyvalent cations such as polylysine.

However, it is clear that cytochrome *c* does affect some properties of the oxidase, e.g. the equilibration during a potentiometric redox titration of highly purified cytochrome a_3 (explaining the results of Tiesjema et al. [4,5]) and the stability of the so-called oxygenated form of the oxidase [6]. Furthermore, Hartzell and Beinert [7] have reported that cytochrome *c* affects reoxidation of NADH-reduced cytochrome aa_3 by ferricyanide in such a way that copper and $g = 6$ signals disappear at higher ferricyanide concentrations.

To study the role of cytochrome *c*, the approach has been adopted that in the reaction:

$$4e + 4\,H^+ + O_2 \xrightarrow{\text{(oxidase)}} 2\,H_2O \qquad (1)$$

ferrocytochrome *c* may be replaced as a source of electrons by ferrocyanide and some other reductants, so that the effects of small amounts of cytochrome *c* on the reaction may be studied. A comparison has been made with the effects of porphyrin cytochrome *c* (cytochrome *c* from which the heme iron has been removed) and of a positively charged protein (clupein), substances which act as competitive inhibitors of the oxidase with respect to cytochrome *c* [8,9].

It is shown that, when ferrocyanide is the source of electrons, cytochrome *c* is needed to sustain oxygen consumption. On the basis of specific effects of the substances mentioned above, a model is proposed to account for the effects of cytochrome *c* on the oxidase. An inhibitory action of ferrocyanide on the oxidase [10] is also briefly discussed.

Methods

Cytochrome *c* oxidase (EC 1.9.3.1) was prepared according to the methods of Fowler et al. [11] and McLennan and Tzagoloff [12] as modified by Van Buuren [13]. Horse-heart cytochrome *c* was prepared by the method of Margoliash and Walasek [14]. Porphyrin cytochrome *c* was a gift of Dr. J. de Kok, who prepared it by the method of Flatmark and Robinson [15], and purified it according to Fisher et al. [16]. Clupein (protamine sulphate from herring, molecular weight 5000) was obtained from British Drug Houses. Tween 80 was obtained from Sigma; other chemicals were purchased from British Drug Houses, Merck or Calbiochem. All chemicals were of analytical grade.

Oxygen consumption was measured polarographically with a Clark-type electrode. Optical spectra and dual-wavelength measurements were made with an Aminco DW-2 UV/visible double-beam spectrophotometer.

Experiments were carried out in a standard medium containing 235 mM

sucrose, 10 mM NaCl, 10 mM sodium-Mops and 1% (v/v) Tween 80. The pH was 7.0 and the temperature 25°C. Potassium ferrocyanide, potassium ferricyanide and sodium ascorbate (pH 7.0) solutions were prepared freshly each day. Oxidase concentrations were determined using an extinction coefficient of 24 $mM^{-1} \cdot cm^{-1}$ at 605 nm [17].

Results

Ferrocyanide as reductant

In Fig. 1, trace A, it is shown that ferrocyanide alone cannot reduce oxygen via cytochrome *c* oxidase. The presence of a small amount of cytochrome *c* is required. The oxygen uptake in the presence of ferrocyanide and cytochrome *c* is slowed down by the production of ferricyanide; this can be seen from the facts that addition of ferricyanide inhibits the oxygen uptake (trace D) and that ascorbate (which has no effect in the absence of cytochrome *c*, not shown) overcomes the inhibition (compare trace D with trace A). Note that trace B shows that conditions are such that oxygen uptake via the pathway ascorbate, cytochrome *c*, oxidase is negligible. Much higher concentrations of cytochrome *c* are required (cf. Ref. 18). Trace C is a control showing that the oxygen uptake indeed takes place via the oxidase.

Fig. 2, trace A, shows that ferrocyanide alone reduces at least part of the heme *a* of the oxidase (maximal absorption at 605 nm). With cytochrome *c*

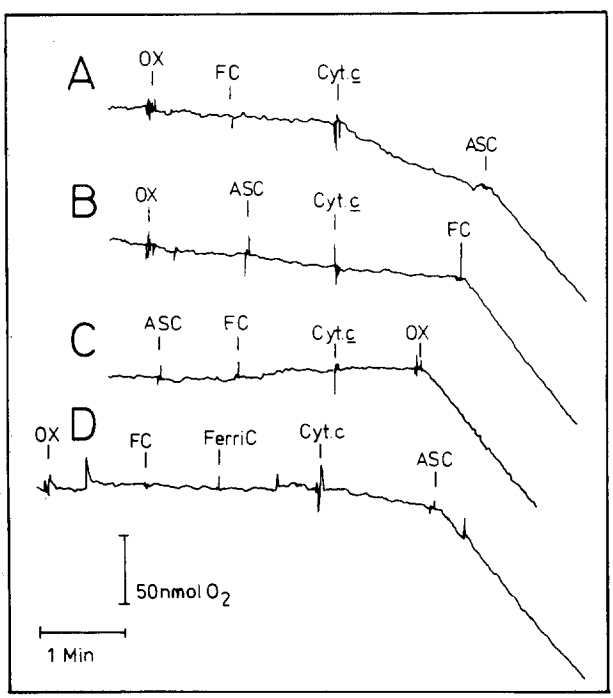


Fig. 1. Reduction of oxygen by ferrocyanide. 4 μM cytochrome *c* oxidase (Ox), 0.17 μM cytochrome *c* (Cyt. *c*), 1.67 mM ascorbate (Asc), 2 mM ferrocyanide (FC) and 0.2 mM ferricyanide (FerriC) were added as indicated to 1.5 ml of the medium described in Methods.

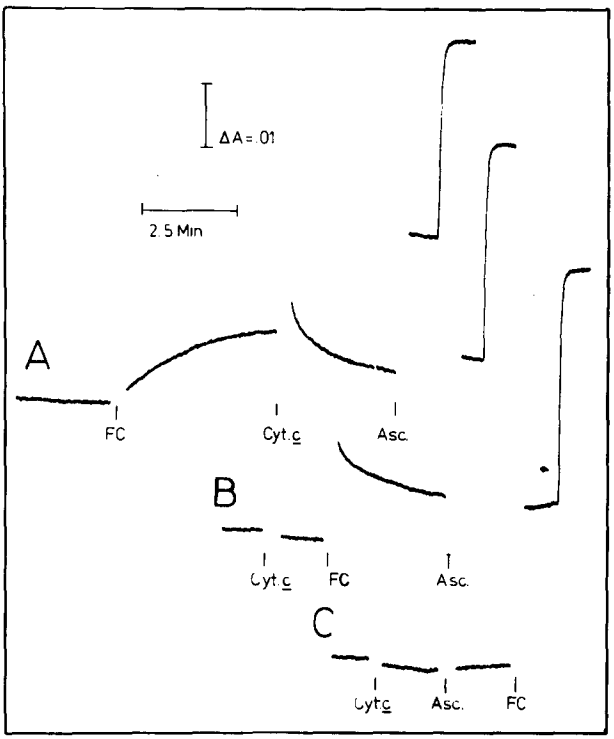


Fig. 2. Reduction of cytochrome *c* oxidase by ferrocyanide. 0.2 μM cytochrome *c* (Cyt. *c*), 1.7 mM ascorbate (Asc) and 5 mM ferrocyanide (FC) were added as indicated to 3 ml of the medium described in Methods containing 4 μM cytochrome *c* oxidase. Heme *a* reduction was measured at 605—590 nm, 1-cm light path.

present the reduction of heme *a* by the ferrocyanide is accelerated (trace B). When both ferrocyanide and cytochrome *c* are added, the redox state of the heme *a* follows the same pattern as the rate of oxygen consumption shown in Fig. 1, namely it decreases unless ascorbate is present. When all oxygen is consumed, the heme *a* is completely reduced. These experiments show that the lack of oxygen uptake with only ferrocyanide present is not due to the fact that ferrocyanide and oxidase do not react. However, the correlation between the reduction level of heme *a* and rate of oxygen uptake which can be observed when both ferrocyanide and cytochrome *c* are present does not hold for the appreciable reduction level obtained by addition of ferrocyanide alone, when there is no detectable oxygen consumption.

Both ascorbate and ferrocyanide in the concentrations used reduce free cytochrome *c* rapidly (within seconds) and completely. Under the conditions used (with the oxidase present), however, only ferrocyanide is able to reduce the cytochrome *c* (absorption peak appearing at 550 nm). Ascorbate does not reduce the heme *a* in this system, either with (Fig. 2, trace C) or without (not shown) cytochrome *c* present. The lack of reduction of cytochrome *c* by ascorbate in the presence of oxidase points to the formation of a complex between the oxidase and cytochrome *c* [18].

The formation of cyano-oxidase

Apart from being a reductant, ferrocyanide also inhibits the oxidase [10]. This is illustrated in Fig. 3, where it is shown that increasing the concentration of ferrocyanide results in a decrease in the rate of oxygen consumption, particularly when the oxidase and ferrocyanide are in contact before the reaction is started (compare traces C and D). Fig. 3E shows the effect of different concentrations of ferrocyanide on the rate of O_2 uptake.

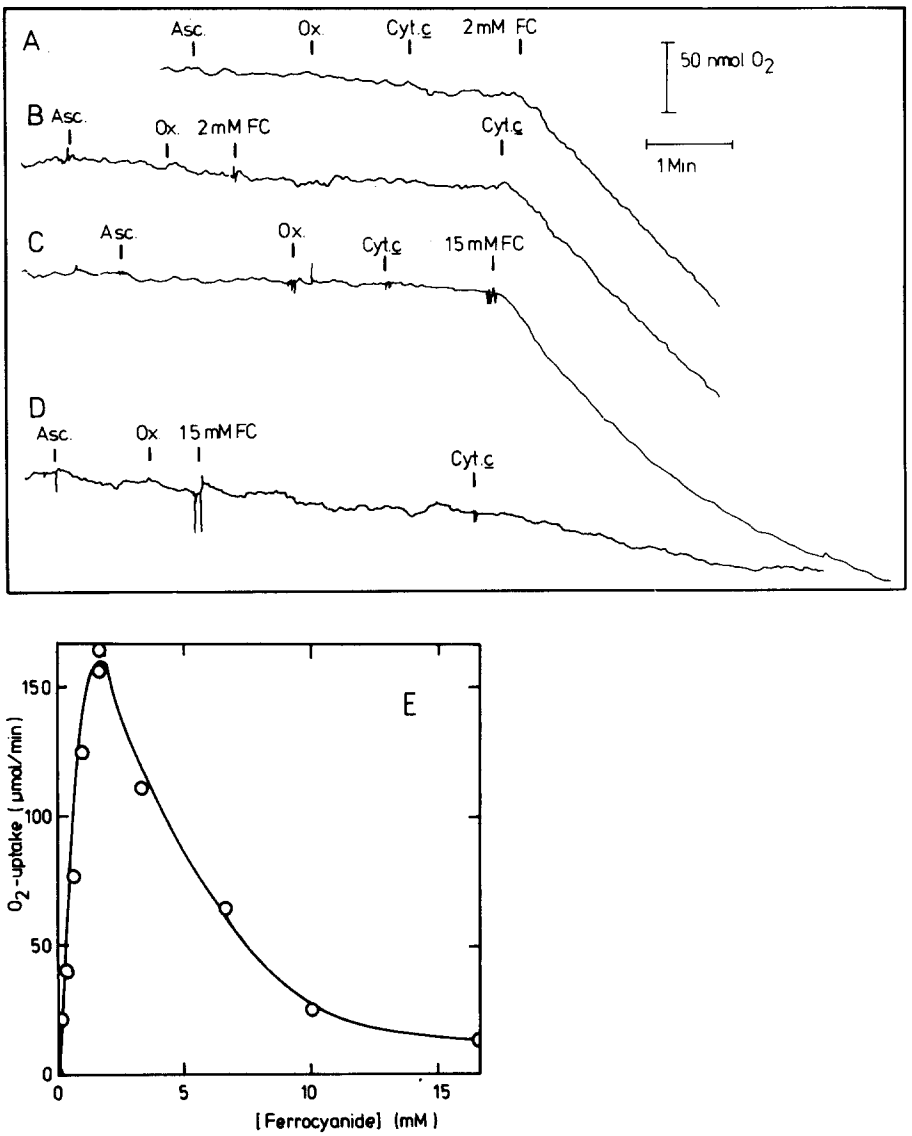


Fig. 3. The effect of ferrocyanide concentration on oxygen reduction in the presence of oxidase and cytochrome *c*. Conditions were as indicated in the legend to Fig. 1, except ferrocyanide concentrations, which are indicated in the figure. E gives the rate of O_2 uptake (measured after addition of cytochrome *c* as in trace 3D) as a function of ferrocyanide concentration.

In Fig. 4 it is shown that the reduced spectrum of the inhibited oxidase is remarkably similar to that of reduced cyano-oxidase (contrast Ref. 10). To check if free cyanide is responsible for the formation of cyano-oxidase, the effect of pyruvate has been tested. This ketoacid can react with cyanide to

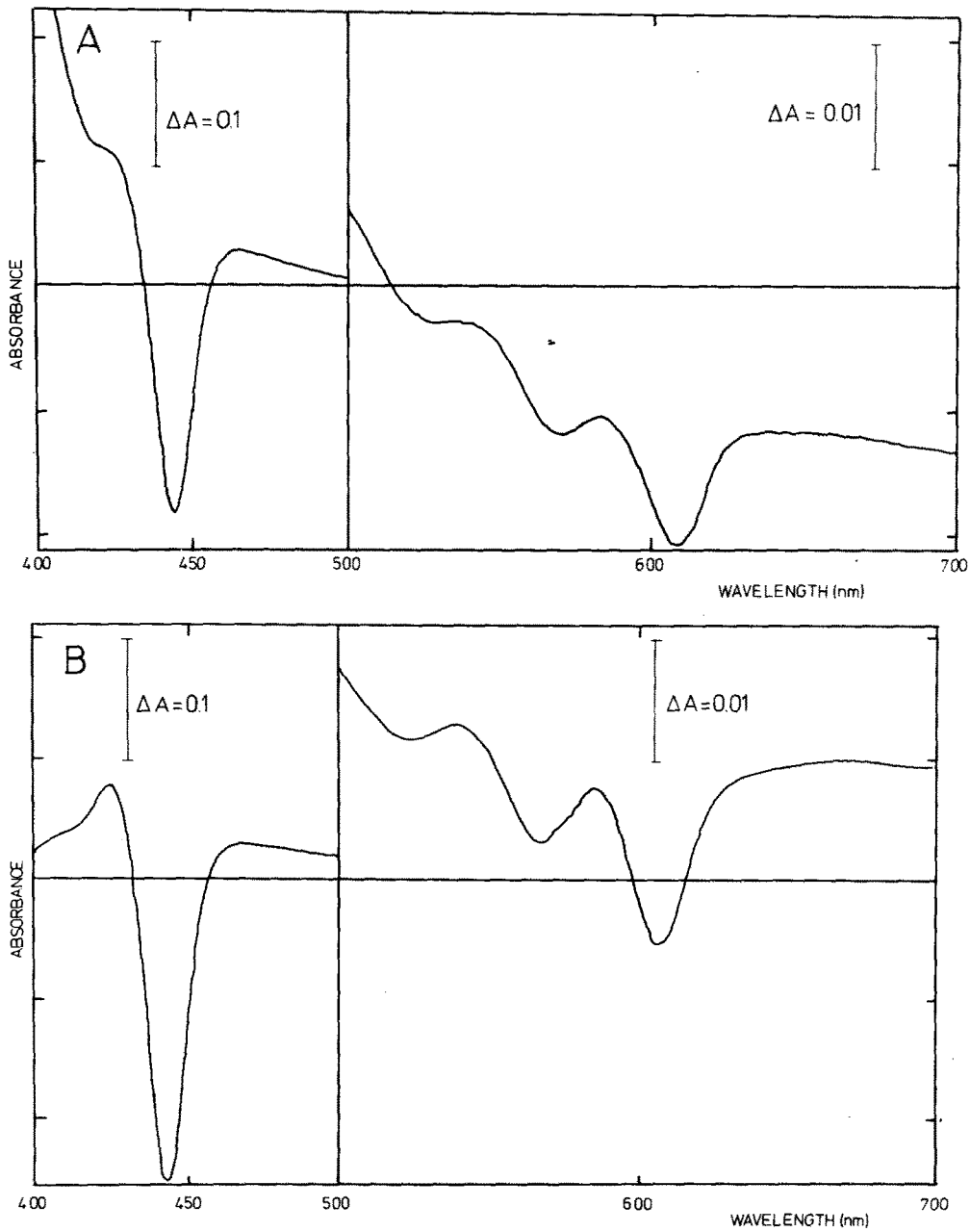


Fig. 4. The effects of ferrocyanide and cyanide on the spectrum of reduced cytochrome *c* oxidase. Oxidase (4 μM) was incubated in the medium described in Methods with 83 mM ferrocyanide (A) or 1.67 mM KCN (B) and then reduced with dithionite. The spectra shown are the measured spectra minus that of dithionite-reduced oxidase. 1-cm light path.

form a cyanohydrin and thus may act as a scavenger for cyanide [19]. Because of the high pyruvate concentration needed, a control with lactate (the corresponding 2-hydroxyacid) has been included. The results are shown in Table I. It can be seen that preincubation of ferrocyanide with pyruvate for 10 min before addition of oxidase diminishes the amount of 'cyano-oxidase' formed, just as is the case when cyanide and pyruvate are preincubated. When ferrocyanide was added to a mixture of pyruvate and oxidase, the same amount of 'cyano-oxidase' was formed as in the absence of pyruvate (not shown). This is presumably because the oxidase is simultaneously reduced by the ferrocyanide, conditions which favour cyanide binding [13,20], so that the scavenger is unable to compete with the oxidase for the cyanide.

The conclusion to be drawn from these results is either that the ferrocyanide contains free cyanide or that a cyanide-binding agent like the oxidase is able to extract cyanide from ferrocyanide. Assuming that the effect reported by Yu and Yu [10] is the same, their finding that recrystallisation of the ferrocyanide did not affect the amount of 'cyano-oxidase' formed favours the latter interpretation.

The effects of porphyrin cytochrome c and clupein

Fig. 5A shows that 8 μM porphyrin cytochrome *c* (about 50 times the concentration of cytochrome *c* in the control experiment) has no effect on oxygen uptake in the ferrocyanide system. 1 μM porphyrin cytochrome *c*, however, markedly stimulates the reduction of oxidase by ferrocyanide, in the absence of cytochrome *c* (Fig. 6, traces A and B). In Fig. 5B it is shown that a high concentration of the positively charged protein clupein is able to replace cytochrome *c* in its stimulating effect on oxygen uptake in the ferrocyanide system. This finding excludes any explanation of the effect of cytochrome *c* in terms of shuttling of electrons between ferrocyanide and oxidase, as clupein obviously cannot do this. Clupein also stimulates reduction of the oxidase by ferrocyanide to the same extent as cytochrome *c* does (Fig. 6, traces C and D).

The stimulating effect on oxygen consumption of a positively charged protein, but not of porphyrin cytochrome *c*, is not unexpected, since a similar

TABLE I

EFFECT OF PYRUVATE AND LACTATE ON FORMATION OF CYANO-OXIDASE WITH FERROCYANIDE AND CYANIDE

The absorbance changes relate to the type of difference spectrum shown in Fig. 4. The time course of the experiment is:

ligand ↓ — X ↓ — 10 min — oxidase ↓ — 5 min — dithionite ↓ — 5 min — spectrum ↑

4 μM oxidase, 5 mM ferrocyanide, 0.1 mM KCN and 40 mM pyruvate or 40 mM lactate.

Ligand	Addition (X) ($\Delta A_{420-444\ nm}$)		
	None	Pyruvate	Lactate
KCN	0.31	0.05	0.32
Ferrocyanide	0.11	0.00	0.14
None	0.00	—0.05	—0.02

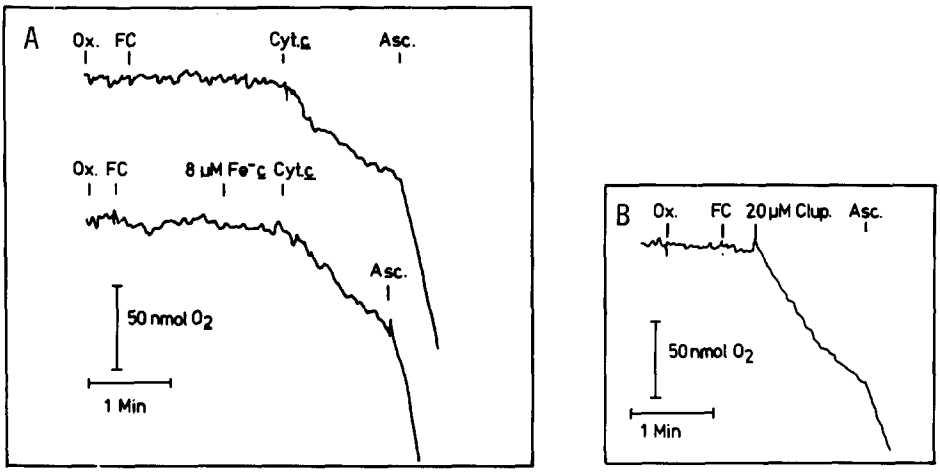


Fig. 5. The effects of clupein and porphyrin cytochrome *c* on the oxygen reduction by ferrocyanide. 4.7 μM oxidase (Ox), 1.7 mM ferrocyanide (FC), 5 mM ascorbate (Asc), and 0.17 mM cytochrome *c* (Cyt. *c*), 8 μM porphyrin cytochrome *c* (Fe^- *c*) or 20 μM clupein (Clup.) were added as indicated to 1.5 ml of the medium described in Methods.

finding has been reported by Jacobs et al. [3] for the oxidase-catalysed oxidation of tetrahalogenated *p*-benzoquinols. It is, however, puzzling that these authors did not find such a requirement for the oxidation of ferrocyanide.

Other reductants

To eliminate the possibility that the requirement of cytochrome *c* for oxygen uptake with ferrocyanide is related to the cyanide-like inhibition demonstrated above, experiments were carried out with ferrous sulphate as reductant. Table II shows that the combination of oxidase and cytochrome *c* catalyses oxidation of ferrous sulphate by oxygen (on top of some autoxidation, catalysed by cytochrome *c* or impurities in the preparation), whereas oxidase alone does not. From Fig. 7 it can be seen that ferrous sulphate, like ferrocyanide, reduces the oxidase in the absence of cytochrome *c*. The oxidation of TMPD or reduced phenazine methosulphate does not require cytochrome *c* and is not inhibited by either clupein or porphyrin cytochrome *c* (not shown).

TABLE II

OXIDATION OF Fe^{2+} BY OXYGEN

1 mM $FeSO_4$, 3.8 μM cytochrome *c* oxidase and 0.67 μM cytochrome *c* in 1.5 ml of the medium described in Methods. The rates are the initial rates after the addition used to start the reaction (oxidase in line 1, cytochrome *c* in lines 2 and 3). Two different experiments are shown.

Oxidase	Cytochrome *c*	Oxygen uptake (nmol O_2/min)	
		I	II
+	—	0	0
—	+	12	27
+	+	22	54

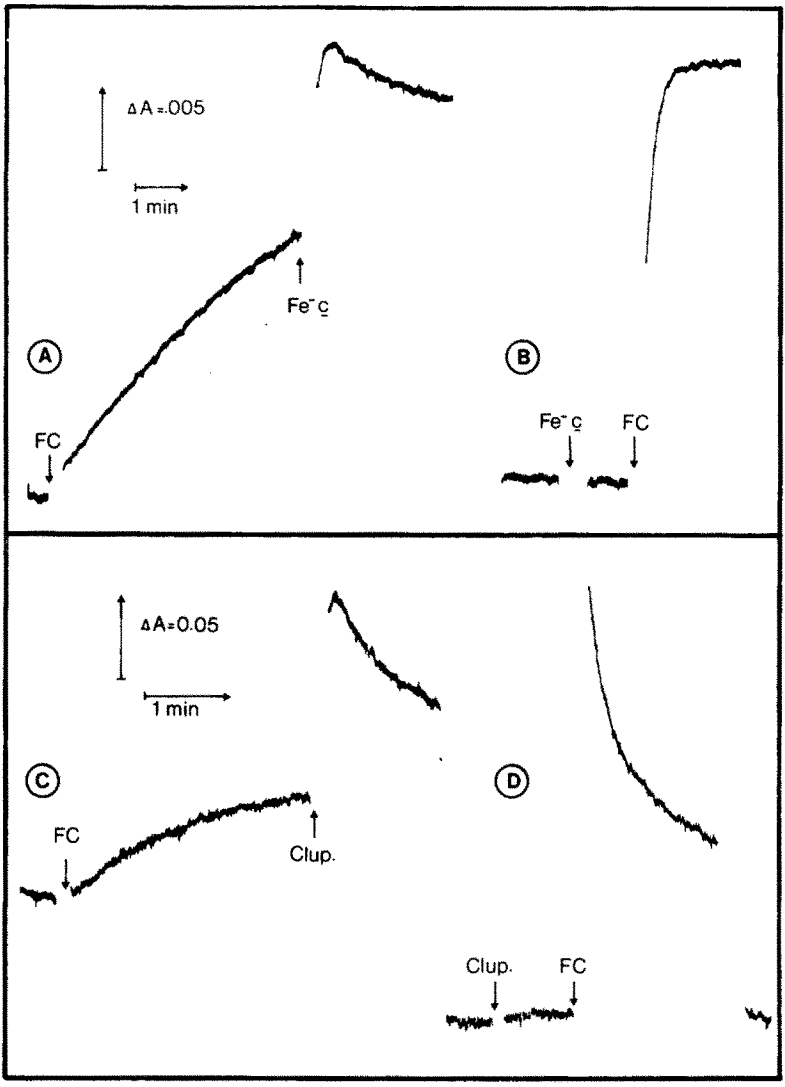


Fig. 6. The effects of clupein and porphyrin cytochrome *c* on the reduction of cytochrome *c* oxidase by ferrocyanide. Traces A, B: 5 mM ferrocyanide (FC) and 1 μM porphyrin cytochrome *c* (Fe^{-} *c*) were added as indicated to 3 ml of the medium described in Methods, containing 4 μM oxidase. Traces C, D: 2.5 mM ferrocyanide (FC) and 10 μM clupein (Clup.) were added as indicated to 3 ml of the medium described in Methods. Heme *a* reduction was measured at 605—590 nm, 1 cm light path.

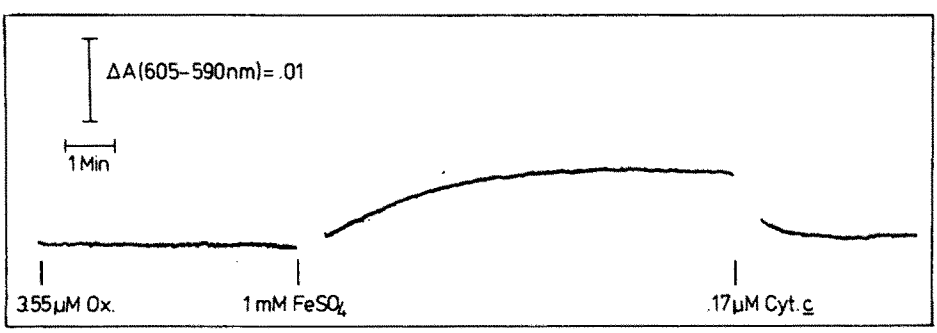


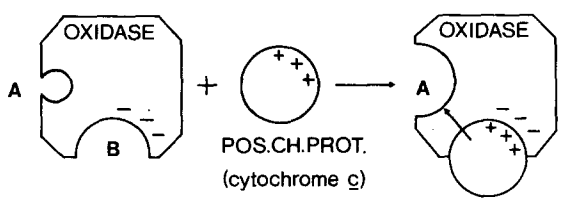
Fig. 7. The reaction between cytochrome *c* oxidase and ferrous sulphate. Concentrations are indicated in the figure; 1-cm light path.

Discussion

When high concentrations of ferrocyanide react with cytochrome *c* oxidase, an inhibited complex is formed with a spectrum similar to cyano-oxidase. The scavenging effect of pyruvate on this process is most easily explained by the assumption that ferrocyanide either contains free cyanide, or produces it in the presence of a cyanide-binding agent. The conclusion of Yu and Yu [10] that ferrocyanide itself is the complexing and inhibiting agent (based on spectral dissimilarities between the effects of ferrocyanide and cyanide) is not supported by the spectra shown here, and it would require a reaction between pyruvate and ferrocyanide itself to explain the results given in Table I. It is possible that the EPR signal reported by Lanne et al. [21] is derived not from a complex between oxidase and ferricyanide (formed upon reaction between ferrocyanide and oxidase), but from a compound formed when oxidase extracts cyanide as well as an electron from ferrocyanide.

Cytochrome *c* stimulates oxygen uptake with ferrocyanide or ferrous sulphate as a source of electrons. Since it may be replaced in this respect by clupein, its effect is not due to cytochrome *c* acting as an electron shuttle between the oxidase and the reductant. Since cytochrome *c*, clupein and porphyrin cytochrome *c* all stimulate reduction of the oxidase by ferrocyanide, this reduction presumably takes place at a site on the oxidase (site A, see Scheme I) which requires binding of a positively charged protein to the oxidase to become readily accessible. The fact that porphyrin cytochrome *c* does not stimulate uptake of oxygen may be explained most easily by the assumption that a second reduction site on the oxidase (site B in the scheme) is required for this process, and that this site is identical to the one that regulates site A. Porphyrin cytochrome *c* binds to this site (thereby opening site A), but in the meanwhile blocks access to ferrocyanide. Clupein also binds to site B, but because of its different structure, it does not block the access of ferrocyanide. Obviously, when cytochrome *c* itself binds to site B, reduction via this route is no problem.

An interpretation of the observed phenomena consistent with this model is the following: Ferrocyanide or ferrous sulphate cannot react with site A, when site B is not occupied by a positively charged protein, but they do reduce the oxidase via the exposed site B (perhaps resulting in the formation of 'oxygenated oxidase [6]). For more hydrophobic reductants like TMPD and reduced phenazine methosulphate both sites A and B are accessible, so neither of these needs the presence of a positively charged protein to be oxidized. The



Scheme I. Model showing a high (B)- and a low (A)-affinity site for cytochrome *c* on cytochrome *c* oxidase. POS.CH.PROT. = positively charged protein.

affinity of porphyrin cytochrome *c* for site B is rather less than that of cytochrome *c*, so that it does not inhibit the effect of cytochrome *c* on ferrocyanide oxidation to any great extent. Also, it can block site B only for hydrophilic reductants, but not for TMPD or phenazine methosulphate. When cytochrome *c* is in excess with respect to the oxidase, cytochrome *c* also supplies the electron for site A. This, however, is competitively inhibited by positively charged proteins [8] and by porphyrin cytochrome *c* [9]. Shuttling of cytochrome *c* between site A and a reductant may well be a rate-limiting step and could be the explanation of the lower K_m for cytochrome *c* with *p*-phenylenediamine than with ascorbate [22].

Although no conclusions can be drawn from this work about the stoichiometry of sites A and B with respect to the oxidase, it is interesting that there is much evidence, both from kinetic [23,24] and direct binding studies [24], that points to the existence of two sites on the oxidase for cytochrome *c* with different properties. It is especially noteworthy that the most successful analysis up to date of the kinetics of cytochrome *c* oxidation involves the oxidation of a second cytochrome *c* molecule catalysed by an oxidase-cytochrome *c* complex [25,26].

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