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REDOX STATE OF THE PARTIALLY REDUCED CYTOCHROME aa_3-CYANIDE COMPLEX

PETER NICHOLLS and VIRGINIA HILDEBRANDT

Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1 (Canada)

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Summary

The low-spin ferric cyanide complex of beef heart cytochrome aa_3 can be partially reduced by stoichiometric additions of ferrous cytochrome *c* or by similar additions of *N,N,N',N'*-tetramethyl-*p*-phenylene diamine. In both cases the initial ratio of cytochrome *c* oxidized : cytochrome *a* reduced or Wurster's Blue : cytochrome *a* reduced approximates the value 2. It is concluded that the binding of a single HCN prevents the reduction of both cytochrome a_3 and its associated EPR-invisible Cu atom.

Carbon monoxide binding to cytochrome *c* oxidase appears to require reduction of both the EPR-invisible copper and the cytochrome a_3 components of the enzyme [1,2], although some workers have claimed that only a_3 reduction is required [3,4]. On the other hand the redox state needed for cyanide binding to the oxidized form is not so clear. Nicholls et al. [5] reported that the product of aerobic oxidation of the species $a^{2+}a_3^{2+}$ HCN was identical with the product of reduction of $a^{3+}a_3^{3+}$ HCN. Yong and King [6] found that the oxidation of the partially reduced ($a^{2+}a_3^{3+}$ HCN) species of ferricyanide required 2 equivalents/mol aa_3. The original titration studies of van Gelder and Muijsers [7], carried out with NADH and phenazine methosulfate at 605 and 445 nm, did indeed identify both a copper and a haem species as nonreducible in the presence of cyanide, but the cyanide-sensitive Cu was also sensitive to EDTA and salicylaldoxime. Moreover, these titrations were not carried to completion, and therefore the total number of reducing equivalents taken up by a given system was never determined. More recently, Hu et al. [8] have claimed that X-ray absorption edge studies show both Cu atoms reduced in the presence of cyanide.

In addition, the presence of cyanide gives rise to spin state change on reduction of the enzyme which may simulate redox effects [5,9], unless the

enzyme and inhibitor are equilibrated for at least 24 h [10].

We have therefore looked at the aerobic reduction of the cyanide complex of mammalian cytochrome *c* oxidase obtained by incubation overnight with 1.85 mM cyanide. Two methods of reduction were employed:

(1) addition of aliquots of reduced cytochrome *c* (cytochrome c^{2+}) and monitoring the subsequent distribution of reducing equivalents in cytochrome *a* at 605—630 nm (ΔEmM = 22.7) and cytochrome *c* at 550—540 nm (ΔEmM = 19.8, reduced; ΔEmM = 2.0, oxidized); and

(2) addition of aliquots of *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD), followed by the monitoring of reduced cytochrome *a* at 605—619 nm (ΔEmM = 18.8) and of Wurster's Blue ($TMPD^+$) at 630—650 nm (ΔEmM = 4.2).

In titrations with TMPD, the appearance of $TMPD^+$ is initially directly proportional to the reduction of cytochrome *a*, as shown in Fig. 2 below. At low levels of added TMPD, this radical one-electron oxidation product is quite stable, and control experiments showed that under our conditions TMPD does not undergo appreciable autoxidation, although at great excess of TMPD a catalysed secondary oxidation cannot be ruled out. If cyanide and enzyme were not preincubated, intermediate states of partial reduction and partial cyanide binding could be seen. Preincubated enzyme, on the other hand, showed no intermediate states that could not be attributed to mixtures of $a^{2+}a_3^{3+}$ HCN and $a^{3+}a_3^{3+}$ HCN.

Fig. 1 illustrates the course of a titration with reduced cytochrome *c*. Under these conditions cytochrome *a* is reduced at an effective redox potential considerably more positive than that of cytochrome *c*. By monitoring

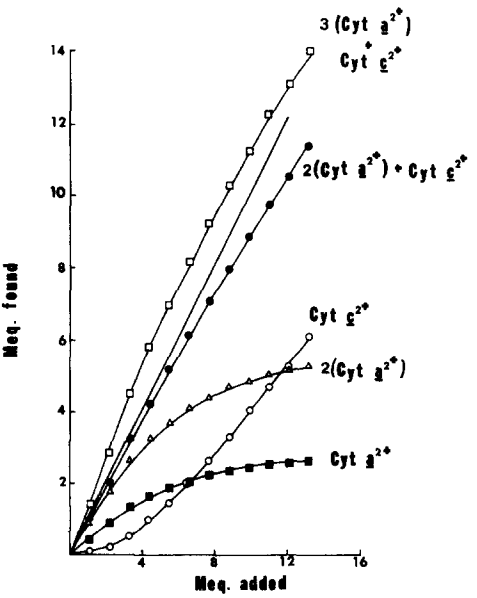

Fig. 1. Titration of cytochrome $a^{3+}a_3^{3+}$ HCN with ferrous cytochrome *c*. 4.5 μM cytochrome aa_3 was incubated overnight with 1.85 mM KCN in 67 mM sodium phosphate pH 7.4, titrated with aliquots of reduced cytochrome *c* at 30°C. Cytochrome *a* reduction monitored at 605—630 nm, cytochrome *c* at 550—540 nm (using correction factor for oxidized cytochrome *c*).

both the disappearance of c^{2+} and the appearance of a^{2+} it is evident that more cytochrome *c* is oxidized (○——○) than cytochrome *a* reduced (■——■). If the latter is doubled, however, (△——△) and added to the remaining cytochrome *c* (●——●) almost all the added equivalents are accounted for. If three electrons entered the oxidase for every equivalent of cytochrome *a* reduced, the upper line (□——□) would be obtained, which is clearly too high until a considerable excess of cytochrome *c* is present.

Fig. 2 illustrates a similar experiment in which TMPD was added to the cytochrome aa_3-cyanide complex. In the absence of ascorbate, the disappear-

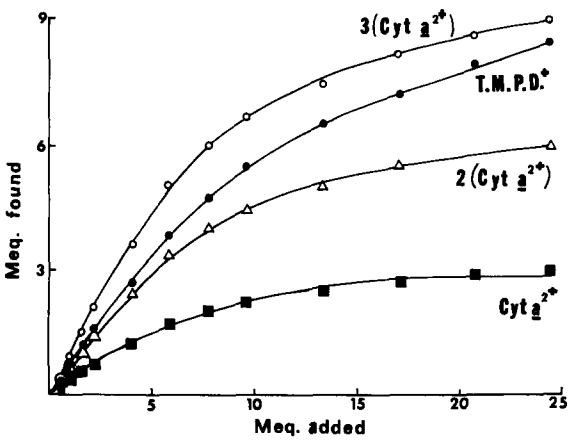

Fig. 2. Titration of cytochrome $a^{3+}a_3^{3+}$ HCN with TMPD. 4.5 μM cytochrome aa_3 incubated overnight with 1.85 mM KCN, 67 mM sodium phosphate pH 7.4, titrated with aliquots of TMPD, standardized against cytochrome *c*. Cytochrome *a* reduction monitored at 605—619 nm, $TMPD^+$ production monitored at 630—650 nm.

ance of reducing equivalents is accompanied by the appearance of $TMPD^+$, Wurster's blue. Again, at low concentrations, the relationship $[TMPD^+] = 2[\text{cytochrome}\, a^{2+}]$ approximately holds. Only at higher levels ($[TMPD)] \geqslant 2[aa_3]$), are extra equivalents taken up. Whether this represents a physiologically active site on the oxidase is doubtful. There are no associated spectroscopic changes.

We conclude that, as previously shown [5,11], only one molecule of HCN is required to block one molecule of cytochrome aa_3, and that the binding of this molecule to the a_3Fe^{3+} iron, evident from the spectroscopic changes seen [9,10], prevents reduction of the associated Cu atom. One must assume that either the $a_3^{3+}Cu_{a_3}^{2+}$ system behaves as a two-electron acceptor, as occurs under some conditions when CO is bound [1,2], or that the reduction of Cu^{2+} requires prior reduction of a_3^{3+} or that the inhibitor blocks Cu^{2+} reduction by some kind of interaction as it blocks a_3Fe^{3+} reduction. As copper and heme do not always go reduced simultaneously, and as $Cu_{a_3}^{2+}$ reduction sometimes seems to precede that of cytochrome a_3Fe^{3+} [12], the simplest assumption is the last one. Some evidence exists [13] for a reaction between inorganic copper and cyanide liganded to the cytochrome *c* haem group. Cyanide may similarly bridge the iron atom and the intrinsic Cu species of the oxidase,

perhaps acting as an analog of the O_2^- species generated in that same milieu during the catalytic reduction of O_2 [14]. The partially reduced complex, we conclude, can correctly be referred to as the 'half-reduced' species, $a^{2+}Cu_a^+a_3^{3+}HCNCu_{a_3}^{2+}$.

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References

1 Wever, R., van Drooge, J.H., Muijsers, A.O., Bakker, E.P. and van Gelder, B.F. (1977) Eur. J. Biochem. 73, 149—154
2 Wilson, D.F. and Miyata, Y. (1977) Biochim. Biophys. Acta 461, 218—230
3 Anderson, J.L., Kuwana, T. and Hartzell, C.R. (1976) Biochemistry 15, 3847—3855
4 Greenwood, C., Wilson, M.T. and Brunori, M. (1974) Biochem. J. 137, 205—215
5 Nicholls, P., van Buuren, K.J.H. and van Gelder, B.R. (1972) Biochim. Biophys. Acta 275, 279—287
6 Yong, F.C. and King, T.E. (1972) J. Biol. Chem. 247, 6384—6388
7 van Gelder, B.F. and Muijsers, A.O. (1966) Biochim. Biophys. Acta 118, 47—57
8 Hu, V.W., Chan, S.I. and Brown, G.S. (1977) FEBS Lett. 84, 287—290
9 Nicholls, P., Petersen, L.C., Miller, M. and Hansen, F.B. (1976) Biochim. Biophys. Acta 449, 188—196
10 van Buuren, K.J.H., Nicholls, P. and van Gelder, B.F. (1972) Biochim. Biophys. Acta 256, 258—276
11 Wrigglesworth, J.M., Baum, H. and Nicholls, P. (1973) FEBS Lett. 35, 106—108
12 Tiesjema, R.H., Muijsers, A.O. and van Gelder, B.F. (1973) Biochim. Biophys. Acta 305, 19—28
13 Nicholls, P. and Mochan, E. (1967) Biochim. Biophys. Acta 131, 397—400
14 Nicholls, P. and Hildebrandt, V. (1978) Biochem. J. 173, 65—72