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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE*

X. SPECTRAL AND POTENTIOMETRIC PROPERTIES OF THE HEMES AND COPPERS

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SUMMARY

1. Potentiometric titrations of highly purified cytochrome *c* oxidase, carried out in the absence of cytochrome *c* at the wavelengths 410, 424, 445 and 605 nm, show that both heme *a* groups act as independent one-electron acceptors with the same midpoint potential ($E_0' = 280$ mV, $n = 1.0$). The titration of the copper atoms at 830 nm suggests an equilibrium between one- and two-electron acceptors ($E_0' = 280$ mV, $n = 1.6$).

2. When cytochrome *c* is present, the copper atoms titrate as single-electron acceptors ($E_0' = 280$ mV, $n = 1.0$). Two heme *a* groups with different midpoint potentials ($E_0' = 370$ mV, $n = 1.0$ and $E_0' = 230$ mV, $n = 1.0$) contribute about equally to the absorbance differences on reduction at 410, 424, 445 and 605 nm.

3. Both in the absence and presence of cytochrome *c* the difference spectrum (reduced *minus* oxidized) of the heme *a* group that is reduced at high oxidation–reduction potentials peaks at 603.5 and 444 nm, whilst that of the heme *a* group that is reduced at low redox potentials peaks at 607 and 446 nm. This indicates that the difference spectrum of one of the heme *a* groups depends on the redox state of the other.

4. No evidence is found for Lemberg's (*Physiol. Rev.* 49 (1969) 48–121) suggestion that cytochrome a_3 absorbs maximally at 411 nm and cytochrome *a* at 423 nm.

5. It is concluded that the heme *a* groups in cytochrome *c* oxidase, in the absence of inhibitors such as CO, azide and cyanide, are formally equivalent.

INTRODUCTION

The functionally active unit of the terminal respiratory chain enzyme cytochrome *c* oxidase (EC 1.9.3.1) consists of two copper atoms and two heme *a* groups[1,2].

* Numbers I through IX of this series have been entitled: Biochemical and biophysical studies on cytochrome aa_3. In view of the possible identity of cytochrome *a* and cytochrome a_3, this series will continue as: Biochemical and biophysical studies on cytochrome *c* oxidase.

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Although the groups of Okunuki[3] and Wainio[4] accept only the existence of cytochrome *a*, most workers in the field agree on the functional difference between these two heme *a* groups, known as cytochrome *a* and cytochrome a_3. This discrimination between cytochrome *a* and a_3, originally made by Keilin and Hartree[5], is based on the reactivity of cytochrome a_3 towards O_2 and inhibitors such as CO, cyanide and azide. Reduction of cytochrome a_3 is blocked by cyanide or high concentrations of azide, whereas oxidation of cytochrome a_3 is prevented by its combination with CO.

On the assumption that cytochrome *a* and cytochrome a_3 may be treated as independent entities, approximate difference (reduced *minus* oxidized) and absolute spectra of cytochromes *a* and a_3 have been calculated from difference spectra (reduced *minus* oxidized) of cytochrome *c* oxidase in the absence and presence of inhibitors[6–9], in combination with the CO action spectrum[10–12]. This approach is open to criticism[13]. Direct or indirect heme–heme interactions can give rise to mutual effects on the spectra of cytochromes *a* and a_3. As a consequence, binding of cyanide, azide or CO to cytochrome a_3 may have an effect on the spectrum of cytochrome *a*. A similar argument holds for heme–copper interactions. Moreover, a contribution of the coppers to the α- and γ-difference absorbance bands can not be excluded[14]. Different results have indeed been obtained with different inhibitors[15]. These considerations have raised doubts concerning the validity of the apparently well-established ratio's of 80/20 and 50/50 for the contributions of the cytochrome *a* and a_3 moieties to the α- and γ-band absorbance difference[6–9,16].

For this reason it was of interest to obtain the difference spectra of the cytochrome *a* and a_3 portions of the isolated enzyme, without the complications introduced by the use of inhibitors. Potentiometric titrations provide such a method where (difference) spectra are related to a potential scale. As was demonstrated by Wilson *et al.*[17] for cytochrome *c* oxidase in mitochondria, this method may serve as a means of obtaining the difference spectra of cytochromes *a* and a_3, not distorted by a possible spectral influence of the binding of inhibitors to cytochrome a_3 on the spectrum of cytochrome *a*. In this paper we will present spectral and potentiometric evidence that the spectra of cytochrome *a* and a_3 are mutually dependent. Whether this is a result of direct heme–heme interactions[18–20,17] or of a conformational change occurring on reduction of one of the heme *a* groups and thus changing the environment of the second heme *a* group is not yet clear.

In previous papers[21,22] of this series the effect of cytochrome *c* on the redox properties of the hemes was examined. This paper extends the observations to the copper constituents of isolated cytochrome *c* oxidase.

METHODS

Beef-heart cytochrome *c* oxidase (5 μmoles cytochrome *c* oxidase or 10 μmoles heme *a* per g protein) was prepared as described elsewhere[23,24]. All concentrations of cytochrome *c* oxidase are based on a catalytically active unit containing 2 hemes per molecule. Horse-heart cytochrome *c* was isolated by the method of Margoliash and Walasek[25].

Spectrophotometric measurements were carried out on a Cary-17 recording spectrophotometer, thermostatted at 22 °C. Potentials were measured with a Philips PW9408 digital pH/mV meter. Calibrations were performed as described in a previous paper[21].

Absorbance coefficients used were for cytochrome *c* (red–ox) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm[26], for cytochrome *c* oxidase (red–ox) 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm[16] and for NADH 6.22 $mM^{-1} \cdot cm^{-1}$ at 340 nm[27,26].

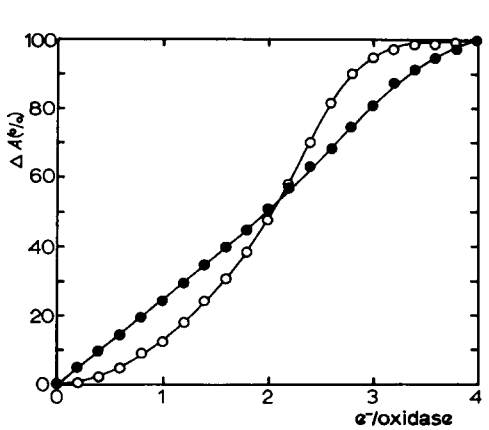
All experiments were carried out in 100 mM potassium phosphate buffer (pH 7.1) with 0.5% Tween-80. Chemicals were mainly Analar Grade, obtained from British Drug Houses.

RESULTS

Determinations of midpoint potentials

Fig. 1 shows a spectrophotometric titration of cytochrome *c* oxidase with NADH and catalytic amounts of phenazine methosulphate under anaerobic conditions, followed at 605 and 830 nm. The line at 605 nm, which is similar to that at 445 nm[21,22], represents the redox state of the hemes, and the line at 830 nm that of the coppers. The hemes and coppers show a clearly different reduction behaviour. Whereas the reduction of the hemes is almost linearly dependent on the amount of NADH added up to complete reduction (4 electron-equivalents per molecule cytochrome *c* oxidase) the reduction of the coppers shows a sigmoidal dependence.

This different behaviour of the hemes and coppers can also be seen in Fig. 2, which shows a Nernst-type plot of a potentiometric titration of cytochrome *c* oxidase in the absence of cytochrome *c*. The results at 410 (not shown), 424 and 445 nm are experimentally indistinguishable from those at 605 nm[21,22]. For both heme groups a midpoint potential of 280 mV is obtained, with $n = 1.0$. The line at 830 nm, representing the redox state of the copper atoms, corresponds to a midpoint potential of 280 mV and an n value of 1.6.



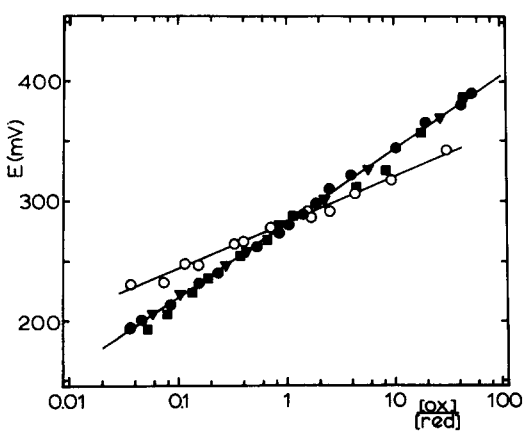


Fig. 1. Anaerobic titration of cytochrome *c* oxidase (10–50 μM) with NADH and catalytic amounts of phenazine methosulphate (0.10–0.50 μM). The abscissa represents electron equivalents added per molecule of cytochrome *c* oxidase. ●—●, 605 nm; ○—○, 830 nm.

Fig. 2. Potentiometric titration of cytochrome *c* oxidase (5–50 μM) in the absence of cytochrome *c*. Mediators: 30–50 μM phenazine methosulphate, 30–50 μM diaminodurene and 120–200 μM $K_3Fe(CN)_6$. ■—■, 424 nm; ▼—▼, 445 nm; ●—●, 605 nm; ○—○, 830 nm. Reduction was brought about by the addition of small aliquots of 10 mM NADH. The points have been corrected for the spectral contribution of the mediators.

A similar potentiometric titration, carried out in the presence of stoicheiometric amounts of cytochrome *c*, is given in Fig. 3. In agreement with earlier observations at 605 nm[21], where two components are found with absorbance contributions of 45 and 55%, the sigmoidal titration line at 445 nm can be separated into two straight lines, representing a high-potential heme *a* species with $E_0' = 370$ mV, $n = 1.0$ and a low-potential heme *a* species with $E_0' = 230$ mV, $n = 1.0$, each contributing about 50% to the total absorbance change on reduction. Titrations at 410 and 424 nm (not shown) show the same behaviour as those at 445 and 605 nm. However, at 830 nm a different behaviour is again found for the copper atoms. The line corresponds to a single-electron acceptor with $E_0' = 280$ mV, $n = 1.0$.

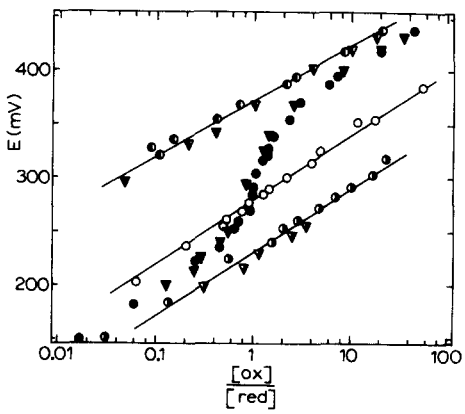


Fig. 3. Potentiometric titration of cytochrome *c* oxidase (5–50 μM) in the presence of stoichiometric amounts of cytochrome *c* on a heme basis (10–100 μM). Mediators as in Fig. 2. Filled triangles, 445 nm; filled circles, 605 nm; open circles, 830 nm, unresolved lines; half-filled (left) triangles, 445 nm; half-filled (left) circles, 605 nm, high-potential component; half-filled (right) triangles, 445 nm; half-filled (right) circles, 605 nm, low-potential component. The points have been corrected for the spectral contributions of cytochrome *c* and of the mediators.

Our results at 830 nm are in close agreement with those of Tzagoloff and MacLennan[28], who reported $E_0' = 284$ mV, $n = 1.0$ in the presence of cytochrome *c*. Wharton and Cusanovich[29], however, reported *n* values of 1.0 in the absence and 1.4 in the presence of cytochrome *c*. We are not able to explain these differences in observation.

Spectra of the components reduced in potentiometric titrations

The difference spectra (reduced–oxidized) obtained at different potentials in the presence of cytochrome *c* are shown in Fig. 4. The spectrum of the high-potential component that is reduced between 425 and 325 mV (0–40% of the total absorbance change at 445 and 605 nm, spectra 1) has symmetrical peaks, the α-band being at 603.5 nm and the γ-band at 444 nm. The low-potential component, reduced between 260 and 100 mV (56–100% of the total ΔA, spectra 3) has an asymmetrical γ-band at 446 nm with a shoulder at higher wavelength and a symmetrical α-band at 607 nm. The intermediate region of the titration from 325 to 260 mV (40–56% of the total ΔA, spectra 2) where both components are reduced shows peaks at the normal wavelengths 445 and 605 nm, as is the case for the summated spectrum from 425 to 100 mV, spectra 4).

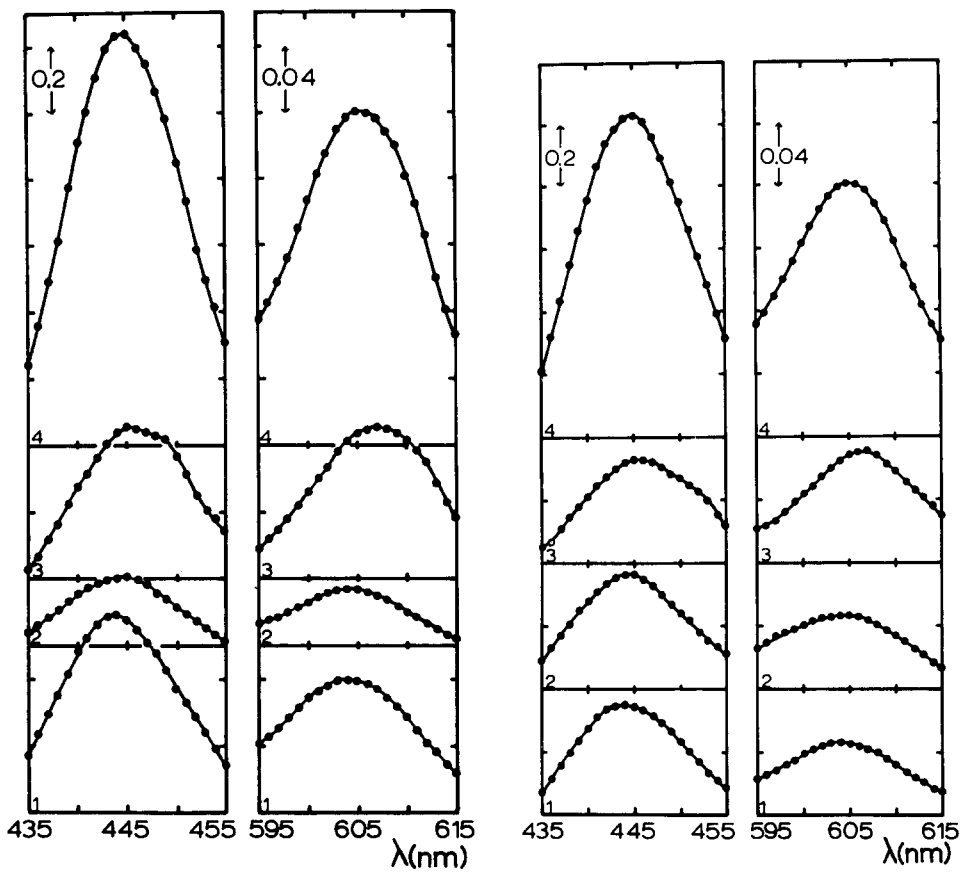


Fig. 4. Difference spectra (reduced *minus* oxidized) of 8 μM cytochrome *c* oxidase during a potentiometric titration in the presence of stoicheiometric amounts (16 μM) of cytochrome *c*. 1, 325 *minus* 425 mV; 2, 260 *minus* 325 mV; 3, 100 *minus* 260 mV; 4, 100 *minus* 425 mV. No correction has been made for the contribution of cytochrome *c* to the absorbance change.

Fig. 5. Difference spectra (reduced *minus* oxidized) of 7 μM cytochrome *c* oxidase during a potentiometric titration in the absence of cytochrome *c*. 1, 295 *minus* 400 mV; 2, 270 *minus* 295 mV; 3, 135 *minus* 270 mV; 4, 135 *minus* 400 mV.

Fig. 5 shows the difference spectra obtained in a potentiometric titration in the absence of cytochrome *c*. Between 400 and 295 mV (0–35% of the total ΔA at 445 and 605 nm, spectra 1) the α-band is at 603.5 nm and the γ-band at 444 nm, from 270 to 135 mV (63–100% of the total ΔA, spectra 3) the peaks are at 605 and 446 nm. The mid-part of the titration from 295 to 270 mV (35–63% of the total ΔA, spectra 2) and the spectrum fully reduced *minus* fully oxidized (spectra 4) again show the normal peak positions at 445 and 605 nm.

The α-band spectra obtained for the high- and low-potential heme *a* components of cytochrome *c* oxidase in the presence of cytochrome *c* with maxima at 603.5 and 607 nm are in good agreement with those reported by Wilson *et al.*[17] for pigeon-heart mitochondria (604 and 605 nm), and by Wikström[30] for rat-liver mitochondria (603.5 and 607.5 nm). The slight difference in peak position can be explained by a

contribution of the high-potential component to the spectrum of the low-potential component and *vice versa*, due to the potential spans used for obtaining these spectra (*cf.* Fig. 3 of ref. 17). This will result in a shift of the peaks of both the high- and low-potential components towards 605 nm. A similar difference between the high- and low-potential species is found in the Soret region, where the high-potential heme *a* component peaks at 444 nm and the low-potential component shows an asymmetrical peak at 446 nm.

In spite of the fact that only one potentiometrically distinguishable heme *a* component is found in the absence of cytochrome *c* (see Fig. 2), essentially the same peak positions and peak shapes are found in the absence of cytochrome *c* as in its presence. The slight differences between the spectra obtained in the presence and absence of cytochrome *c* are due to a smaller overlap of the spectra in the presence of cytochrome *c*.

DISCUSSION

Potentiometric titrations and the effect of cytochrome c

Our results at 410, 424 and 445 nm in the absence and presence of cytochrome *c* confirm our earlier observations at 605 nm[21,22]. The $n=1.0$ line in the absence of cytochrome *c* indicates that the two heme *a* groups of isolated cytochrome *c* oxidase are at least potentiometrically equivalent. The identity of the titrations at 410, 424 and 445 nm is in contrast to Lemberg's suggestion[31] that the cytochrome a_3 absorbance maximum is at 411 nm and the cytochrome *a* maximum at 425 nm.

For our observations at 830 nm several explanations are possible, since it is not yet clear whether the 830-nm band is caused by both or only one of the copper atoms[14,28]. Moreover, a contribution of heme *a* to this band can not be excluded[32].

Two possible explanations can be given for the $n=1.6$ line found for the coppers. If 60% of the copper titrates with $n=2$, and the remaining 40% with $n=1$, $n=1.6$ will be obtained. Since the cupric–cuprous couple involves a one-electron step, $n=2$ means that reduction of part of the copper is obligatory coupled to the reduction of another group in the enzyme molecule. This other group can not be one of the heme groups, because these titrate with $n=1$. We therefore suggest a coupling between the two copper atoms in some of the cytochrome *c* oxidase molecules in such a way that these two coppers together behave as a two-electron acceptor. Support for an interaction between the copper atoms comes from EPR spectroscopy, where the copper signal with $g_{\|}=2.18$ and $g_{\perp}=2.03$ accounts for only 40% of the total enzymic copper[33].

In an alternative explanation at least one of the heme *a* groups contributes about 40% to the absorbance change on reduction at 830 nm. In this case all the copper titrates with $n=2$, and the resultant $n=1.6$ line is obtained by interference with an $n=1$ line of a heme group. The absence of a component with $E_0'=370$ or 230 mV in a titration at 830 nm in the presence of cytochrome *c* makes this explanation unlikely.

In the presence of cytochrome *c* an $n=1.0$ line with $E_0'=280$ mV is found at 830 nm. This means that in the presence of cytochrome *c* the coupling between the copper atoms is broken and that at least one of the coppers acts as a one-electron acceptor with a midpoint potential in between that of the two heme groups. Preliminary EPR experiments, however, did not show a significant increase of the copper

TABLE I

MIDPOINT POTENTIALS AND *n* VALUES OF THE HEMES AND COPPERS OF CYTOCHROME *c* OXIDASE

	*c absent**	*c present**	*In situ***	$\%\,\Delta A_{445}$	$\%\,\Delta A_{605}$
a	280 mV, $n=1.0$	370 mV, $n=1.0$	365 mV, $n=1.0$	50	45
a		230 mV, $n=1.0$	220 mV, $n=1.0$	50	55
Cu	280 mV, $n=1.6$	280 mV, $n=1.0$	250 mV, $n=1.0$	–	–

* Data taken from this paper and refs 21 and 22.
** Data taken from refs 17 and 34–38.

signal at $g=2.03$ on addition of cytochrome *c*, indicating that the antiferromagnetic coupling between the copper atoms or between copper and heme is still present. Although this may be due to the difference in temperature at which the optical and EPR spectroscopy were carried out, no sufficient explanation is available at present.

A comparison of the results described in this paper and the values for mitochondrial cytochrome *c* oxidase as reported by Wilson and co-workers[17,34–38] is given in Table I. It is clear that the redox properties of the hemes and coppers in highly purified cytochrome *c* oxidase in the presence of cytochrome *c* are similar to those found for particulate cytochrome *c* oxidase.

From the effect of cytochrome *c* on the redox potentials it may be concluded that, in the absence of cytochrome *c*, cytochrome *c* oxidase is in a metastable conformation, stabilized by the high activation energy for its transition to a more favourable conformation. Cytochrome *c* catalyses a conformational change in the cytochrome *c* oxidase molecule that changes the redox properties of the heme and copper components.

These changes can not be due to a permanent binding of cytochrome *c* since they are also found with sub-stoicheiometric amounts of cytochrome *c*[21,22]. Additional evidence for such a catalytic effect of cytochrome *c* comes from experiments with cyanide (Van Buuren, K. J. H., personal communication) where the k_{on} for the binding of cyanide to oxidized cytochrome *c* oxidase increases 2 orders of magnitude on the addition of only 5% of the stoicheiometric amount of cytochrome *c*, without a further increase on addition of more cytochrome *c*. We must therefore conclude that different forms of oxidized cytochrome *c* oxidase exist, the interconversion of which is catalysed by cytochrome *c*. Reversible slow structural changes in cytochrome *c* oxidase are not unusual as shown in our laboratory[23,39].

A similar effect of cytochrome *c* on the properties of one of the hemes of cytochrome *c* oxidase has already been suggested by Orii and Okunuki[40] in 1963. Whether it directly catalyses this conformational change in cytochrome *c* oxidase or only promotes such a change which then is stabilized by reduction of a heme group or binding of cyanide is open for further research.

Spectra

Both in the absence of cytochrome *c*, where only one potentiometrically distinguishable heme *a* compound is found, and in the presence of cytochrome *c*,

where two different heme *a* compounds can be demonstrated, similar peak positions and peak shapes are found. This means that at least in the absence of cytochrome *c* the different spectra can not *a priori* be attributed to different heme *a* species, *in casu* cytochrome *a* and cytochrome a_3, and by inference the same may be the case in the presence of cytochrome *c*.

The two sets of spectra have in common that in the high-potential range one heme *a* group is reduced, which means that the spectrum in this region is mainly due to $Fe^{2+}Fe^{3+}$ *minus* $Fe^{3+}Fe^{3+}$ (where Fe^{3+} stands for ferric, and Fe^{2+} for ferrous heme *a*). At low potentials both heme *a* groups are reduced, resulting in a difference spectrum $Fe^{2+}Fe^{2+}$ *minus* $Fe^{2+}Fe^{3+}$. The difference spectrum Fe^{2+} *minus* Fe^{3+} then depends on the redox state of the other heme *a* group.

The observation that the large change in midpoint potentials of the two heme *a* groups on addition of cytochrome *c* is not accompanied by any spectral change indicates that the change in midpoint potential is caused by long-distance effects on interaction of cytochrome *c* with cytochrome *c* oxidase, such as a change in dissociation constants of the amino acid side chains. The small differences between the difference spectra obtained on reduction of the first and second heme *a* group should be ascribed to a conformational change of the protein in the vicinity of the second heme *a* group on reduction of the first heme *a* group rather than to large differences in the ligand fields of the two heme *a* groups, such as would be expected by a change in liganding groups from the protein. In the latter case much larger spectral differences should be expected.

In the original concept of Keilin and Hartree[5] cytochrome *c* oxidase consists of "two distinct but closely allied and intimately connected compounds, *a* and a_3", although "the differences may also be of such a nature as not to exclude the interconvertibility of these two compounds". Our experimental data point into the direction of their last statement. We see as a working hypothesis that, in the absence of cytochrome *c*, cytochrome *c* oxidase is a symmetrical molecule with two equivalent heme *a* groups and two equivalent copper atoms with symmetrical interaction. In this symmetrical dimeric molecule no distinction can be made between cytochrome *a* and cytochrome a_3. Which one of these two equivalent heme *a* groups is first reduced is governed only by statistics, not by thermodynamics. On the addition of cytochrome *c*, or in mitochondria, a different conformation of cytochrome *c* oxidase is induced resulting in a higher midpoint potential for the first group to be reduced and a lower midpoint potential for the second group.

Wilson and collaborators[17] found a midpoint potential of 250 mV for the only titrable heme *a* group in the presence of CO. Because this value is closer to the E_m of 220 mV for the low-potential heme *a* than to the 370 mV for the high-potential heme *a*, they concluded that CO binds to the high-potential heme *a* species and indentified that species with cytochrome a_3.

However, as proposed by Greenwood and Gibson[41] in 1967, on binding of ligands to or reaction of oxygen with one heme group "a conformation change may occur, which prevents the other heme group from following suit". This negative cooperativity between the two heme *a* groups of cytochrome *c* oxidase, and between the two copper atoms as well, implies that in the absence of ligands no discrimination can be made between cytochrome *a* and cytochrome a_3. The distinction between cytochromes *a* and a_3, as proposed by Keilin and Hartree in 1939, should then only be

applied in the presence of ligands, where cytochrome a_3 is that part of the oxidase molecule where the ligand is bound.

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