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HAEM—HAEM INTERACTIONS IN CYTOCHROME aa_3 DURING THE ANAEROBIC-AEROBIC TRANSITION

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Summary

A biphasic response is seen at both 445 and 605 nm as the ascorbate—cytochrome *c*—cytochrome aa_3 system is taken slowly from the anaerobic to the aerobic state. At low oxygen tensions the 445 nm band is more reduced while at high oxygen tensions the 605 nm band is more reduced. It is suggested that the redox potential for cytochrome *a* (contributing 70% at 605—630 nm and 50% at 445—455 nm) is a function of the redox state of cytochrome a_3. This model can account for both the aerobic/anaerobic data and for observations of interactions in the anaerobic system alone (Leigh, Jr, J.S., Wilson, D.F., Owen, C.S. and King, T.E. (1974) Arch. Biochem. Biophys. 160, 476—486).

Leigh et al. [1] have recently presented EPR evidence for haem—haem interaction between ferrous cytochrome a_3 and ferric cytochrome *a*. On the basis of these and other studies involving anaerobic redox titrations [2], they propose that carbon monoxide binding to reduced cytochrome a_3 modifies the spin state of ferric cytochrome *a* and also increases the extinction coefficients of ferrous cytochrome *a*. At the same time they claim that an alternative model of such interactions [3, 4, 5], in which the formation of mixed ferric and ferrous species is thermodynamically favoured, is rendered improbable by their results. This alternative model, based largely on the behaviour of the enzyme under aerobic conditions in the presence and absence of inhibitors [3, 6], postulates that it is the redox potentials rather than the spectra of cytochromes *a* and a_3, that are interdependent.

In an attempt to reconcile the apparent differences in redox and spectral properties of the cytochrome aa_3 system in the aerobic steady state [3, 4, 6]

and under conditions of anaerobic titrations [2], we have examined the response of the enzyme as the system containing ascorbate, oxidase and cytochrome *c* becomes aerobic, using the method devised by Degn and Wohlrab [7] and employed recently by Petersen et al. [8] in a study of mitochondrial respiration.

Cytochrome *c* oxidase was isolated from beef heart essentially according to van Buuren [9]. The uptake of oxygen in the presence of cytochrome *c* and ascorbate was then monitored while the oxygen tension was slowly increased from zero [7, 8]. At the same time the absorbance at 551—540 nm (cytochrome *c*), 445—455 nm (cytochrome aa_3), or 605—630 nm (cytochrome aa_3), could be followed with a Perkin-Elmer/Hitachi 356 dual wavelength spectrophotometer. At very low dissolved oxygen tensions, the rate of respiration is directly proportional to the oxygen tension in the gas phase [7]. If the latter is therefore increased in a linear fashion, a plot of percentage reduction against gas phase O_2 tension is equivalent to a plot of reduction against velocity (electron flux). In the case of the ascorbate—cytochrome *c* system, this is subject to the check that the flux should also be proportional to the product of reductant and ferricytochrome *c* concentration (Eqn 1):

$$\text{ascorbate} \xrightarrow{k_3} c \xrightarrow{k_2} aa_3 \xrightarrow{k_1} O_2 \qquad (1a)$$

$$v = k_3[\text{ascorbate}][c^{3+}] = k_2[c^{2+}][aa_3{}^{3+}]^* = k_1[O_2][a_3{}^{2+}] \qquad (1b)$$

*probably $[a_3{}^{3+}]$ rather than $[a^{3+}]$ (see below).

As illustrated for two different cytochrome *c* concentrations in Fig. 1, this relationship is obeyed, the amount of oxidized *c* present in the steady state increasing linearly with gas phase O_2 tension until the tension in the liquid phase becomes appreciable (> 0.1 μM O_2).

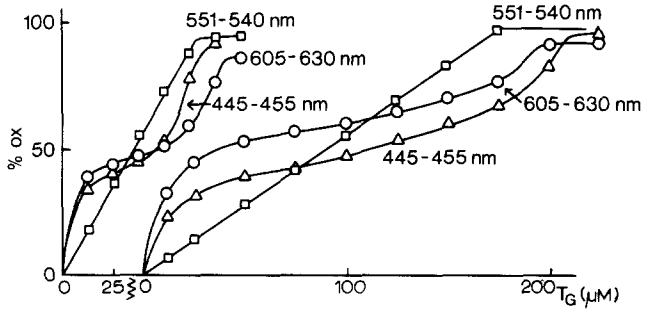


Fig. 1. Percentage reduction at three wavelength pairs as oxygen tension increases. T_G (O_2 tension in gas phase) plotted against differential absorbance at 551—540 nm (□—□), 605—630 nm (○—○) and 445—455 nm (△—△). Left hand data with 1.65 μM cytochrome *c*, right hand data (displaced from origin for clarity) with 5.0 μM cytochrome *c*. 0.975 μM cytochrome aa_3 (1.95 μM haem *a*), 15 mM ascorbate, 70 mM potassium phosphate pH 7.3, 30° C. 100% ≡ oxidation level in the absence of ascorbate. Note. T_L (O_2 tension in liquid phase) becomes measurable only when T_G > 200 μM; below this value T_L < 0.1 μM, above this value T_L increases rapidly [7, 8].

In contrast to the behaviour of the enzyme when undergoing anaerobic redox titrations [10], during the anaerobic—aerobic transition the absorbances at the oxidase α and γ bands behave quite differently. Both bands show the presence of a component oxidized at low tensions and another at high O_2 tensions. Neither component can be identified with one of the two classical cytochromes, a and a_3, whose spectra have been defined on the basis of inhibitor studies. At high $[O_2]$, as in the normal aerobic steady state, the percentage reduction at 605 nm is approximately twice that at 445 nm. But at low $[O_2]$, the percentage reduction at 445 nm exceeds that at 605 nm (i.e. there is always a kind of 'cross over point' between the two bands as oxygen increases). The 'early oxidized' component thus has an apparent redox potential more negative than that of c (cf. anaerobic 'cytochrome a') [2] while the 'late oxidized' component has an apparent redox potential greater than that of c (cf. 'cytochrome a' in the aerobic steady state [4, 6]). Somewhat similar results have been reported by Holton [11] with submitochondrial particles, and by Oshino et al. [12] with yeast, but without detailed interpretations. It may be noted that the 'early oxidized' species is not simply identifiable as cytochrome a_3, as its appearance in the ferric form is not accompanied by any directly proportional increase in respiration rate (cf. Chance [13]).

An attempt can be made to analyse the data of such experiments according to the classical concept of separate cytochromes a and a_3. Fig. 2 summarises the result of an experiment at high $[c]$ and low phosphate (aa_3 is

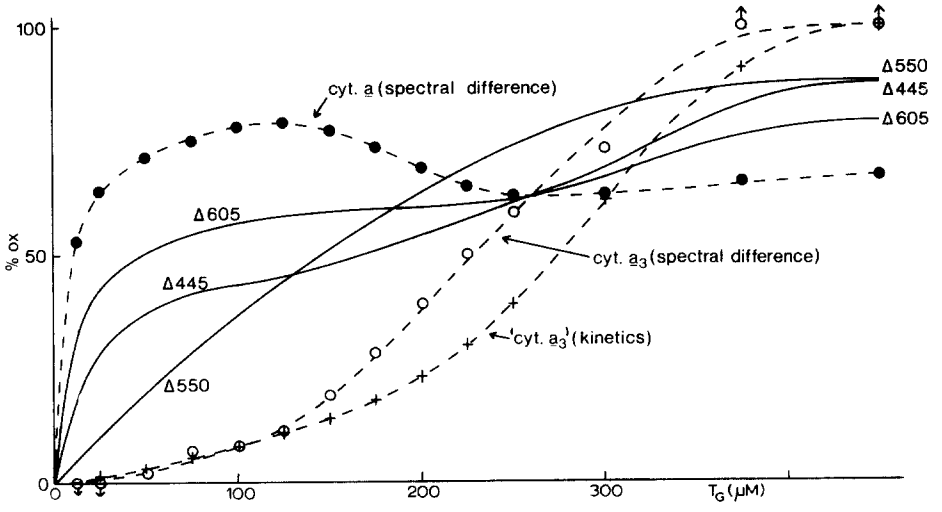
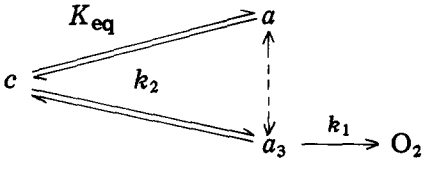


Fig. 2. Calculated percentage reduction of the three cytochromes with increasing oxygen tension. Data plotted as in Fig. 1, but with 11 μM cytochrome c and 5 mM potassium phosphate pH 7.3. 'Δ550' is equal to % reduction of cytochrome c. Cytochrome a (•- - -•) is obtained as (0.7 Δ445—0.5 Δ605)/0.2; cytochrome a_3 (○- - -○) is obtained 'spectroscopically' as (0.5 Δ605—0.3 Δ445)/0.2; cytochrome a_3 (+- - -+) is obtained 'kinetically' as equal to $v/[c^{2+}]$, that is: Δ551 (1—Δ551 max)/Δ551 max (1—Δ551). 100% ≡ oxidation level in the absence of ascorbate. Note. T_L was ≈ 0.1 μM at T_G = 300 μM increasing steeply thereafter.

largely in the form of the cytochrome *c*—cytochrome *c* oxidase complex). If it is assumed that cytochromes *a* and a_3 contribute equally to the Soret absorption (445—455 nm) but that cytochrome *a* is responsible for 70% of the absorption at the α-band region (605—630 nm) then their apparent redox behaviour is that given in Fig. 2. Cytochrome 'a_3' is highly reduced at low oxygen tensions, and becomes more oxidized as the flux increases. This is the behaviour expected of a terminal oxidase largely oxidized in the aerobic steady state [13]. Cytochrome '*a*', however, behaves in an anomalous way: at low oxygen tensions, when 'a_3' is largely reduced, '*a*' is largely oxidized (apparent $E'_0 \leqslant +220$ mV); but at higher oxygen tensions, when 'a_3' is largely oxidized, '*a*' becomes more reduced (apparent $E'_0 \geqslant +280$ mV). A type of haem—haem interaction in which the redox state of a_3 affects the redox potential of *a* is needed to explain such results [2] provided that the classical $a + a_3$ picture is retained.

If the analysis given here is correct, then Fig. 2 also shows that the second reaction of Eqn 1, governed by k_2, cannot involve the transfer of electrons to cytochrome '*a*' from ferrous cytochrome *c*. As in the aerobic steady state [6, 14], there is no correlation between respiration rate and either the product $[c^{2+}] \times [a^{3+}]$ or the product $[a^{2+}] \times [a_3{}^{3+}]$. On the other hand, if it is assumed that the rate-limiting step involves the transfer of electrons from ferrous *c* to ferric cytochrome 'a_3', then an alternative estimate of $a_3{}^{2+}$ can be obtained by simply dividing flux by $[c^{2+}]$, assuming that back reactions are negligible in the steady state (E'_0 for a_3 always $\gg E'_0$ for *c*). The resulting curve (+- - -+) in Fig. 2 resembles that derived by spectroscopic analysis but lies below it. The difference may be attributed to our neglect of back reactions in the kinetic calculation.

We suggest that the results of Figs 1 and 2 can be accommodated by a scheme such as that of Eqn 2; a modification of the original proposals of Yakushiji and Okunuki (see Okunuki [15]) and Yonetani [6, 16]:



(2)

Here the solid arrows indicate electron transfer and the dotted arrow the haem—haem interaction. From experiments at saturating reductant and cytochrome *c* levels, we have found $k_1 \approx 3$ to $5 \cdot 10^7$ $M^{-1} \cdot s^{-1}$. To account for the oxidase turnover [9], k_2 must equal 240 s^{-1} (if 4 electrons go by this route) or 60 s^{-1} (if only 1 electron goes this way) at pH 7.4, 30°C. The value of K_{eq} ($[c^{3+}][a^{2+}]/[c^{2+}][a^{3+}]$) is about 4.0 when a_3 is ferric, and 0.1 when a_3 is ferrous.

The existence of such an interaction must be reciprocal. That is, if reduction of a_3 makes reduction of *a* more difficult, the reverse must also be true. A mixture of half reduced forms ($a^{2+}a_3{}^{3+}$ and $a^{3+}a_3{}^{2+}$) will therefore tend to

accumulate in the mid range of redox potentials. Under the conditions of the anaerobic titration, there is therefore almost no difference in the behaviour of the α and γ peaks [2, 5]. Leigh et al. [1] have now found that the EPR spectrum of the partially reduced intermediate is a composite of the spectra of two heams in equilibrium, one in a very asymmetric environment and the other with a more symmetric environment. Their data are consistent with the presence of a rapidly equilibrating mixture of $a^{2+}a_3{}^{3+}$ and $a^{3+}a_3{}^{2+}$, because under these conditions CO and azide (reacting with $a_3{}^{2+}$ and $a_3{}^{3+}$) appear to be competitive. The alternative idea [2], that azide reacts with cytochrome a, is difficult to reconcile with aerobic steady state results [14, 17]. Moreover, the addition of CO to the partially reduced oxidase is seen to induce a shift from high to low spin among the haems [1]. All these observations can be explained by a scheme such as that given in Eqn 3, where the competition between CO and HN_3, and the high $\rightleftharpoons$ low spin changes, are secondary consequences of the primary haem—haem interaction involving redox potentials.

$$
\begin{array}{ccccccc}
 & & & & a^{3+}a_3{}^{3+} & & \\
 & & & & \Big\uparrow\Big\downarrow\ e^- \quad E_0' > 280\ \text{mV} & & \\
 & & & \overbrace{\phantom{a^{3+}a_3{}^{2+}}}^{c} & & & \\
a^{3+}a_3{}^{2+}CO & \underset{CO}{\rightleftharpoons} & a^{3+}a_3{}^{2+} & \rightleftharpoons & a^{2+}a_3{}^{3+} & \xrightleftharpoons{HN_3} & a^{2+}a_3{}^{3+}HN_3 \\
 & & & \underbrace{\phantom{a^{3+}a_3{}^{2+}}} & & & \\
 & & & & \Big\uparrow\Big\downarrow\ e^- \quad E_0' < 220\ \text{mV} & & \\
 & & & & a^{2+}a_3{}^{2+} & &
\end{array}
\qquad (3)
$$

Although in the anaerobic state in the presence of cytochrome c [2, 10], the central equilibrium (Eqn 3) may thus play a crucial role, in the presence of oxygen under turnover conditions the two half-reduced species can occur without rapid interconversion. Fig. 2 suggests that at high $[O_2]$ the predominant half-reduced form is $a^{2+}a_3{}^{3+}$ while at low $[O_2]$ an appreciable amount of $a^{3+}a_3{}^{2+}$ is present. Greenwood et al. [18] have generated the latter species by photodissociation of $a^{3+}a_3{}^{2+}CO$ and shown that it is catalytically competent.

We conclude that the apparent discrepancies between the calculated extinction coefficients and redox potentials for cytochrome a can be reconciled in a model involving haem—haem interactions dependent on the redox state of cytochrome a_3, evidence for which has been obtained by following the absorbance changes that occur during the transition to aerobiosis.

Acknowledgment

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