Review Letter

A RE-EVALUATION OF THE SPECTRAL, POTENTIOMETRIC AND ENERGY-LINKED PROPERTIES OF CYTOCHROME *c* OXIDASE IN MITOCHONDRIA

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1. Introduction

In this paper we analyze recent results on the spectral, potentiometric and energy-linked properties of cytochrome *c* oxidase. It is demonstrated that the current hypothesis in which cytochromes *a* and a_3 are assumed to contribute equally to the α-band at 605 nm disagrees with spectral information, and that this band is mostly, perhaps entirely, due to cytochrome *a*. The two hemes of cytochrome oxidase interact strongly, this interaction being reflected extensively in the redox properties of the hemes (midpoint redox potentials, E_m) and also in the spin state and light absorption spectra. The effects of the ligands to cytochrome *c* oxidase are discussed in some detail in view of the new information. Special attention is given to the implications of the heme–heme interaction for the mechanism of action of the enzyme and its control by the mitochondrial energy state.

1.1. *The classical view of cytochrome c oxidase*

According to the original proposal by Keilin and Hartree [1] cytochromes *a* and a_3 are different components of cytochrome *c* oxidase with quite different reactivities toward ligands (only cytochrome a_3 reacts with O_2, cyanide, CO, azide, etc.) and widely different spectroscopic properties. The α-band at 605 nm of the reduced enzyme was proposed to be due mainly to cytochrome *a* with little contribution from cytochrome a_3. On the other hand, the Soret band at 445 nm was proposed to be composed of both cytochromes *a* and a_3 with roughly equal contribution. This interpretation, subsequently adopted by most workers in the field (see review by Lemberg, [2]), stems from the well-known finding that the binding of a ligand such as CO or cyanide to cytochrome a_3, abolishing the spectral contribution of this cytochrome to the reduced minus oxidized difference spectrum at 605 nm, decreased the absorption only a little at this wavelength, while the decrease at 445 nm is about one-half.

1.2. *The 50/50 hypothesis of cytochrome c oxidase*

More recently the above classical view was changed as a result of data obtained from anaerobic potentiometric titration of cytochrome oxidase both in intact mitochondria [3] and with the isolated enzyme [4]. It was demonstrated that cytochrome aa_3 titrates as two potentiometrically separate components with E_m values at pH 7 of approx. 380 and 220 mV respectively, each with n = 1, and with approx. 50/50 spectral contribution to both the α and the Soret band. The two components were proposed to be the two chemically distinct but interacting hemes, cytochrome a_3 (E_m 380 mV) and cytochrome *a* (E_m 220 mV) respectively [3]. The fact that in the presence of CO or other ligands such

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Abbreviations: E_m, midpoint oxidation–reduction potential usually at pH 7 if not otherwise specified, relative to the Normal Hydrogen Electrode. FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone. HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate. TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

as sulphide and cyanide only one component (cytochrome *a*) is seen at 605 nm with a spectral contribution of more than 80% of the total reduced minus oxidized difference spectrum was specifically explained as being due to a large increase in the extinction coefficient of reduced minus oxidized cytochrome *a* upon binding of a ligand to cytochrome a_3 (by heme–heme interaction, see [5]). However, for the case of azide Wilson et al. [3,6] proposed binding of the ligand to ferricytochrome *a*, and not a_3 (contrast [1,2,7,8]), and a concomitant large increase in the extinction coefficient of reduced minus oxidized cytochrome a_3 (heme–heme interaction) as well as a decrease in the extinction of cytochrome *a* upon binding of this ligand to the enzyme.

It should be realized that the 50/50 hypothesis relies heavily on these postulated large (>60%) changes in the extinction coefficients of cytochromes *a* and a_3 and that the hypothesis can be tested on the basis of these postulates.

Although heme–heme interaction in cytochrome oxidase has recently been discussed at great length [3–10] in terms of effects on absorption spectra, e.p.r. spectra and ligand-induced E_m shifts, little account has been given to the possibility of a type of heme–heme interaction that would affect the midpoint redox potential of one of the hemes upon reduction of the other. As pointed out by Malmström in a recent review [11], see also [12], the assignment of the potentiometrically separable components of cytochrome oxidase to cytochromes *a* and a_3 respectively is by no means a unique interpretation of the data. Heme–heme interaction involving the redox midpoint potentials (i.e., the intrinsic reducibility) of the hemes can affect the system in such a way as to simulate two potentiometrically distinguishable components even if only one single heme is spectroscopically visible (see section 3 and [11,12]). A similar proposal was also recently offered by Nicholls [13,14] and will be analyzed in some detail in the present paper.

1.3. *The neo-classical hypothesis of cytochrome* c *oxidase*

Nicholls [13,14] has recently outlined an alternative explanation of the data obtained from the potentiometric titrations. This hypothesis is based on the original (classical) interpretation of the spectral properties of cytochromes *a* and a_3 (see section 1.1), the two being both chemically and spectrally quite distinct entities. Heme–heme interaction was proposed to occur, but to affect the midpoint redox potentials of the hemes rather than their spectra (contrast the 50/50 hypothesis where heme–heme interaction is proposed to be the basis for the large increase in extinction coefficient of one of the hemes upon binding a ligand to the other). The interaction was proposed to be such that reduction of one of the two hemes would cause a decrease in the E_m of the other heme. In addition, the half-reduced cytochrome aa_3 was suggested to be a mixture of ferrous *a* and a_3, i.e., an equilibrating mixture of the species $a^{2+}a_3^{3+}$ and $a^{3+}a_3^{2+}$. As pointed out by Nicholls, this model accounts for some of the weak points of the 50/50 hypothesis, such as the finding of apparently different degrees of reduction of the hemes as determined from measurements of cytochrome oxidase in the Soret and α regions of the spectrum both under steady state and pre-steady state conditions (see [13]). Moreover, Nicholls and Petersen [14] have presented evidence with the isolated enzyme that the midpoint potential of cytochrome *a* is a function of the redox state of cytochrome a_3, in line with the neo-classical model.

2. Spectral and potentiometric properties of cytochrome *a*

It is clear from the above description that a study of the properties of cytochrome *a* may greatly contribute to our understanding of cytochrome oxidase and should also help us decide between and test the above models.

2.1. *The dissociation of carbon monoxide from cytochrome* c *oxidase*

It is well known that the bond between ferrous cytochrome a_3 and carbon monoxide is photosensitive (see e.g. [15,16]). It is also known that the species $a_3^{2+} \cdot CO$ cannot be oxidized by ferricyanide in uncoupled mitochondria [3] or with isolated cytochrome oxidase [17]. The addition of excess ferricyanide to the fully reduced enzyme in the presence

of excess carbon monoxide results in quantitative oxidation of cytochrome *a* with a half-reduction potential of 255 mV [3], the end result being the species $a^{3+}a_3{}^{2+} \cdot CO$.

Thus, by comparison of the difference spectra of CO dissociation from the species $a^{2+}a_3^{2+} \cdot CO$ and $a^{3+}a_3^{2+} \cdot CO$ respectively, it should be possible to determine if binding of CO to the enzyme induces a drastic increase in the extinction coefficient of ferrous minus ferric cytochrome *a* as postulated by Wilson et al. [3].

Fig.1 shows the photolysis difference spectra of cytochrome *c* oxidase of beef-heart mitochondria in the mixed valency and reduced configurations (1A and 1B respectively) and in the absence of a significant amount of oxygen. The results shown in fig.1 were also confirmed in experiments where the suspension was titrated to various redox potentials by the method

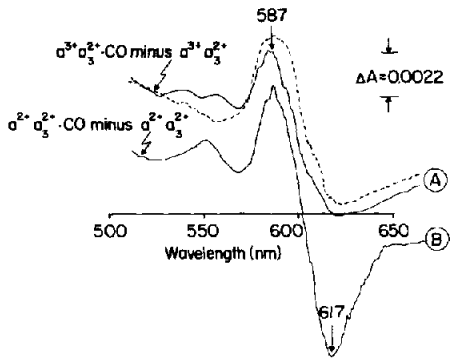


Fig.1. Difference spectra of carbon monoxide dissociation from cytochrome oxidase in the mixed valency and fully reduced state. Beef heart mitochondria [58] (15 mg protein/ml) were suspended in buffer containing 100 mM sucrose, 10 mM Tris-HCl, 20 mM phosphate buffer and 30% ethylene glycol, pH 7.4. The suspension was saturated with carbon monoxide and in (B) 10 mM succinate was also present. In (A), 5 μl of CO-saturated potassium ferricyanide (10 mM) was further added at −30°C (to a volume of 0.5 ml) with minimal admission of oxygen. Both samples (A and B) were subsequently frozen at −80°C in the dark. The bottom part of the tube was subsequently illuminated for 15 sec in bright light after recording the baseline (top minus bottom) at −140°C, after which the difference spectrum, top minus bottom, was immediately recorded in a split beam spectrophotometer. An upward deflection in the figure represents a higher absorption in the top part of the tube. The dotted spectrum of (A) represents a second experiment under the same conditions.

described by Dutton [18] under strictly anaerobic conditions and in the presence of redox mediators, and by kinetic experiments shown below (fig.2).

At low redox potentials (fig.1B) photolysis produces the well-known difference spectrum with a peak at approx. 590 nm due to $a_3^{2+} \cdot CO$ and a well-defined trough at approx. 617 nm, which generally has been believed to be due to free ferrous cytochrome a_3 in the difference spectrum (but see below). At high redox potentials, however, under conditions where cytochrome *a* is highly oxidized, only the peak at 590 nm due to the ferrous cytochrome a_3–CO compound is visible without the sharp trough at the longer wavelength (fig.1A). Instead a shallow and very broad trough centered at about 630 nm appears. The change in response to photolysis from one type of spectrum (fig.1A) to the other (fig.1B) occurs with half-maximal transition at approx. 255 mV and follows a slope of 60 mV/decade (not shown), showing that the change in spectral response correlates with the redox state of cytochrome *a*, which has an E_m of 255 at saturating CO concentrations [3].

From these data it may first be concluded that the dissociation of CO does not induce a large decrease in the extinction coefficient of ferrous minus ferric cytochrome *a* (contrast [3]) since such an effect would have led to a less extensive trough in the 615 nm region (or a more extensive peak) for the case of the fully reduced enzyme (fig.1B) as compared to the half-reduced enzyme (fig.1A). As seen by comparison of the two difference spectra, the result is opposite to this prediction. This finding is inconsistent with the central postulate of the 50/50 hypothesis.

Even more important, as shown in fig.1A, free ferrous cytochrome a_3 which is produced upon photolysis, does not appear to absorb at all in the 605 nm region, since no sharp trough appears on photolysis. The trough at 617 nm observed with the fully reduced enzyme (fig.1B), and which is related to the presence of ferrous cytochrome *a* (see above), may therefore best be interpreted as a *small increase* in the extinction coefficient of ferrous minus ferric cytochrome *a* upon *dissociation* of CO from cytochrome a_3 (or conversely, a small decrease in the extinction coefficient of cytochrome *a* upon binding of CO to cytochrome a_3, opposite to the postulate

of the 50/50 hypothesis. The alternative explanation, that ferrous minus ferric cytochrome a_3 absorbs in the α-region (less than 20% of the total peak) only when cytochrome *a* is reduced, is less probable and does not agree with other experimental data (see section 2.3.).

The shallow and very broad trough in fig.1A, which is centered around 630 nm, is reminiscent of the charge-transfer band typical of high spin ferric hemoproteins (see [2] and section 3.1.).

Fig.2 shows the kinetics of change at 594 and 610 nm (with reference to 635 nm) upon flash photolysis of fully reduced and half-reduced carbonmonoxi-cytochrome oxidase. In the fully reduced enzyme (fig.2B) the absorption decreases abruptly at 594 nm and increases at 610 nm consistent with the difference spectrum shown in fig.1B. In the mixed valency state, however, only the 594 nm change is present (fig.2A cf. spectrum of fig.1A). In the experiment of fig.2, which was performed at a higher temperature than that of fig.1, recombination of CO with the enzyme is also visible as the traces return to their starting positions. No changes are observed in the near-infrared band at 830 nm in these experiments verifying that no significant oxygen contamination

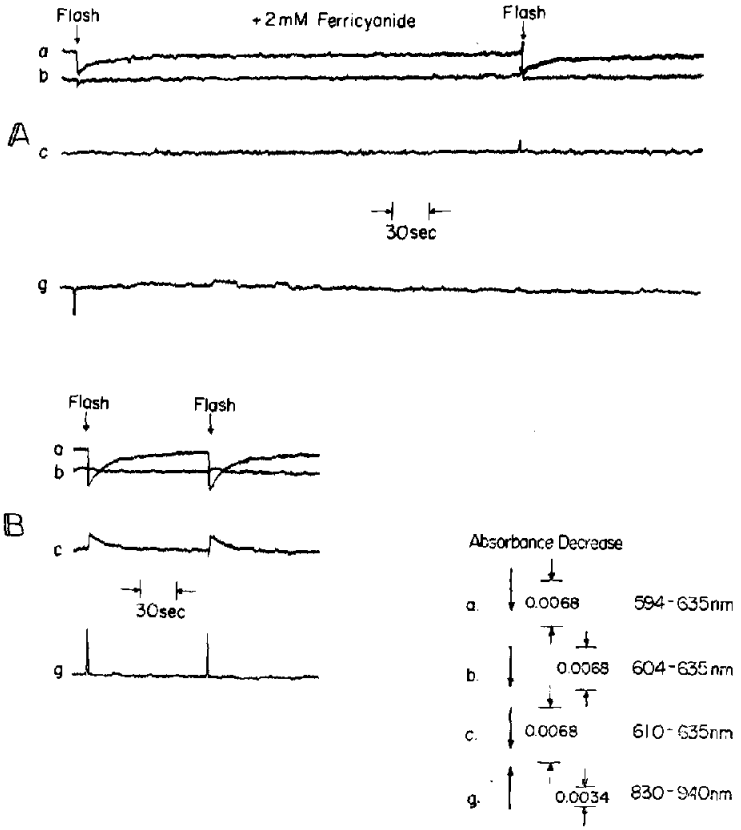


Fig.2. Carbon monxide dissociation from half-reduced and reduced cytochrome oxidase. Pigeon-heart mitochondria [59] (approx. 15 mg protein per ml) were suspended in a medium containing 75 mM sucrose, 30 mM Tris-HCl and 40% ethylene glycol, pH 7.2 and in addition 10 mM succinate and 10 mM glutamate. The mitochondrial suspension was saturated with carbon monoxide and transferred into e.p.r. tubes fitting a multiwavelength spectrophotometer [19,20]. The tubes were subsequently frozen at −80°C unitl use. In (A) 2 mM potassium ferricyanide was further added at room temperature with minimal admission of oxygen, followed by freezing to −80°C. The experiments were performed by firing a liquid dye laser [19,20] and appropriate readout at wavelength couples indicated in the figure. The experiment was performed at −97°C.

was present. In the presence of oxygen there is a very rapid increase of absorption at 830 nm which has been attributed to oxidation of copper [19].

The CO dissociation spectra reported by Greenwood et al. [16] for the isolated enzyme at room temperature are very similar to the spectrum of fig.1B both with the fully reduced and the half-reduced enzyme. We have noted, however, that if some oxygen is present upon flash photolysis of the half-reduced enzyme (unpublished experiments with pigeon heart mitochondria) there is in addition to the rapid decrease in absorption at 591 nm (cf. figs.1 and 2) an increase of absorption at around 610 nm in the difference spectrum, but the latter change is much slower than the former when measured at temperatures at or below $-100°C$ (contrast the fast change at 610 nm in fig.2). The extent of this slow change is oxygen dependent and is presumably due to the formation of 'Compound C' as described by Chance et al. [19], a species that may be similar to the 'oxygenated' from as described by Greenwood and co-workers [16], see also section5.

In summary, these data strongly suggest that free ferrous *minus* ferric cytochrome a_3 contributes very little, if at all, to the absorption band at 605 nm, and that this band is consequently mostly, perhaps entirely, due to cytochrome *a*. The slight (<20%) decrease in intensity of this band upon ligand binding to cytochrome a_3 is probably due to a small decrease in the extinction coefficient of ferrous cytochrome *a* as a result of heme–heme interaction. In the following analysis we are adopting this interpretation. The less probable alternative, as indicated above, would be that cytochrome a_3 contributes 20% or less to the 605 band, but absorbs here only when cytochrome *a* is reduced.

Thus our picture is not far from the original interpretation by Keilin and Hartree [1] and suggests that the hemes of cytochromes *a* and a_3 are spectrally very different.

2.2. *The calcium-induced shift in the spectrum of ferrocytochrome* a

As recently demonstrated by Wikström and Saari [21], calcium ions specifically shift the absorption band of ferrocytochrome *a* to the red in uncoupled mitochondria, submitochondrial particles and even in isolated cytochrome oxidase. This shift is indistinguishable from the red shift observed upon energization of coupled mitochondria by ATP. The fact that both the calcium- and ATP-induced spectral shifts are nearly unaffected both in wavelength dependence and extent by ligands to cytochrome a_3 [21–25] strongly suggests that the spectral properties of ferrocytochrome *a* can be only little affected by the binding of a ligand to cytochrome a_3. This furnishes additional strong evidence against the postulated large increase in extinction coefficient of ferrocytochrome *a* upon ligand binding to a_3 but is consistent with a minor decrease in the extinction coefficient of ferrous cytochrome *a* upon binding of a ligand to cytochrome a_3.

2.3. *Redox titration of cytochrome* a *in the aerobic steady state*

Since it has been clearly shown by Chance and collaborators [26,27] that electron transfer between cytochromes *c* and *a* (in both directions) is much faster than the overall rate of respiration, the two cytochromes are expected to be close to redox equilibrium with each other (see also [28]) even in the aerobic state.

Fig.3 shows an experiment where mitochondria have been titrated with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) aerobically in the presence of an excess of ascorbate as reductant. The higher the concentration of TMPD, the faster the rate of oxygen consumption and the higher the steady state levels of reduction of cytochromes $c + c_1$ and *a*. The redox states of these cytochromes have been correlated by a logarithmic plot in the figure. The best straight line correlation is obtained assuming that the measured cytochrome (at 605 nm) is the only species absorbing at this wavelength. Any contribution less than 100% gives a less satisfactory fit in the plot. This, again, suggests that cytochrome a_3 has little or no contribution to this absorption band.

Fig.4 shows the difference spectrum at $77°K$ of the species reduced under the conditions of the experiment of fig.3 i.e., aerobically in the presence of TMPD plus ascorbate. The split Soret band as well as the red shift in the α-band induced by calcium (fig.4B), which is here of the same magnitude as in the fully reduced enzyme, have been suggested to be

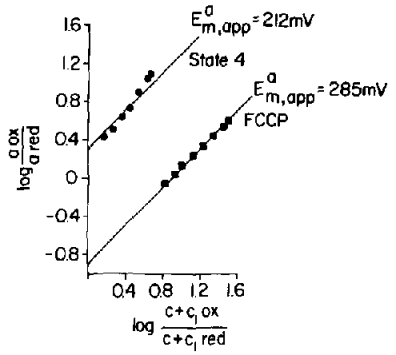


Fig.3. Titration of the redox states of cytochromes $c + c_1$ and a in aerobic respiring mitochondria. Rat liver mitochondria (2 mg protein per ml, isolated as described in [21]) were suspended in a medium containing 200 mM sucrose, 20 mM KCl, 20 mM HEPES buffer pH 7.2. In addition 3 μM rotenone, 0.2 mM EDTA and 6.7 mM ascorbate were present. Respiration was initiated by varying concentrations of TMPD in the absence ('State 4') or presence ('FCCP') of the uncoupler FCCP (1 μM). Dual wavelength measurements were done at 24°C at 550–540 nm (cytochrome $c + c_1$) and 605 minus 630 nm (cytochrome aa_3). The change in absorption at 605 nm from the aerobic to the anaerobic state was assumed to be entirely due to cytochrome a and a similar assumption was made for cytochrome $c + c_1$ at the other wavelength couple. At each concentration of TMPD, the log of oxidized/reduced cytochrome has been plotted in the figure. The apparent midpoint potentials for cytochrome a ($E^a_{m,app}$) have been calculated assuming redox equilibrium between cytochrome a and $c + c_1$ (see text) and an E_m for cytochrome $c + c_1$ of 230 mV [29].

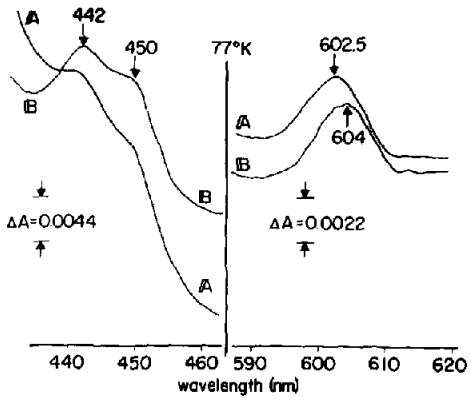


Fig.4. Difference spectra of cytochrome a in the aerobic steady state. Conditions essentially as described in the legend to fig.3. 0.5 μM FCCP was present. The mitochondrial suspension was divided into two batches and a sample was frozen at 77°K into the reference cuvette. Then 0.2 mM TMPD was added to the rest of the sample followed by quick freezing of the sample into the measuring cuvette. (A) shows the difference spectrum sample minus reference. In (B) the EDTA was omitted from the reaction mixture and replaced by 100 μM $CaCl_2$.

characteristic for cytochrome a. Thus complete reduction at 605 nm does not appear to introduce any new species (cytochrome a_3) that would not show the calcium-induced spectral shift.

Assuming then that cytochromes c and a are in redox equilibrium under aerobic conditions, the E_m of cytochrome a may be calculated from the data of fig.3 using the known E_m value for cytochrome $c + c_1$ as a reference [29]. Since the E_m of cytochrome $c + c_1$ is unaffected by the mitochondrial energy state [30], the midpoint potential of cytochrome a can be estimated cf. [22] both in the uncoupled state and in state 4. By this method it may be calculated that the apparent E_m of cytochrome a is about 285 mV in uncoupled aerobic mitochondria (cf. Nicholls and Petersen, [14]) and about 212 mV in state 4 (fig.3). Thus energization of the mitochondria apparently decreases the E_m of cytochrome a by approx. 70 mV in the aerobic state. This may be compared with similar findings in non-respiring mitochondria [22,24,30]. This is the first demonstration of an apparent energy-linked shift in the E_m of cytochrome a aerobically, in the absence of added ligands.

As will be shown below, the reported ATP-linked *increase* in the apparent E_m of cytochrome a as inferred from anaerobic potentiometric titrations [25,31] is a result of incorrect assignment of the two potentiometric transitions to cytochromes a and a_3 respectively (50/50 hypothesis). Energization of the mitochondrial membrane always induces an apparent decrease in the E_m of cytochrome a both anaerobically and aerobically and in the presence and absence of ligands added to the enzyme (see also below).

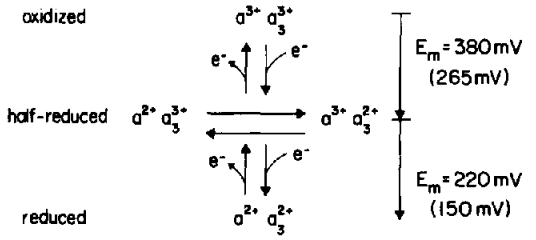
2.4. *The cross-over point between cytochromes* c + c_1 *and* a

The energy-linked E_m shift in cytochrome a as discussed above is most probably the basis for

the cross-over point observed by Chance and Williams [32] between cytochrome $c + c_1$ and a in the state 4 to state 3 (and vice versa) transition. Upon addition of ADP or uncoupling agents to mitochondria in state 4, cytochrome $c + c_1$ becomes more highly oxidized due to the increased flux of reducing equivalents from this component to oxygen. Since cytochrome a is in redox equilibrium with $c + c_1$ the former cytochrome would also a priori be expected to become more oxidized. However the simultaneous apparent increase in the E_m of cytochrome a (fig.3) would tend to make this component more reduced. Thus, in cases where the change in E_m of cytochrome a is larger than the change in redox potential (E_h) of cytochrome $c + c_1$ (and a) a cross-over point is observed between these redox carriers. However, when the shift in E_h is larger than the shift in E_m, both cytochromes $c + c_1$ and a become more oxidized in state 4 to state 3 transition (the cross-over is then 'beyond' cytochrome a, see [33]). Thus the cross-over point identifies an energy-controlled step of electron transfer in the respiratory chain (not necessarily a step of energy conservation, [34] and section 5.1.), in this case due to the energy-linked shift in the midpoint potential of cytochrome a.

3. Heme–heme interaction in cytochrome *c* oxidase

In the sections above we have arrived at the conclusion that the α-band of reduced cytochrome oxidase is due to cytochrome a with no more than 20%, contribution from cytochrome a_3. This picture is similar to the original interpretation by Keilin and Hartree ([1], see Introduction). The question then arises how the potentiometric titrations under anaerobic conditions can show *two* distinguishable components with widely different E_m values in the 605 nm band, and with 50/50 contribution to the band. This is, however, entirely possible if the heme–heme interaction between cytochromes a and a_3 affects the redox properties of hemes (i.e., the E_m values) as demonstrated by Malmström [11] and as proposed by Nicholls [13,14] in his recently published 'neoclassical' model of cytochrome oxidase. As pointed out by Malmström, the possibility of reduction of one of the hemes causing a shift in the



Scheme 1. The neo-classical model of cytochrome *c* oxidase. The scheme only refers to the behaviour of the two hemes, the copper atoms of the enzyme are not discussed in this article. For details, see text. Midpoint potentials in parentheses refer to the values found at a high phosphate potential [31].

E_m of the other by heme–heme interaction has been largely neglected in considering the results obtained from potentiometric titrations of the enzyme. Our main objective in this paper is to show that the assumptions required to maintain the 50/50 hypothesis are untenable, and that a model encompassing heme–heme interaction of the type described above is the only alternative explanation for the bulk of experimental data accumulated so far.

In the following sections the effects of heme–heme interaction in cytochrome oxidase on e.p.r. behavior, potentiometric properties, optical spectra and ligand binding will be analyzed as well as the effects of energization of the mitochondrial membrane.

Scheme 1 represents the 'neoclassical' model of cytochrome oxidase advanced by Nicholls [13,14]. This is the framework upon which the succeeding analysis will be based. The following postulates are inherent in this model and differ only slightly from those put forward by Nicholls:

(a) The hemes of cytochromes a and a_3 have quite different spectral properties and affinities to added ligands. The α band is entirely* due to cytochrome a (see above) and the Soret band is presumable

* Even a 20% contribution (which we consider very unlikely) of ferrous *minus* ferric cytochrome a_3 to the alpha band measured at 605 minus 630 nm would change the developed model only little. It would mainly cause small changes in the E_m values shown in tables 1 and 2, but would not alter the direction of heme–heme interaction or ATP-linked shifts in the E_m values.

composed of both cytochromes a and a_3 in roughly equal proportions. Cytochrome a_3 but not a reacts with ligands such as O_2, cyanide, CO, sulphide, etc. We see no reason to postulate that azide would be any different in this respect (see section 4.3).

(b) Heme–heme interaction occurs between the cytochrome a and a_3 hemes affecting the midpoint redox potentials as well as optical and e.p.r. properties. The major heme–heme interaction is such that reduction of one of the hemes induces a decrease in the E_m of the companion heme. This may be looked upon as being equivalent to a negative cooperative effect where an increase in ligand concentration (here electrons, i.e., a decrease in redox potential, E_h) results in a decreased affinity for the ligand (here a more negative E_m value of the heme).

(c) Half-reduction of cytochrome c oxidase results in an equilibrium mixture of ferrous cytochrome a and a_3 (i.e., $a^{2+}a_3^{3+} \leftrightarrow a^{3+}a_3^{2+}$, see scheme 1 and below).

3.1. *e.p.r. spectroscopy*

The e.p.r. studies of Leigh and collaborators [6,10,35] support the model presented in scheme 1. Their demonstration that only one kind of e.p.r.-detectable low spin ferric heme is present in the fully oxidized enzyme (see also [7,8]) may suggest that the ferric hemes a and a_3 are similar. However, alternatively only one of the two hemes is visible under the experimental conditions as indicated by Hartzell and Beinert (see also [11] and section 4.3.).

Half-reduction of the enzyme, both in the isolated form [7,8] and in submitochrondrial particles [6,10,35] results in the appearance of high spin ferric heme. As shown by Leigh et al. [6,10,35], two kinds of high spin ferric heme can be distinguished in an approx. 1:1 ratio, one in a much less symmetric environment than the other. The appearance of these high spin signals occurs with a half-reduction potential of about 380 mV in anaerobic redox titrations, thus coinciding with the apparent E_m found for the appearance of the high-potential component in titrations of the optical bands. This behavior agrees very well with scheme 1 where half-reduction of the enzyme (E_m = 380 mV) results in an equilibrium mixture between the species $a^{2+}a_3^{3+}$ and $a^{3+}a_3^{2+}$, i.e., *two different ferric hemes.* The high spin signals disappear again upon further reduction, with an E_m of approx. 220 mV, again in agreement with the light absorption data (reduction of the low-potential component). It should be noted that proponents of the 50/50 hypothesis have postulated two equilibrating different forms of ferric cytochrome a to account for these e.p.r. data [6,10,35], while we are proposing that the two high spin signals may be due to ferric cytochrome a and a_3 respectively in the mixed valency state of the enzyme complex. We suggest that reduction of one of the hemes in the transition from the fully oxidized form to the mixed valency state induces a transition from low to high spin in the companion ferric heme by heme–heme interaction. This effect is also reflected upon the E_m value of the latter heme and its spectral properties (see below). The effect of ligands on the e.p.r. spectra is further discussed in section 4.3.

3.2. *Potentiometric titrations in the uncoupled state*

Half-reduction of cytochrome oxidase results in the appearance of approximately 50% of the reduced minus oxidized absorption difference in the 605 nm region [3,4,37]. Since this band is entirely* due to cytochrome a (section 2) we can conclude that the equilibrium constant of the central equilibrium (scheme 1) is close to unity in agreement with the e.p.r. data (section 3.1.).

$$K_{eq} = \frac{(a^{3+}a_3^{2+})}{(a^{2+}a_3^{3+})} = 1 \qquad (1)$$

This means that cytochromes a and a_3 have very similar, perhaps identical, E_m values in the transition from fully oxidized to half-reduced as well as in the transition from half-reduced to fully reduced enzyme.

From the observed apparent E_m values in the potentiometric titrations (380 and 220 mV respectively) and the equilibrium constant (eqn. 1) we may now calculate the true E_m values for cytochromes a and a_3 respectively (table 1). It is seen that the two hemes have initially quite positive and similar E_m values, and that reduction of one of the hemes

* See footnote on p. 265.

Table 1
Midpoint potentials of cytochromes a and a_3 in uncoupled mitochondria

	Cytochrome a	Cytochrome a_3
High E_m	362 mV	362 mV
Low E_m	238 mV	238 mV

The E_m values have been calculated from the two half-reduction potentials found experimentally at pH 7.2 [3] as described in the text on the basis of the model shown in scheme 1.

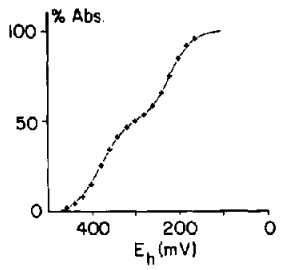


Fig.5. Simulated potentiometric titration of the 605 nm band of cytochrome oxidase under anaerobic conditions in uncoupled mitochondria. The simulation is based on the model described in scheme 1. Cytochrome a is assumed to contribute 100% to the absorption change at 605 minus 630 nm upon reduction of cytochrome oxidase while cytochrome a_3 has no contribution. The midpoint potentials of cytochromes a and a_3 calculated from the two E_m values found experimentally [3] have been used (see table 1) as well as the equilibrium constant K_{eq} for the central equilibrium (eqn. 1). The appearance of the 605 nm absorption peak (in per cent of total) has been plotted against applied redox potential. The simulation was performed using a programme applied to a Hewlett-Packard model 25 calculator.

(irrespective of which) induces a decrease of approx. 124 mV in the E_m value of the other heme. This change in E_m thus expresses the extent to which heme–heme interaction affects the redox properties of the enzyme under uncoupled conditions. The effect is reversible so that half-maximal oxidation of the fully reduced enzyme is thermodynamically a much more favorable reaction than subsequent complete oxidation of the half-reduced enzyme, the energy difference being of the order of 2.8 kcal/mole. The heme–heme interaction is also expressed upon the e.p.r. properties of the hemes (section 3.1.) and their optical properties (sections 3.4. and 3.5.).

Fig.5 shows a simulated potentiometric titration of the 605 nm band using the E_m values of table 1, the system of scheme 1 and the assumption that cytochrome a_3 does not contribute* to this absorption band. Note that the result is indistinguishable from the published titrations (cf. [31]) although no contribution of ferrous minus ferric cytochrome a_3 has been assumed for the α-band.

3.3. *Potentiometric titrations in the ATP-supplemented state*

The energization of mitochondria by ATP induces two important changes in cytochrome aa_3 as revealed by anaerobic potentiometric titrations [31]. Again two apparent E_m values are found (approx. 265 and 150 mV respectively) although very different from those in the uncoupled state. In addition, the spectral contribution of the high and low potential component is changed to 75 and 25% respectively (cf 50/50 in the uncoupled state), but the extent of the total reduced minus oxidized difference spectrum is unaffected by ATP. The ATP-linked change in the spectral proportions of the potentiometrically distinguishable components has been interpreted [5,31] as an ATP-induced increase in the extinction coefficient of ferrocytochrome a and a decrease in the extinction of ferrocytochrome a_3 (cf. postulated changes in ε upon ligand binding, sections 1 and 2). However, we consider it rather unlikely that such changes in the extinction coefficients would add up exactly to an unchanged overall extinction coefficient for the fully reduced minus fully oxidized transition. Our finding that the 605 nm band is due to cytochrome a makes this interpretation untenable.

In the present model the changed proportions of absorption upon half-reduction of the enzyme can be explained as an energy-linked shift in the central equilibrium of scheme 1. The new equilibrium constant in the presence of a high phosphate potential may again be calculated from the extent of absorption change upon half-reduction of the enzyme (ΔA is approx. 75% of total) in the same way as done above for the uncoupled state. The new equilibrium

* See footnote on p. 265.

constant in the 'energized' state (a very high ATP/ADP · P_i ratio) is then approximately,

$$K'_{eq} = \frac{(a^{3+}a_3^{2+})}{(a^{2+}a_3^{3+})} = 25/75 = 0.33 \qquad (2)$$

This obviously means that cytochrome a_3 has now become a better reductant than cytochrome a. Again, from the K'_{eq} and the apparent E_m values found experimentally, the 'real' E_m values of cytochromes a and a_3 may be calculated respectively. These are summarized in table 2. By comparison with table 1 we note the following changes induced by ATP:

(a) The high E_m values of cytochromes a and a_3 have apparently been decreased by 104 and 133 mV respectively, and the low E_m values (for the transition of the mixed valency state to fully reduced enzyme) by 52 and 81 mV respectively. As a result, cytochrome a_3 is now the better reductant of the two hemes though both hemes do become better reductants as a result of energization of the mitochondrial membrane. Note that the effect of ATP is greater on the oxidized to mixed valency state transition than on the transition from the latter to the fully reduced enzyme.

(b) The extent of heme–heme interaction has been reduced from 124 mV (table 1) to 72 mV (table 2), or by approx. 1.2 kcal/mole.

Thus ATP induces a decrease in the apparent E_m values of both cytochrome a and a_3 (see also [22,24])

Table 2
Midpoint potentials of cytochromes a and a_3 in mitochondria energized by a very high phosphate potential

	Cytochrome a	Cytochrome a_3
High E_m	258 mV	229 mV
(Effect of ATP, ΔE_m)	(−104 mV)	(−133 mV)
Low E_m	186 mV	157 mV
(Effect of ATP, ΔE_m)	(− 52 mV)	(− 81 mV)

The apparent E_m values were calculated for the highly energized state (a very high ATP/ADP · P_i ratio) as described in the text. The numbers in parentheses give the decrease in the respective E_m from the values given in table 1.

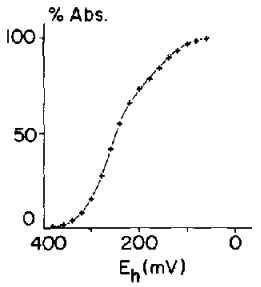


Fig.6. Simulated potentiometric titration of the 605 nm band of cytochrome oxidase under anaerobic conditions in mitochondria energized with ATP. The simulation was performed as described in the legend to fig.5, but using the E_m values of table 2 and K'_{eq} of eqn. 2.

and it need not be postulated that the direction of E_m shift in cytochrome a caused by ATP is any different in the presence and absence of ligands to cytochrome a_3 (see e.g., [22]).

Fig.6 shows a simulation of a potentiometric titration of the 605 nm band in the presence of ATP using the E_m values of table 2 and the K'_{eq} from eqn. 2. The result is again indistinguishable from the published potentiometric titrations at a high phosphate potential, (cf. [29]).

3.4. *Optical spectra*

Wilson and collaborators [3] have reported spectra of mitochondrial cytochrome oxidase taken during the course of anaerobic potentiometric titrations. These results have shown that the difference spectrum of half-reduced minus oxidized enzyme (measured E_m = 380 mV) peaks at a relatively short wavelength in the α-band (603–604 nm at room temperature), while the transition from the mixed valency state to the fully reduced enzyme produces a peak around 606 nm. This was also demonstrated in the isolated enzyme by Tiesjema et al. [4] who also showed that the former transition exhibited a single peak in the Soret region while the latter transition gave a split peak.

It seems to us that the positions of the light absorption peaks in the α-band are mainly dependent on whether one is looking at the difference spectrum between the mixed valency state and the oxidized state of the enzyme or the fully reduced minus mixed valency state difference spectrum, rather than

being dependent on whether the main reduced minus oxidized species is cytochrome *a* or a_3. This is similar to the conclusion made by Tiesjema et al. [4], who suggested that the difference spectrum Fe^{2+} minus Fe^{3+} depends on the redox state of the other heme group. We suggest that this effect on the optical spectra reflects the same heme–heme interaction which is also revealed as a transition to high spin in the ferric heme upon reduction of the other heme and as a decrease in the E_m of the former upon the reduction of the latter. Thus the heme–heme interaction in cytochrome oxidase appears to involve at least three physico-chemical properties of the enzyme, the spin state, the half-reduction potentials and the optical spectra, all three measurable effects being linked to one another in a fashion which fits the present model quite well. The interpretation of the heme–heme interaction on the optical properties of cytochrome aa_3 is supported by several experimental findings:

(a) Half-reduction of the ferric enzyme produces a peak of cytochrome *a* at wavelengths below 605 nm (room temperature) in several different cases, e.g., half-reduction of ferric aa_3 in the presence of ATP [22,31], reduction of cytochrome *a* aerobically in the presence of cyanide (e.g., [13]) or anaerobically in the presence of azide [3], and half-reduction of ferric aa_3 in the aerobic or anaerobic uncoupled state ([3] and fig.4).

(b) Reduction of the mixed valency state to fully reduced aa_3 gives rise to a peak at wavelengths higher than 605 nm. Examples of this are partial oxidation of aa_3 in the anaerobic uncoupled state (606 nm, [3,4]), oxidation of cytochrome *a* in CO-saturated mitochondria (606 nm, [25], cf. the shorter wavelength extreme when cyanide or azide have liganded *ferric* cytochrome a_3), and ATP-induced partial oxidation of cytochrome aa_3 in anaerobic mitochondria (608 nm at room temperature, [22,24,31] and fig.7).

Fig.7 shows the difference spectrum at 77°K of the partial oxidation of cytochrome aa_3 induced by ATP in anaerobic mitochondria, cf. [22,24,31]. The trough in the α-band is at very long wavelength as shown previously, but a clear split Soret band is shown here for the first time. Note that the position of the split trough (445 and 450 nm) differs from that attributed to cytochrome *a* in the aerobic

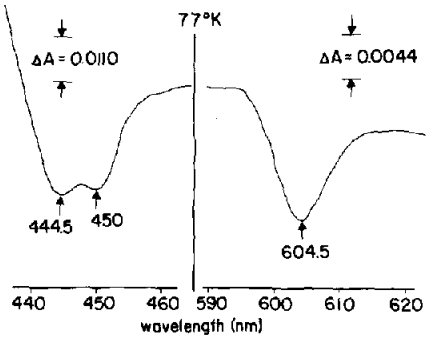


Fig.7. Energy-linked oxidation of cytochrome aa_3 in anaerobic mitochondria. Rat liver mitochondria [21] (approx. 2 mg protein/ml) were suspended in 0.2 M sucrose–20 mM KCl–20 mM HEPES buffer, pH 7.2, and supplemented with 3 μM rotenone, 0.2 mM EDTA and 6.7 mM succinate. After the suspension became anaerobic it was divided into two test tubes. 1.7 mM ATP was added to both samples and 1 μM FCCP to the 'reference sample' after which the two samples were frozen in liquid nitrogen into two 0.2 cm cuvettes. The spectrum represents the difference ATP-treated minus ATP + FCCP-treated mitochondria. A decrease in absorption in the sample (ATP alone) cuvette is reflected as a downward deflection in the difference spectrum.

steady state (442 and 450 nm, fig.3). The E_m values in the presence of ATP (table 2) suggest that under the conditions described in fig.7, there may be oxidation of both cytochromes *a* and a_3 in an approximate ratio of 1:2. Thus the difference spectrum (fig.7) is

$$a^{2+}a_3^{3+} + a^{3+}a_3^{2+} \text{ minus } a^{2+}a_3^{2+}$$

2 : 1

This is consistent with the split trough in the Soret region, which would not be expected from oxidation of cytochrome a_3 alone under the influence of ATP, an interpretation proposed by Lindsay and Wilson [31]. In this connection it may also be noted that the relative decrease in absorption of the Soret band upon ATP addition at room temperature to anaerobic mitochondria is greater than the decrease in the 605 nm (see e.g. figs.1 and 2 of [38]), once more suggesting that the proportions of cytochromes *a* and a_3 absorption are very different in these two spectral regions.

3.5. *ATP-linked spectral shift in ferrous cytochrome* aa_3

We have previously reported [22,24,38] that ATP induces a red shift in the spectrum of ferrous cytochrome *a* and that this shift can be mimicked by calcium ions in uncoupled mitochondria and in isolated cytochrome oxidase [21,24]. In view of the present data this red shift is thus the most probable reason why the ATP-linked partial oxidation of reduced cytochrome aa_3 is seen as a trough at an unusually high wavelength in the α-region (approx. 608 nm at room temperature, (see [22,23] and fig.7), a wavelength 1–2 nm longer than the long-wavelength maximum of the half-reduced to fully reduced transition of cytochrome *a* (section 3.4).

Lindsay and Wilson [31] have maintained that the ATP-linked red shift is in ferrous cytochrome a_3 but in order to do this, additional very complicated spectral changes introduced by ATP must be postulated. Thus, in order to explain why the ATP-linked shift is unaffected by cyanide and CO, it must be postulated that a red shift in cytochrome *a* occurs only in the presence of ligands to cytochrome a_3 but not in their absence. In addition, in order to explain the very long wavelength position of the ATP-linked oxidation of cytochrome aa_3, Lindsay and Wilson [31] assumed that ATP shifts the peak position of ferrous *a* towards the *blue* in the absence of ligands to cytochrome a_3. None of these postulates have been experimentally verified by any independent method. Moreover, as discussed in section 2.2, the 50/50 hypothesis has no explanation for the fact that the ATP- (and calcium-) linked shifts are almost identical in the presence and absence of various ligands to cytochrome a_3, which according to this model should cause a drastic increase in the extinction coefficient of ferrocytochrome *a*.

In view of the very simple explanation for these phenomena offered by the present model we feel confident that it may be close to the actual behavior of cytochrome *c* oxidase. The spectral properties of the enzyme are summarized in table 3.

Table 3
Spectral properties of cytochrome aa_3 in the 605 nm band

	λ_{max} (nm, room temp.)
Reduced minus oxidized	605
Half-reduced minus oxidized	603–604
Reduced minus half-reduced	606–607
+ ATP	608–609

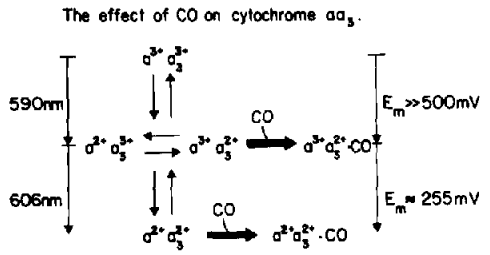
According to the present model cytochrome *a* is the only absorbing species in the 605 nm band. The absorption maximum is dependent upon whether cytochrome a_3 is oxidized (<605 nm) or reduced (>605 nm). For details, see text.

4. Effect of ligands to cytochrome aa_3

4.1. *Carbon monoxide*

The presence of a saturating concentration of carbon monoxide in the medium results, as shown by Lindsay and Wilson [3,25,31], in the finding of only one potentiometrically distinguishable species absorbing at 606 nm (cytochrome *a* by definition) which contributes more than 80% to the total reduced *minus* oxidized difference spectrum (without CO) and with a midpoint potential of approx. 255 mV. The large spectral contribution of this species was explained as being due to a very large increase in the extinction coefficient of ferrous minus ferric cytochrome *a* whenever cytochrome a_3 is liganded (similar findings have been made with other ligands such as cyanide, azide and sulphide, although the found E_m values differ, see [5]). However, as we have demonstrated in this paper, this explanation does not appear valid since there is no observable increase in the extinction coefficient of cytochrome *a* on binding of CO (section 2).

The reason for the shift of the E_m value of cytochrome *a* to 255 mV from the values reported in table 1 is apparent qualitatively from the modification of scheme 1 presented below (scheme 2). In the presence of saturating concentrations of CO the high-potential redox transition is usually lost experimentally for all practical purposes due to the stabilization of cytochrome a_3 in the ferrous form (a_3 attains a very positive apparent E_m value and is not oxidizable by ferricyanide, the oxidant used in potentiometric titrations [25]. The high affinity of the $a^{3+}a_3^{2+}$ species for CO (Greenwood et al., [16]) ensures moreover that the concentration of the



Scheme 2. The effect of carbon monoxide on cytochrome *c* oxidase. Modification of Scheme 1. Values to the left refer to absorption maxima at room temperature for the respective transitions. For details, see text.

species $a^{2+}a_3^{3+}$ is negligible under these experimental conditions. Thus we are left with the redox transition $a^{3+}a_3^{2+} \cdot CO \leftrightarrow a^{2+}a_3^{2+} \cdot CO$, the E_m value of which should be shifted from the E_m of the $a^{3+}a_3^{2+} \leftrightarrow a^{2+}a_3^{2+}$ transition (table 1) by an amount that depends on the relative affinity of the half-reduced and fully reduced enzyme for CO. Given the appropriate K_d values for CO (Greenwood et al., [16]) we may calculate the expected E_m value in the presence of an excess of CO according to methods described in detail by Clark [39]:

$$E_m^{CO} = E_m + \frac{RT}{nF} \ln \frac{K_{ox}}{K_{red}} + \frac{RT}{nF} \ln \frac{K_{red} + (CO)}{K_{ox} + (CO)} \quad (3)$$

which simplifies to:

$$E_m^{CO} = E_m + 60 \log \frac{K_{ox}}{K_{red}} \quad (4)$$

at saturating CO concentrations, pH = 7 and 25°C. Insertion of the published values for K_{ox} (0.59 μM), the dissociation constant for the $a^{3+}a_3^{2+}$ species, and K_{red} (0.33 μM), gives an E_m value of 238 + 15 = 253 mV for cytochrome *a* in the presence of CO, in excellent agreement with the E_m of 255 mV found experimentally. This quantitative fit must be considered very good support for the present model. In contrast to this interpretation, Wilson and collaborators [3,5] have somewhat arbitrarily suggested that the shift in E_m value of cytochrome *a* on CO binding to ferrous a_3 is the result of 'heme–heme interaction' and have not considered the different affinities of CO for the fully reduced and half-reduced enzyme.

4.2. *Cyanide*

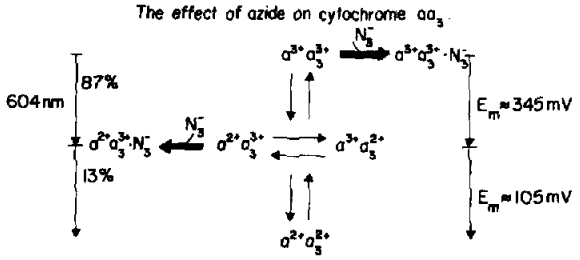
A good fit is also obtained for the case of cyanide, but this case is more complicated due to binding of the ligand to both the reduced, the oxidized and the half-reduced forms of the enzyme [40]. A quantitative test is difficult to perform for this case at present since the affinities of the different forms for cyanide are not known for the same kind of preparation. However, since it is known that the affinity of the oxidized enzyme is much greater than that of the fully reduced enzyme in mitochondria [41], and that the half-reduced form may have the highest affinity of all forms [40], $a^{3+}a_3^{3+} \cdot CN \leftrightarrow a^{2+}a_3^{3+} \cdot CN$ is expected to be the dominating redox transition, which would be shifted to somewhat lower values than the high E_m of cytochrome *a* (table 1). This is qualitatively in agreement with the found E_m of approximately 300 mV for cytochrome *a* in the presence of cyanide [5,42].

4.3. *Azide*

The potentiometric and spectral behavior of cytochrome oxidase in the presence of azide is very interesting. An increase in the azide concentration not only gradually decreases both the high and low apparent midpoint potentials in an anaerobic titration, but induces a drastic increase in the absorbance of the high-potential component (from 0.5 to 0.88, see Wilson et al., [3]) and a concomitant decrease in the absorbance of the low-potential component (from 0.5 to 0.12), the sum of the two components being constant. Wilson et al. [3,6] interpret these data to show that azide binds to cytochrome *a* in conflict with the traditional view of azide binding to cytochrome a_3, and with the finding of competition [41,43] between cyanide and azide towards cytochrome aa_3. The latter phenomenon was instead explained as being due to cytochrome a_3–*a* interaction [41] rather than simple competition for the same site (cytochrome a_3). Heme–heme interaction was also offered as the explanation for the large changes in absorbance of the high- and low-potential components of cytochrome oxidase in potentiometric titrations in the presence of azide [3] and for the observed changes in the measured E_m values (cf. interpretation of effect of CO). Thus it was suggested that the binding of azide to

ferric cytochrome *a* induces an increase in the extinction coefficient of cytochrome a_3 by heme–heme interaction. However, if it is simply assumed that azide combines with ferric a_3 (the traditional interpretation, see above), the effect of the ligand on the measured midpoint potential species in the presence of azide should differ from the high E_m in the absence of ligands by an amount that depends on the ratio of affinities of azide for the ferric and half-reduced enzyme. The found E_m at high azide concentrations [3], 345 mV, is consistent with this idea providing azide has a slightly higher affinity for the ferric than for the half-reduced enzyme (see Nicholls, [12]). The other component contributing only 13% to the absorption in the presence of excess azide has an E_m value of 105 mV [3]. Taking the K_i of 125 μM used by Nicholls [13] for the half-reduced enzyme and assuming that the fully reduced enzyme does not bind azide (or has a very high K_i), we arrive at an E_m value of 106 mV for the low-potential species at an azide concentration of 20 mM. This is again in excellent agreement with the E_m of 105 mV found experimentally [3]. The reason for the large shift in relative absorption of the high and low potential components by azide is also immediately apparent from scheme 3: due to the stabilization of the species $a^{2+}a_3^{3+}$ by azide, the high-potential transition results mainly in the species $a^{2+}a_3^{3+} \cdot N_3^-$ and in very little $a^{3+}a_3^{2+}$. Hence nearly all of cytochrome *a* is already reduced after the high-potential transition, resulting in the very large change in absorption. The remaining 13% of the absorption which appears with a very low midpoint potential is probably due to a slight increase in the extinction coefficient of ferrous cytochrome *a* upon release of the ligand concomitant with reduction of a_3 but might also be due to some contribution of ferrous cytochrome a_3, although the latter is certainly very small (see section 2).

The effect of azide on the e.p.r. spectrum of cytochrome *c* oxidase is also very interesting [6,43]. At high redox potentials a low-spin ferric heme signal is seen at g = 3 and is unaffected by azide. This signal can be attributed to approximately one-half of the heme present [36] and may thus be from ferric cytochrome *a* (see scheme 3). Upon half-reduction of the enzyme in the presence



Scheme 3. The effect of azide on cytochrome *c* oxidase. Modification of Scheme 1. Values to the left refer to the absorption maximum of the oxidized to half-reduced transition, and the per cent spectral contribution of the two transitions to the 605 nm band in the presence of an excess of azide. For details, see text.

of azide a new low-spin ferric heme signal appears at g = 2.9, not observed in the absence of the inhibitor, while the g = 3 signal disappears. This could then well be explained as being due to the appearance of the ferric cytochrome a_3–azide complex ($a^{2+}a_3^{3+} \cdot N_3^-$, see scheme 3) where cytochrome *a* is reduced (hence the disappearance of g = 3). At still lower redox potentials the g = 2.9 signal disappears as well, in agreement with reduction of the enzyme and release of azide. The redox potentials at which these different events occur are in good agreement with the E_m values of the optical spectra. In addition to these features of azide-binding, no high-spin ferric heme is seen in the presence of this ligand. This is again consistent with scheme 3 (cf. scheme 1 and section 3.1.) as the only ferric heme in the half-reduced state (a_3) is liganded to azide. Thus we feel that the present model offers a very simple explanation for the e.p.r. behavior of cytochrome aa_3. In the fully oxidized enzyme only low-spin ferric cytochrome *a* is visible. Upon half-reduction an equilibrium is established between two ferrous/high-spin ferric forms of the enzyme ($a^{2+}a_3^{3+}$ and $a^{3+}a_3^{2+}$). The strong heme–heme interaction is reflected as an effect of the reduction of one heme upon the companion ferric heme forcing the latter into a high-spin state with less affinity for an electron (lowered E_m, table 1) and a slightly shifted light absorption spectrum upon reduction (section 3.4.). This strong interaction between the two hemes suggests that they may be very close to one another as also

indicated by the results of Leigh et al. [44], but is of course not necessarily so. However, recent data on the first intermediates of the cytochrome oxidase oxygen reaction measured at very low temperatures (Chance, Harmon and Wikström, unpublished) suggest that cytochromes a and a_3 may indeed exchange electrons rapidly.

The reduced minus oxidized difference spectrum of the high-potential component in the presence of azide peaks at a short wavelength [3] (604 nm) as expected for a transition of oxidized to half-reduced aa_3 (see section 3.4.). However, as reported by Wilson [45], the reduced minus oxidized spectrum of cytochrome a peaks at 602 nm in the azide-inhibited aerobic steady state at room temperature. This anomalous behavior of azide deserves further study. It may be possible, for instance, that azide reacts under such conditions with a species of cytochrome oxidase not present during anaerobic potentiometric titrations, perhaps an oxygen compound of the aa_3 system [19,20].

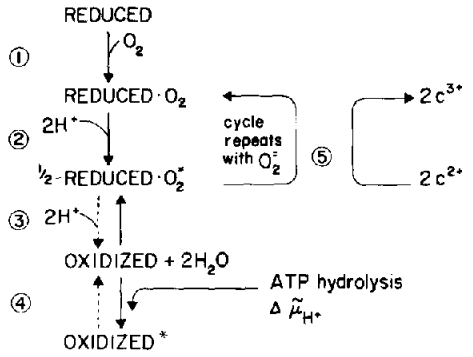
4.4. *Comparison of the effects of azide and membrane energization*

An extremely interesting facet of the effect of azide on the potentiometric and spectroscopic behavior of the 605 nm band is its rather close analogy to the effect of 'energization' by ATP. In both cases there is an apparent increase in the relative absorption of the high-potential component at the expense of the low-potential component. By using the azide effect as a model (scheme 3 and section 4.4.) we might then propose that energization of the mitochondrial membrane stabilizes the fully oxidized form of the enzyme and the $a^{2+}a_3^{3+}$ species of the half-reduced form (the latter is less stabilized than the former) over the fully reduced form. It must be noted, however, that this analogy is not quantitatively accurate. Thus 'energization' apparently stabilizes the fully oxidized species more with respect to the half-reduced species as compared to azide (large shifts in the high E_m values, table 2), but stabilizes the half-reduced species less with respect to the fully reduced enzyme, again in comparison with azide. The effect of 'energization' is in any case consistent with binding of a ligand with high affintiy to $a^{3+}a_3^{3+}$, the same or another ligand with lower affinity to $a^{2+}a_3^{3+}$, and much less stabilization of $a^{2+}a_3^{2+}$ and $a^{3+}a_3^{2+}$. This suggests, as in the case of azide, that the ligand has high affinity to forms of the enzyme which involve ferric cytochrome a_3. The energy-linked spectral shift in the ferric cytochrome aa_3 observed and studied recently [10,46–50] is consistent with this picture as is the apparent decreased affinity of $a^{3+}a_3^{3+}$ for cyanide upon energization of the mitochondrial membrane and the similarity between the spectral effects of energization and cyanide binding to the $a^{3+}a_3^{3+}$ species.

5. The mechanism of action of cytochrome aa_3

The introduction by Chance, Saronio and Leigh [19] of the low temperature trapping technique for studying the oxygen intermediates of cytochrome oxidase and their kinetics has made it possible to identify the reaction sequence of the enzyme. This work has indicated that the enzyme cycles between its fully reduced form, the 'oxy' form ($a^{2+}a_3^{2+} \cdot O_2$), and the half-reduced 'peroxy' form during activity without participation of the free ferric form [20]. More recent work has suggested that the 'peroxy' form of cytochrome oxidase (or Compound 'B') may actually be an equilibrium mixture of the species $a^{2+}a_3^{3+}O_2^{2-}$ and $a^{3+}a_3^{2+}O_2^{2-}$ (cf. scheme 1) rather than the first species alone, suggesting that cytochrome a is readily equilibrating with cytochrome a_3 at temperatures below $-100°C$.

The reason for the 'peroxy' intermediate not being readily oxidized to the ferric enzyme plus water may be deduced from the effect of heme–heme interaction described above (scheme 1 and table 1). The transition of the enzyme from the fully reduced to the half-reduced form occurs with a low midpoint potential (i.e., the hemes are relatively good reductants): the 'peroxy' species is formed. Further electron transfer from the second heme may now be hampered by the fact that the midpoint potential for this transition is 124 mV more positive (see table 1) and the free ferric enzyme may therefore be formed only to a slight extent. Instead, the enzyme preferentially accepts two more electrons from ferrocytochrome c to be able to, in the next step, reduce the bound O_2^{2-} ligand to water by the thermodynamically more favorable fully reduced to half-reduced transition (scheme 4).



Scheme 4. Tentative scheme of the function and control of cytochrome *c* oxidase. The enzyme is depicted to function normally via steps 1, 2 and 5. Under 'energized' conditions the oxidized form is stabilized (reaction 4) with inhibition of enzyme activity. For details, see text.

It is of great interest that a similar 'reluctance' of the enzyme to form ferric aa_3 is observed upon reacting the half-reduced species $a^{3+}a_3^{2+}$ with oxygen. As shown by Greenwood et al. [16] and by Chance et al. [19] there is an increase of absorption around 605 nm upon flash photolysis of, $a^{3+}a_3^{2+} \cdot CO$ in the presence of oxygen, the new species formed being clearly different spectroscopically from the ferric enzyme (see fig. 2A). Greenwood et al. [16] call attention to the spectral similarities of this compound with the so-called oxygenated form of cytochrome oxidase [51]. Chance et al. [19] have suggested that the heme of cytochrome a_3 may be oxidized to the ferryl state in this species (compound C). Another possibility may be partial reduction of cytochrome *a*, i.e., formation of $a^{2+}a_3^{3+}$ under these experimental conditions. In any case, again it appears clear that the transition of half-reduced cytochrome oxidase to the free ferric form is 'forbidden' in the mechanism of oxygen reduction (cf. scheme 4). One of the reasons for this may be the thermodynamic ineffectiveness of this transition which is apparent from the analysis above.

5.1. *The effect of membrane energization by ATP*

The behavior of cytochrome oxidase described above is expected to change drastically upon energization of the mitochondrial membrane at high phosphorylation potentials (high ATP/ADP · P_i ratios). Both hemes become far better reductants and the effect of heme–heme interaction on the E_m values is diminished (table 2). Under such conditions it is to be expected, at least from the thermodynamic standpoint, that a larger proportion of the enzyme would exist in the free ferric form ($a^{3+}a_3^{3+}$) as compared to the non-energized state. It is of great interest that the ferric cytochrome oxidase is indeed affected both in its spectroscopic properties and in its reactivity toward cyanide by the mitochondrial energy state [46–50]. Thus, as was pointed out by Wilson and collaborators [52], there is independent evidence for the relative stabilization of the ferric enzyme other than the observed shift in midpoint potentials. The stabilization of the ferric enzyme at high phosphorylation potentials (equivalent to a decrease in E_m values, table 2) would lead to inhibition of enzyme activity (see scheme 4) since the ferric species may not be involved in the normal catalytic cycle. In addition, as proposed by Owen and Wilson [53], the decrease in the concentration of ferrous cytochrome a_3 would be expected to severely limit respiration kinetically. Such a kinetic component of respiratory control, in addition to the widely accepted equilibrium type of control at 'coupling sites 1 and 2' may be necessary at cytochromes oxidase to ensure irreversibility and hence rapid electron flow in the active respiratory state [48–50].

Wikström et al. [48–50] have recently shown that the conformational changes observed in cytochrome oxidase (as evidenced by the energy-linked spectral shifts and change in affinity for cyanide) may be the response of the membraneous enzyme complex toward the establishment of an electrical gradient across the mitochondrial membrane. The mechanism of respiratory control at cytochrome *c* oxidase proposed by Mitchell [55] simply assumes that the electric field across the membrane would interact directly with transmembrane electron transport from cytochrome *c* to cytochrome a_3. Since no energy-linked conformational changes of the hemes are expected a priori from such kind of a mechanism we would propose as an alternative that the electric field and the pH gradient influences the kinetic and thermodynamic properties of the hemes via a conformational change of the complex. Recent studies have shown that the spectral shift in ferric cytochrome aa_3 is actually sensitive to the pH

gradient as well as to the membrane potential, i.e., sensitive to the entire electrochemical proton gradient across the membrane [49,50,54]. The reason for this is presumably that the conformational change involves binding of protons on the C-side and release on the M-side of the membrane [49,50]. Whether the observed 'high-energy' forms of cytochrome aa_3 are in addition directly involved in energy transduction as proposed by Wilson et al. [52] is presently uncertain and is under investigation [50].

In addition to the above evidence of a structural change in cytochrome oxidase in response to energization of the mitochondrial membrane, we have recently found [20] that energization of mitochondria at room temperature alters the kinetic behavior of cytochrome oxidase and cytochrome *c* at low temperatures in the frozen state (−25°C to −120°C). These effects involve acceleration of CO and O_2 binding by the enzyme and of the transfer of the first two electrons to bound oxygen with formation of the second easily recognizable intermediate, presumably a 'peroxy' form. Further reactions, such as oxidation of ferrocytochrome *c* and c_1 are partially inhibited after energization by ATP at room temperature. This data certainly strengthens the view that true structural reorganization occurs in the cytochrome oxidase complex in response to membrane energization. It is thus quite possible in view of this evidence that the potentiometrically observed energy-dependent shifts in the E_m values are 'real' in this case and not apparent, being due to electrode equilibration via a 'coupling site'. This contrasts greatly to the case of cytochromes *b* where there is little or no evidence at present for true changes in structure and conformation upon energization [56,57].

6. Summary and conclusions

Evidence has been presented which suggests that cytochromes *a* and a_3 are chemically different components of cytochrome *c* oxidase in situ with very different spectroscopic properties. However, in unperturbed systems (no ligands added, uncoupled mitochondria) the two cytochromes appear to have similar redox properties. The chemical difference between the two hemes may be located at the sixth coordination position of the heme iron, a point that appears to be open to attack by ligands in the case of cytochrome a_3 but not in cytochrome *a*.

The two hemes interact strongly so that reduction of one heme forces the other into a high spin state with a drastically decreased affinity of the latter for an electron (decrease in E_m). This interaction is also reflected on the absorption spectra so that the difference spectrum peaks at wavelengths just below 605 nm for the oxidized to half-reduced transition, but just above 605 nm for the half-reduced to reduced transition of the enzyme. The half-reduced enzyme appears to be a mixture of $a^{2+}a_3^{3+}$ and $a^{3+}a_3^{2+}$ in rapid equilibrium.

The spectral and potentiometric effects of ligands to cytochrome a_3 can be accounted for qualitatively and in some cases quantitatively from the different affinities of the ligand to the different forms of the enzyme. The effect of energization of the mitochondrial membrane involves a decrease in the E_m values of both hemes with a greater effect on cytochrome a_3 than on *a*, and on the oxidized to half-reduced transition as compared to the half-reduced to oxidized transition. In addition, ATP apparently decreases the extent of heme–heme interaction, i.e., the extent to which reduction of one heme affects the reducibility of the other heme.

It is proposed that the heme–heme interaction in cytochrome *c* oxidase has great bearing on the mechanism and control of oxygen reduction by the enzyme allowing it to cycle only between its oxygenated, reduced and half-reduced forms but not normally involving the fully oxidized form. In the energized mitochondrial membrane the oxidized form of the enzyme is stabilized with respect to both half-reduced and reduced forms in accordance with the shifts in E_m values. This would then shift part of the cytochrome oxidase molecules to a form not involved in the catalytic cycle and hence inhibit activity (respiratory control). Whether this 'inhibited' form is also an intermediate in energy coupling is presently uncertain.

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