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Energy-dependent reversal of the cytochrome oxidase reaction

(respiration/energy conservation/oxidative phosphorylation)

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ABSTRACT **Energization of isolated rat liver mitochondria with ATP under conditions in which cytochrome *c* is poised in a highly oxidized state shifts the state of cytochrome oxidase (cytochrome *c* oxidase; ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) from fully oxidized to two new spectroscopically distinguishable states depending on the applied phosphorylation potential and redox potential at cytochrome *c*. Both new states are spectrally similar or identical to two previously described intermediates in the reaction between reduced enzyme and O_2. The data suggest that the energy-dependent transitions are due to reversed electron transfer from water to ferricytochrome *c* linked to accumulation of intermediates of O_2 reduction at the catalytic heme a_3/copper center. Titrations with redox potential indicate that each transition is a one-electron step, a finding that would identify the second observed compound as enzyme-bound peroxide or its equivalent. This is consistent with this compound being spectrally identical to "Compound C," previously described as the reaction product between half-reduced oxidase (two electrons) and O_2. On the basis of these data a catalytic scheme of O_2 reduction is proposed for the heme a_3/copper center of cytochrome oxidase.**

Cytochrome oxidase (EC 1.9.3.1) catalyzes electron transfer from cytochrome *c* to O_2, with consequent reduction of O_2 to water. Electron transfer is linked to proton translocation across the mitochrondrial membrane (1). The minimal functional unit of cytochrome oxidase contains two hemes (*a* and a_3) and two coppers (Cu_A and Cu_B). Heme a_3 and Cu_B form a binuclear center of O_2 reduction, but cytochrome *a* and Cu_A are usually thought merely to aid electron transfer between cytochrome *c* and this center (2–6).

The introduction of the "triple-trapping" low-temperature technique by Chance *et al.* (7) rendered the mechanism of O_2 reduction experimentally testable. However, of observed intermediates only the first, namely the "oxy" species Fe^{II}—O_2 (or compound A), has been unambiguously identified. The structures of subsequent intermediates and the catalytic mechanism of O_2 reduction are still obscure (see refs. 7–11). It has even been suggested that some of the observed intermediates may be artefacts due to the use of CO in the triple-trapping technique (12).

In this contribution it will be demonstrated that the reduction of O_2 to water can be partially reversed by energization of isolated mitochondria with ATP under favorable conditions. These observations provide a simple explanation for the ATP-linked shifts in the oxidized enzyme described previously (1, 13) and an independent basis for identification of intermediates in the oxygen reaction. On the basis of these data, as well as earlier reports in the literature, a catalytic scheme of O_2 reduction by cytochrome oxidase is proposed.

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METHODS AND MATERIALS

Rat liver mitochondria were isolated as described (14). The concentration of mitochondria is reported as concentration of cytochrome aa_3. This was determined from the reduced minus oxidized (reduction by dithionite) absorption difference at the wavelength couple 605–630 nm, using a millimolar extinction coefficient of 27 cm^{-1} (15).

Split-beam and dual-wavelength spectrophotometry was performed at room temperature with DBS-1 (Johnson Research Foundation Workshops, University of Pennsylvania) and Aminco DW2 spectrophotometers. Cuvettes with a 1-cm light path were used throughout. Some spectra were also recorded at 77K (data not shown), using the low-temperature attachment of the DW2 spectrophotometer.

Experimental conditions are described in the legends to the figures. All reagents used, except carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), were commercial products of the highest purity available. The FCCP was kindly provided by P. G. Heytler.

RESULTS AND DISCUSSION

Experimental Rationale. When mitochondria are poised in a highly oxidized state by the action of ferricyanide through cytochrome *c*, the addition of ATP induces a large spectral change in ferricytochrome a_3 (13). The Soret band is shifted toward the red, and a broad increase of absorption is centered near 580 nm (see Fig. 1). The extent of change is a function of the $[ATP]/[ADP][P_i]$ ratio, and the effect is prevented and abolished by uncoupling agents, by oligomycin, and also by cyanide (13, 16, 17). This energy-dependent spectral shift has been attributed to generation of a "high-energy" state of cytochrome a_3 that may be directly involved in primary energy conservation (13, 18), or more specifically, in proton translocation (1, 19, 20).

It has not been excluded, however, that this effect may be a consequence of energy-linked reversed electron transfer (see ref. 21). Although O_2 generation from water has not been demonstrated despite numerous attempts (see, e.g., ref. 22), a partial reversal of the O_2 reaction remains possible. In such a case transfer of less than four electrons from water to cytochrome *c* may be expected to generate states of the a_3/Cu_B center that are identical with "oxygen intermediates" in the forward reaction between the reduced center and O_2. If so, large spectral shifts in cytochrome a_3 are indeed to be expected.

The phenomenon of reversed electron transfer may be described by the relationship (see, e.g., ref. 23)

$$E_2 - E_1 = \frac{\Delta G_p}{nF}, \qquad [1]$$

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

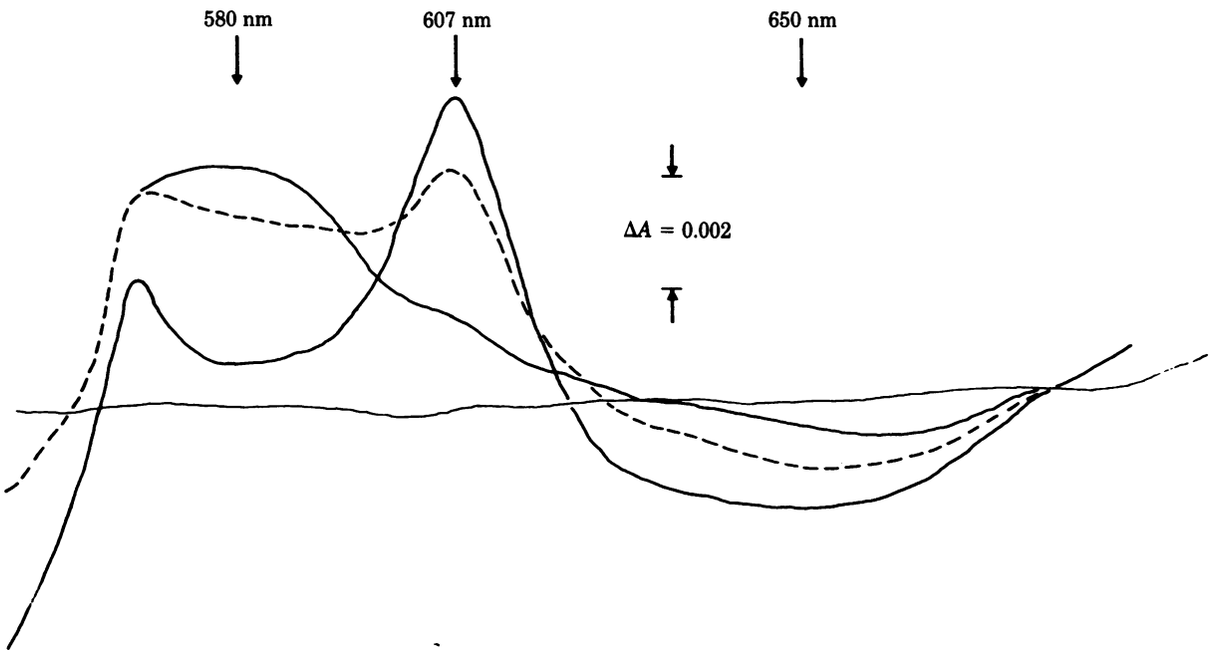

FIG. 1. ATP-linked spectral shift in ferric aa_3 at high pH in rat liver mitochondria. The mitochondria were suspended at 0.86 μM aa_3 in 0.2 M sucrose/20 mM KCl/30 mM Hepes–Tris buffer (pH 7.8), supplemented with 2 μM rotenone, 0.2 mM EDTA, and 1 mM potassium ferricyanide. A baseline (thin continuous line) was recorded by split-beam spectrophotometry. Then 2 mM of Tris ATP was added to the sample cuvette and the difference spectrum was recorded immediately from 500 to 700 nm at a speed of 5 nm/sec (solid line with 607-nm peak). This was immediately followed by two further sequential scans (broken line, and solid line peaking at 580 nm, respectively). Upward deflection corresponds to an increase of absorption in the sample, relative to the reference.

in which E_1 and E_2 are the respective redox potentials on the low- and high-potential sides of the energy-conservation "site," ΔG_p is the phosphorylation potential (equivalent to the Gibbs free energy change in ATP synthesis from ADP and P_i), F is the Faraday constant, and n is the number of electrons that need to be transferred from E_1 to E_2 for synthesis of one ATP molecule. This relationship is strictly valid only at equilibrium. However, due to the high degree of coupling between respiration and phosphorylation (see, e.g., ref. 24), it may still be a good approximation for near-equilibrium states such as those under the experimental conditions reported here (see ref. 25). In these conditions there is no detectable net electron transfer between cytochrome c and the O_2/H_2O couple.

According to Eq. 1 an increase in ΔG_p yields an increase in E_2 when E_1 is held constant (by a ferri–ferrocyanide redox "buffer"). The increased E_2 may then be sufficient to shift redox equilibria in the sequence of O_2 reduction to water. If the observed effect is indeed due to reversed electron transfer it should be enhanced independently by a rise in E_1 at constant ΔG_p.

Properties of the Energy-Linked Spectral Changes. In Figs. 1 and 2 it is shown that the ATP-induced 580-nm species (see ref. 13 and above) is replaced by a compound with intense and sharp absorption near 607 nm (ε_{max} = 10–12 mM^{-1} cm^{-1} on an aa_3 basis relative to the state prior to ATP) when the pH of the medium is raised. There was no accompanying dramatic change in the Soret band (not shown), which remained red-shifted relative to the state prior to ATP addition. Fig. 1 shows that the 607-nm state decays spontaneously in about 80 sec into the 580-nm state (and further to the state prior to ATP). This is due to slow ATP hydrolysis and reduction of ferricyanide by endogenous substrates. Both states were generated (depending on pH) to a maximal extent in 1–2 sec, either on addition of ATP (Figs. 1 and 2) or on changing pH by addition of alkali in the presence of ATP (not shown). Generation of both states was completely blocked by cyanide, uncoupling agents, and oligomycin (cf. ref. 13). Antimycin had no effect. In contrast, a spectral change seen near 560–570 nm (Fig. 1) was insensitive to cyanide and was modified by antimycin. The latter effect is thus not due to cytochrome a_3 but presumably arises from cytochrome b. Erecińska *et al.* (13) reported that the 580-nm state also forms anaerobically. This was confirmed and extended to include also the 607-nm compound. However, all experiments shown in this paper were done aerobically.

A rise in pH, particularly above pH 7, enhances ΔG_p (see ref. 26) and should therefore favor reversed electron transfer (Eq. 1). If this is the effect of pH here, the data suggest that formation of the 607-nm compound may require a higher ΔG_p than does formation of the 580-nm compound. This interpretation may be confirmed by studying the absorption changes at 580 and 607 nm as a function of [ATP]/[ADP][P_i] at constant pH (results to be published elsewhere; see also refs. 16, 17, and 19). It was indeed found that formation of the 607-nm compound required a ΔG_p about 2.2 kcal (9.2 kJ)/mol higher than formation of the 580-nm compound at constant pH and ferri-/ferrocyanide ratio.

If, as suggested above, the spectral changes are indeed the result of reversed electron transfer, their extents should be increased by raising the ferri-/ferrocyanide ratio at constant ΔG_p (*Experimental Rationale*). Moreover, formation of the 607-nm state should require a higher ferri-/ferrocyanide ratio than required to form the 580-nm state. Data pertinent to these predictions are shown in Fig. 3. The extent of both spectral changes is a function of redox potential, as anticipated (note that the 607-nm state was titrated at a higher pH to permit comparison at similar redox potentials). The slopes of the lines fitted through the points are each close to unity, suggesting that each transition corresponds to a one-electron transfer process. Note that the 607-nm transition, as plotted in Fig. 3, measures predominantly the conversion from the 580- to the 607-nm state, because the contribution of the original fully oxidized state is negligible in the titration range. This is due to the comparatively large difference in "driving force" required to generate the two states.

Taken together, these data indicate that the oxygen reaction may be reversed, starting from the fully oxidized heme a_3/Cu_B

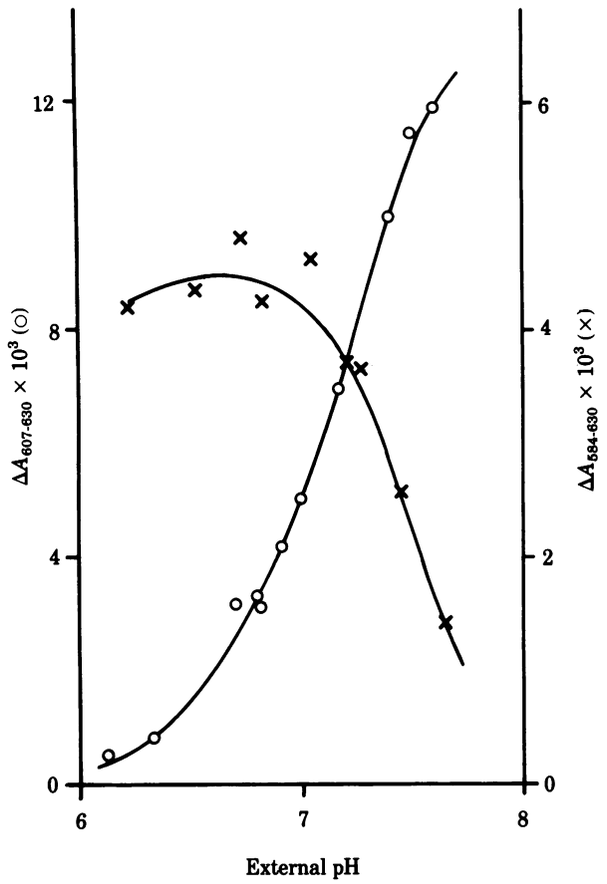

FIG. 2. pH dependence of the extent of absorption changes at 580 and 607 nm. Experimental conditions were similar to those for Fig. 1, except that the aa_3 content was 1 μM and the ATP-induced changes were measured immediately after 1 sec of stirring by dual-wavelength spectrophotometry at the indicated wavelength pairs. The pH was adjusted by addition of small portions of HCl and KOH prior to addition of the mitochondria and was checked before and after the addition of ATP.

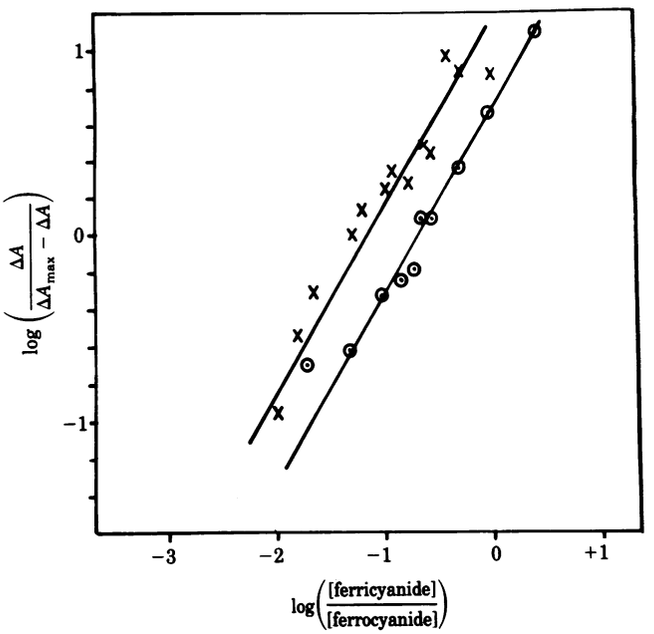

FIG. 3. Redox potential dependence of absorption changes. Conditions as described for Fig. 1, but measurements were done at 584 − 620 (X) and 607 − 630 (○) nm at pH 7.2 and 7.7, respectively. ΔA refers to the extent of the ATP-induced change in absorption at the indicated ferri-/ferrocyanide ratio. ΔA_{max} is the corresponding change in the presence of ferricyanide (3 mM) alone.

center plus water, by two sequential one-electron steps. The electrons are apparently transferred in an energy-dependent fashion from water, via cytochrome *c*, to the redox buffer. The final product of water oxidation may hence be peroxide that remains tightly bound to the a_3/Cu_B center. No further reversal appears possible, at least with ferricyanide as oxidant, and with the phosphorylation potentials achieved by addition of ATP.

Possible Structures of "Oxygen Intermediates". "Compound C" was previously described as the main product in the reaction between the half-reduced enzyme (a_3 and Cu_B reduced) and O_2. Formation of compound C is associated with a quite unusual spectral change, namely a large *increase* in absorption near 605–610 nm even though *oxidation* of the enzyme is expected (7, 8, 10, 12, 27). The spectral properties of the 607-nm compound are very similar, if not identical, to those of compound C. Difference spectra of the former at 77 K (not shown) are consistent with this identity. The increase in absorption at 607 nm in our conditions is also most unusual, as it is more enhanced the more oxidizing the conditions. Formation of the 607-nm compound by (stepwise) two-electron oxidation of the "oxidized" a_3/Cu_B center plus water provides further strong support for the identity of this species with compound C.

Preferential generation of compound C at highly oxidizing conditions (Fig. 3) is inconsistent with the idea that it contains ferrous heme a_3 (8, 10, 11). The lack of a sizable Soret band (see ref. 8) also contradicts this possibility. The lack of the 655-nm band typical of the oxidized enzyme (see Fig. 1) is a weak argument for ferrous a_3, because this band is also absent under some conditions in which a_3 is unambiguously in the ferric state (28). The possibility that compound C is a μ-peroxo species with ferric a_3 and Cu_B^{II} (ref. 29; see structure 1 in Fig. 4) is supported by the likelihood that reduction of O_2 to peroxide occurs by concerted two-electron transfer. The reasons for this are energetic (29) and structural (4, 6), as well as functional (5). Alternatively, the "peroxy" structure may be one with heme iron in the ferryl (Fe^{IV}) state and copper as Cu_B^I (30, 31).

If the "peroxy" state is indeed an important intermediate in the reduction of dioxygen, it should also be observed in the reaction between the fully reduced enzyme and O_2. We ascribe the intermediate designated "II" by Clore *et al.* (9) to this state. However, II is transient (9), because its formation is quickly followed by electron transfer from both heme *a* and Cu_A (30, 31).

"Oxygen intermediates" with spectral properties similar to those of the 580-nm species have been described by Orii and King (ref. 32; their "Compound II") and by Shaw *et al.* (33). In agreement with our data, this species indeed appears to be a "later" intermediate than compound C in the forward reaction. Our results suggest that this may be a one-electron oxidation product of the oxidized a_3/Cu_B center plus water. Because it may be considered unlikely that the reactive HO$^\cdot$ radical is formed in the catalytic sequence, it is proposed that this species may have the structure shown as no. 2 (Fig. 4). As shown, this would permit a mechanism in which the O_2 is reduced to water in two concerted two-electron steps, but in which the heme a_3/Cu_B center nevertheless receives electrons from cytochrome *a* in discrete one-electron steps.

The intermediate described by Shaw *et al.* (33) exhibits unique EPR resonances at $g = 5$, 1.78, and 1.68, which suggests a straightforward test of the proposed identity with our 580-nm compound. Meanwhile, it may be tentatively proposed that intermediate 2 (Fig. 4) could give rise to such a spectrum, due to antiferromagnetic coupling between Fe^{IV} ($S = 1$ or 2) and Cu^{II} ($S = \frac{1}{2}$), and a resultant binuclear $S = \frac{3}{2}$ center.

In the model of Fig. 4 the "oxy" species (compound A) is

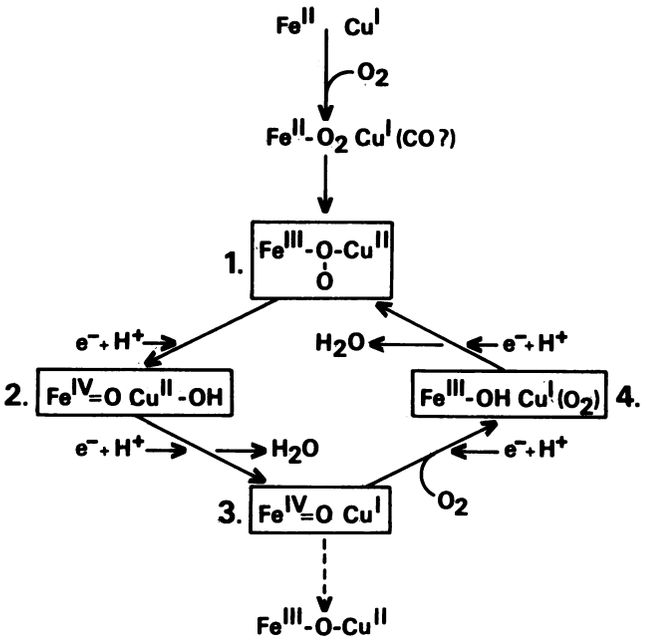

FIG. 4. Proposed catalytic scheme of O_2 reduction to water by the heme a_3/Cu_B center of cytochrome oxidase. Fe^{II} Cu^{I} and Fe^{III}-O-Cu^{II} represent the fully reduced and "oxidized" states of the heme a_3/Cu_B center, respectively. For discussion of structures 1–4 and possible alternatives, see the text.

considered to have very low probability in the aerobic steady state (cf. ref. 29). Accumulation of this compound at low temperatures after photodissociation of Fe^{II}-CO in the presence of O_2 (7) might be due to binding of the CO to Cu_B^I, preventing concerted two-electron transfer. Recent infrared data showing Co binding to copper at low temperatures (34) support this possibility, and also that Cu_B^I may be the primary O_2-binding site. Fig. 4 also shows two possible isoelectronic structures of the fully oxidized heme a_3/Cu_B center [state 3 and the μ-oxo compound (6), respectively]. More states than those shown may be required in the sequence between nos. 3 and 1. State 4 with high-spin heme iron might account for the rhombic $g = 6$ EPR signal observed in aerobic steady states (35).

It is concluded that the energy-dependent spectral shifts in ferric heme a_3 find a rational explanation from a partial reversal of catalytic steps of O_2 reduction, without implicating a direct role of cytochrome a_3 in energy conservation. The reason why O_2 reduction is not fully reversible may be thermodynamic irreversibility of the two-electron step that generates the "peroxy" intermediate. Together with previous considerations (see ref. 1) our data favor the possibility that cytochrome *a* rather than a_3 may be intimately involved in proton translocation. Thus transfer of each electron from cytochrome *c* to the heme a_3/Cu_B center via heme *a* is suggested to be linked to translocation of 1 H^+ across the mitochondrial membrane. In addition, 1 H^+/e^- is taken up from the mitochondrial matrix phase in the reduction of O_2 to water, as shown in Fig. 4 (1). More direct evidence for the role of cytochrome *a* as redox element of the proton pump in cytochrome oxidase will be given elsewhere (30, 31).

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