Resolution of cytochrome oxidase into two component complexes
(electron transfer complex/ion transfer complex/energy coupling system/exergonic and endergonic centers/mechanism of coupling)

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ABSTRACT Cytochrome c oxidase (ferrocytochrome c: oxygen oxidoreductase, EC 1.9.3.1) has been resolved into a pair of complexes of unequal molecular weight. The larger complex (electron transfer complex) contains exclusively the oxidation–reduction proteins characteristic of cytochrome oxidase; the smaller complex (ion transfer complex) shows exclusively the capability for cation-dependent induction of the fluorescence of 8-anilino-1-naphthalenesulfonic acid—a capability demonstrable in preparations of cytochrome oxidase. The duplex nature of cytochrome oxidase has important implications for the mechanism of energy coupling.

The mechanism of energy coupling is still one of the most baffling problems of biology. [See a recent review (1) for the epitomization of present uncertainties.] Whatever the principle of energy coupling, it is obvious that the structure of an energy coupling system must express with fidelity this principle and the mechanism of energy coupling has to be deeply rooted in the structure of the system. The ultimate test, therefore, of the validity of any proposed principle of energy coupling is that the principle should provide the key for the systematic resolution, description, and rationalization of the energy coupling system.

Because energy coupling depends upon the coupled interaction of driving and driven systems (2), it necessarily follows that energy coupling systems should be resolvable into a moiety that effectuates the driving reaction and a moiety that effectuates the driven reaction. Cytochrome oxidase, one of the four complexes of the mitochondrial electron transfer chain, has been shown in several laboratories to be an energy coupling system by various criteria (3–6). The ease of preparation of cytochrome oxidase makes it an ideal system for probing the structural parameters of energy coupling. The present communication is one of a series addressed to the systematic resolution of an energy coupling system and it deals specifically with the demonstration that cytochrome oxidase is a duplex cleanly resolvable into an electron transfer complex (ETC) and an ion transfer complex (ITC). These two derivative complexes have their own characteristic subunit compositions.

Elsewhere we have shown that Complex III is similarly resolvable into ETC and ITC complexes (7).

EXPERIMENTAL PROCEDURES

Preparation of Cytochrome Oxidase. The method of Fowler et al. (8) as modified by Capaldi and Hayashi (9) was used routinely. We have introduced the following purification step at the end of the Capaldi–Hayashi method. The suspension of cytochrome oxidase was dialyzed to remove cholate and ammonium sulfate, and the particles were resuspended at a final protein concentration of 10 mg per ml in 0.1 M phosphate, pH 7.4/2% potassium cholate. Further removal of impurities occurred with addition of neutral ammonium sulfate to 25% saturation. After incubation at 0°C for 4 hr, the mixture was centrifuged for 15 min at 30,000 rpm in a type 30 Spinco rotor. The purified cytochrome oxidase was precipitated at 33% saturation with ammonium sulfate and dissolved in water. The average yield of cytochrome oxidase from 28 g of protein of a frozen suspension of beef heart mitochondria was in the range of 1–1.5 g. The final preparation of cytochrome oxidase based on protein determination by the biuret method consistently had a heme content of 10–12 nmol/mg of protein and an equal or greater amount of bound copper.

Estimation of Heme. A modification of the pyridine hemochromogen methods of Basford et al. (10) and Rawlinson and Hale (11) was used for the determination of a heme. An aliquot of a hemoprotein suspension containing no more than 1 mg of protein was mixed first with alkali and then with pyridine (after clarification) to a final volume of 1 ml. The final concentration of NaOH was 0.10 M and of pyridine, 50%. The heme was then reduced by addition of Na2S2O4. The difference in absorption between 585 and 650 nm was measured; the mM extinction coefficient for this differential was taken to be 21.7. Absorption referable to turbidity is compensated for in this measurement of the differential.

Estimation of Copper. The method of Felsenfeld (12) was used for the extraction of copper from cytochrome oxidase and its determination. Adventitious copper was removed by exposing the suspensions to an aqueous medium containing EDTA and then removing the EDTA by successive washes.

Estimation of Protein. The biuret (13) and Lowry (14) methods were used routinely to determine protein with bovine serum albumin as the standard. The protein samples were solubilized with deoxycholate and where necessary alkali was added to compel solubilization.

Estimation of the Cation-Induced Fluorescence of 8-Anilino-1-naphthalenesulfonic Acid (ANS). The method of Feinstein and Felsenfeld (15) as modified by Kessler et al. (3) was followed.

Gel Electrophoresis. The method developed by Swank and Munkres (16) for sodium dodecyl sulfate/urea/gel electrophoresis in highly crosslinked gels was employed for the analysis of the subunit composition of cytochrome oxidase, ETC, and ITC. Sample preparation, buffer solutions, gel composition, and staining and destaining procedures employed were identical to those recommended by Downer et al. (17) with the exception that the staining time was doubled, and it was necessary to add dilute alkali to samples of ITC in order to effectuate complete solubilization. Electrophoresis was carried out in a Bio-Rad model 150A gel electrophoresis cell at a constant current of 2.5 mA per gel for 22–24 hr. Densitometric traces of the gel were made at 550 nm in a Gilford linear transport and recorder apparatus.

Abbreviations: ETC, electron transfer complex; ITC, ion transfer complex; ANS, 8-anilino-1-naphthalenesulfonic acid.
The ETC and ITC fractions were prepared as described in the text except that the pellets were not washed. Protein was determined by the Lowry method (14).

METHOD OF RESOLUTION OF CYTOCHROME OXIDASE

Preparations of cytochrome oxidase were first washed twice by dilution (1:10) in 0.05 M Tris-HCl (pH 7.4) and resuspended in 0.01 M Tris-HCl (pH 7.4) to a protein concentration of approximately 30 mg per ml. To 1 vol of cytochrome oxidase suspension (reduced with a trace of sodium dithionite) was added 6 vol of a 4:1 mixture of t-butanol and methanol, 0.12 M in HCl and 0.02 M in CaCl₂. The mixture was immediately vortex-mixed for 30 sec and incubated at 37°C. After 15 min the white precipitate of ITC was removed by centrifugation. The tight pellet was washed once with 5 vol of the same acid/ alcohol mixture, washed twice with 10 vol of 0.05 M Tris-HCl (pH 7.4), and then resuspended in a minimal volume of the 0.05 M Tris-HCl buffer.

The colored supernatant from which the ITC precipitate was removed was neutralized to pH 7.0 with Tris-base. After being thoroughly mixed the suspension was incubated at 37°C. After 15 min the green precipitate of ETC was removed by centrifugation, washed twice with 10 vol of 0.05 M Tris-HCl (pH 7.4), and then resuspended in a minimal volume of the buffer.

Table 1 is a summary of the yields of ETC and ITC achieved by the resolution method described. The ratio of ETC protein to ITC protein was 1.2:1. As shown in Table 2 the heme and copper contents of the ETC were both about 1.9 times the respective values for the unresolved cytochrome oxidase. These values are clearly rationalized in terms of the ETC to ITC protein ratio. No heme content was measurable in the ITC, and, although a small amount of copper was present, this was clearly the result of crosscontamination which was easily removed by washing with an EDTA solution (Table 3).

Spectral analysis of the ETC and ITC confirmed the exclusive localization of cytochrome a in the ETC, and also indicated that the method of resolution did not produce any modification in the spectral characteristics of this cytochrome (Fig. 1).

Cytochrome oxidase from beef heart mitochondria has been shown to contain at least seven subunits ranging in molecular weight from 35,000 (subunit I) to 4400 (subunit VII) (17). Fig. 2 contains the gel electrophoretic profiles of cytochrome oxidase, ETC, and ITC. Subunits I and III appeared to be exclusive to the ITC, whereas subunit II was common to both ETC and ITC fractions. The “chain-subunits” (IV–VII) so well defined in ETC were present to a marginal extent in ITC. We conclude from the gel profiles that subunits IV, V, VI and VII are intrinsic to ETC but not to ITC.

Cytochrome oxidase has been shown (3) to induce the cation-dependent fluorescence of ANS in an aqueous medium. This capability has been demonstrated to be a reflection of the presence of electrogentic ionophores. Table 4 contains the data establishing that the ITC, but not the ETC, contains the components that augment the cation-dependent fluorescence of ANS. The activity of the ITC was approximately 2.5-fold greater than that for cytochrome oxidase (using the same amounts of protein of each). When suspensions of ETC and ITC were mixed together, solubilized with alkali to pH 12, and then neutralized, the resulting particles behaved indistinguishably

![Fig. 1](image-url)
from cytochrome oxidase. When solubilization with alkali was omitted, the two populations of particles were easily demonstrable. (ITC is insoluble in the acid/alcohol mixture used for resolution whereas cytochrome oxidase is initially soluble.) This simple experiment demonstrates that the resolution of cytochrome oxidase is a reversible process.

**DISCUSSION**

Kessler et al. (3) were the first to recognize the ETC–ITC arrangement of cytochrome oxidase but the methodology for the quantitative resolution of cytochrome oxidase into its two derivative complexes had yet to be developed. The present communication documents a method for the clean resolution of cytochrome oxidase into two component complexes. The ETC contains exclusively the components necessary for oxidation–reduction reactions, whereas the ITC contains exclusively the components necessary to mediate cyclical cation transfer (2).

Wikström (6) has recently shown that electron transfer in cytochrome oxidase is coupled to protonic changes on the two sides of the membrane, which he interprets in terms of the coupling of electron and proton flow. Wikström is in agreement with our position that cytochrome oxidase is a duplex energy coupling system. We are proposing coupling of electron flow to cation transfer; Wikström proposes coupling of electron flow to proton transfer.

Based on the molecular weights of the subunits in each complex (17), ITC (subunits I, II, and III) has a molecular weight of 80,500, and ETC (subunits II, IV, V_a, V_b, V_l_a, V_l_b, and VII), a molecular weight of 90,800. The doublets for subunit V are designated as V_a and V_b; the doublets for subunits VI, as V_l_a and V_l_b. The molecular weights for these four species were assumed to be 14,600 (V_a), 12,400 (V_b), 10,300 (V_l_a), and 8,200 (V_l_b)—the values for V_a and V_l_a being estimated by extrapolation. The predicted molecular weight of cytochrome oxidase is accordingly 171,300 with a heme and copper content of 11.7

**Table 4. Cation-dependent augmentation of ANS fluorescence by cytochrome oxidase, ETC, and ITC**

<table>
<thead>
<tr>
<th>Complex</th>
<th>No cation</th>
<th>Ca^{2+}</th>
<th>K^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome oxidase</td>
<td>10</td>
<td>38</td>
<td>25</td>
</tr>
<tr>
<td>ETC</td>
<td>3</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>ITC</td>
<td>51</td>
<td>100</td>
<td>70</td>
</tr>
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Fluorescence measurements were made in a Perkin-Elmer MPF-3 fluorescence spectrophotometer at 22°C with an excitation wavelength of 380 nm. The sample (4 ml) was 10 mM in Tris-HCl (pH 7.5), 50 μM in ANS, and 0.2 M in the chloride salt of either Ca^{2+} or K^+.

**Table 5. Heme contents of cytochrome oxidase and ETC and the ETC to ITC protein ratio**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Heme content, nmol/mg protein*</th>
<th>ETC to ITC ratio, mg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome oxidase</td>
<td>10.8</td>
<td>1.17</td>
</tr>
<tr>
<td>ETC</td>
<td>21.1</td>
<td>22.0</td>
</tr>
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</table>

* The values for protein were determined by the Lowry method and, depending upon the preparation, these could be higher or lower than those determined by the biuret method. The range of variation is usually within 10%.
nmol per mg of protein, given two molecules of heme and two atoms of copper per unit of molecular weight. The correspondence of theoretical and observed heme and copper values for cytochrome oxidase and ETC is satisfactory (see Table 5).

In mitochondria electron flow can be coupled to ATP synthesis, active transport of cations, transhydrogenation, or cyclical transport of cations. How can these options be reconciled with the commitment in cytochrome oxidase to the coupling of electron flow to cation transport? A control mechanism involving a Ca\(^{2+}\)-induced transition determines the coupling options in mitochondria according to Hunter et al. (18), and thus the coupling of electron flow can be switched to ATP synthesis from cyclical cation transfer by virtue of this controlled transition in coupling modes.

The duplex nature of cytochrome oxidase establishes beyond peradventure that coupling is direct—i.e., that electron flow is directly coupled to ion transfer. Models such as the chemiosmotic model that postulate indirect coupling between electron flow and cation transfer via a membrane potential are incompatible with the experimental facts.

How general is this ETC–ITC arrangement of electron-transfer complexes? Does it apply to other complexes of the mitochondrial electron transfer chain? Elsewhere we have described the resolution of Complex III into ETC and ITC (7) by an identical method to that described above for the resolution of cytochrome oxidase. Furthermore, this concept has been shown to apply to Complex II (unpublished data). As we expected, the ETC–ITC structure of electron transfer complexes may be general for energy coupling systems in the sense that each such system is a composite of a driving reaction center and a driven reaction center.

Phan and Mahler (19, 20) have isolated a derivative form of the electron transfer chain from cytochrome oxidase of both yeast and beef heart mitochondria. The electrophoretic gel profile of this fragment closely resembles that of the ETC reported above and contains the same set of five subunits including the doublet for V and VI.

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