RESPIRATORY CONTROL AND OXIDATIVE PHOSPHORYLATION OF THE CYTOCHROME c-CYTOCHROME OXIDASE COMPLEX

by

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SUMMARY--Our reconstituted cytochrome c-cytochrome oxidase complex showed an average respiratory control index of 4 in the classical ADP reaction and 3 in the oligomycin-FCCP system. Respiratory control was obscured or abolished upon the addition of exogenous cytochrome c or by treating the complex with such membrane-breaking agents as cholate, Triton X-100, or bilirubin. Phosphorylation from the oxidation was demonstrated, in addition to ADP/O, by $^{32}P_i$ uptake, light emission in the presence of luciferase and luciferin, and the reduction of TPN by the hexokinase-glucose-6-phosphate dehydrogenase system. There were no requirements for external phospholipids or other factors in order to obtain respiratory control and oxidative phosphorylation exhibited by the cytochrome c-cytochrome oxidase complex.

Although the existence of a cytochrome c-cytochrome oxidase complex can be soundly deduced from modern concepts of the respiratory chain, its actual isolation has not been successful mainly because of the solubility characteristics of the components. However, reconstitution of this segment of the respiratory chain has been successful (1). The cytochrome c in the reconstituted cytochrome c-cytochrome oxidase complex is more active than the free form and approximates the properties of endogenous c (2). Recently we have found that the cytochrome c-cytochrome oxidase complex possesses a capability of controlled oxidation of substrates as manifested by respiratory control in the classical ADP reaction of Lardy and Wellman (3) or in the uncoupling system as defined by Lee and Ernster (4). Moreover, the complex also shows oxidative phosphorylation without the addition of any external coupling factors or phospholipids. Since the system is so simple and well defined, the results thus obtained certainly lend a new approach to, if not a new perspective of, the long eluded mechanisms of a basic biochemical

reaction--oxidative phosphorylation.

EXPERIMENTAL--Cytochrome oxidase was prepared by the ammonium sulfate-cholate-Emasol method of this laboratory (1) with modifications to be published. The modifications enabled us to obtain cytochrome oxidase with the highest heme to protein ratio, to our knowledge, ever reported, i.e. 11 nmoles heme a per mg protein and showed enzymic activity of 200 electron equivalents per sec per molecule of heme a. The oxidase preparations by our original method (1) showed the same properties as those of the modified preparation described in this paper. Cytochrome c was crystallized from beef heart (5) or procured from Sigma. In the latter case, it was exhaustively dialyzed before use. The cytochrome c-cytochrome oxidase complex was prepared as detailed previously (1, 2) in 0.01 M phosphate buffer, pH 7.4, and 0.025% Emasol. The reaction mixture was separated on a Sephadex G-200 column.

Oxygen uptake was determined polarographically in a Gilson oxygraph with a Clark oxygen electrode at room temperature (about 22°). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was kindly supplied by Dr. P. G. Heytler and used in a 50% ethanolic solution. A stock solution of bilirubin was prepared as before (6) and Triton X-100 was dissolved in 0.01 M phosphate buffer, pH 7.4, containing 0.025% Emasol-1130. Emasol was generously donated by the Kao Soap Co., Tokyo. The extent of oxidative phosphorylation was determined by the ADP/O ratio (7), the formation of ATP by the luciferase-luciferin method (8), the reduction of TPN by the hexokinase-glucose-6-phosphate dehydrogenase system (9) and by the incorporation of $^{32}P_i$ (10). Radioactive inorganic phosphate was purchased from New England Nuclear and purified by repeated treatment with $Mg(NO_3)$, hydrolyzed in HCl and finally recrystallized as the magnesium salt.

RESULTS AND DISCUSSION--The cytochrome c-cytochrome oxidase complex was eluted from a Sephadex column as a single band and was completely devoid of free cytochrome c. The difference spectrum of a typical preparation of the complex is shown in Fig. 1. The ratio of the oxidase in terms of heme a

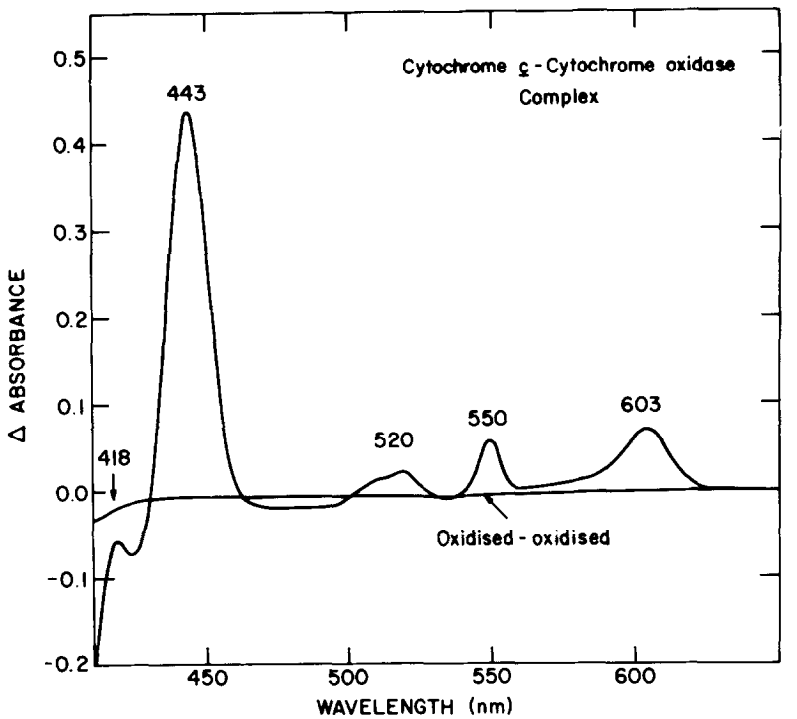

Fig. 1. The difference spectrum of the reduced minus the oxidized cytochrome c-cytochrome oxidase complex in 0.01 M phosphate, pH 7.4, and 0.025% Emasol-1130. The spectrum was obtained by the conventional way; i.e., the dithionite reduced preparation (with a moderate excess of $Na_2S_2O_4$) was used as the reduced and the "as prepared" complex as the oxidized.

to cytochrome c of the complex was found to be 2 (cf. Ref. 2) and

$(A_{278}-A_{480})/(A_{414}-A_{480}) = 2.0$.

The complex showed cytochrome oxidase activity using ascorbate as a substrate without addition of exogenous cytochrome c, although the latter was stimulatory (see below). Ascorbate cannot be oxidized by cytochrome oxidase alone. The catalytic oxidation by the complex was somewhat inhibited by oligomycin. In the presence or absence of the antibiotic, oxygen consumption increased immediately upon addition of a low concentration of FCCP (Curves 1 and 2 in Fig. 2). Respiratory control between 2 and 6 was observed in more than twenty preparations. In spite of the increase in the rate of oxygen uptake by FCCP, addition of exogenous cytochrome c still further increased

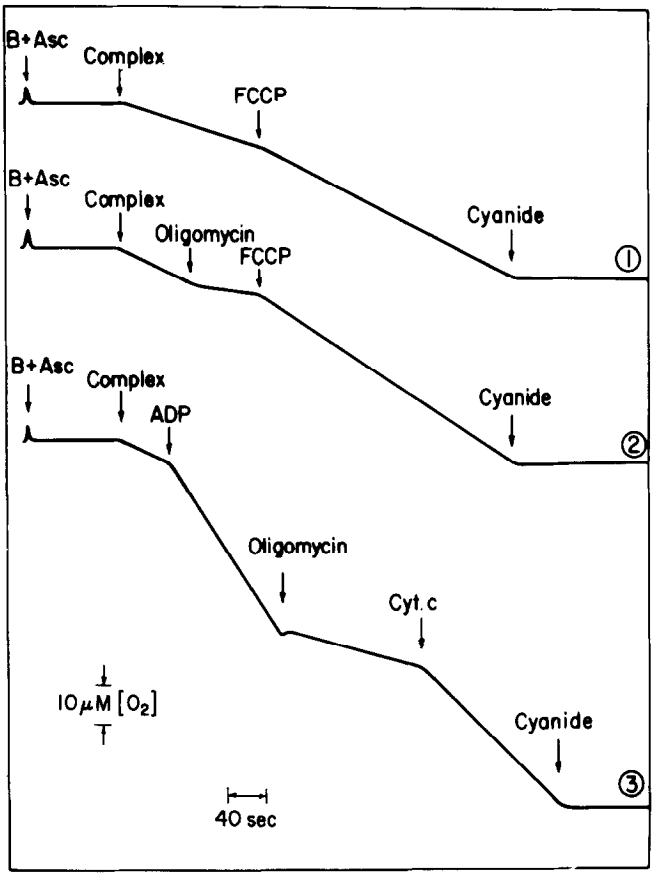

Fig. 2. Protocols of respiratory control and oxidative phosphorylation in the cytochrome c-cytochrome oxidase complex. The basal medium at pH 7.4 consisted of 9.3 mM Tris-maleate, 168 mM sucrose, 4.6 mM $MgCl_2$, 9.4 mM Na/K-phosphate, 31 mM ascorbate (Asc) and 0.2 mM EDTA. The complex in an amount of 0.5 μM bound cytochrome c and 1.0 μM cytochrome oxidase (in terms of heme a), 5 μg oligomycin, 50 ng FCCP, 0.2 μmole ADP, 10 nmoles exogenous cytochrome c and 30 nmoles KCN were added where indicated. Total volume was 1.96 ml and temperature 23°.

the rate of oxidation. The rapid consumption of oxygen in the presence of a large excess of cytochrome c is evidently artificial, not dissimilar to other respiratory enzymes using artificial electron carriers such as ferricyanide and indeed also external cytochrome c as in the isolated DPNH dehydrogenase systems (11). At any rate, oxidation either with or without FCCP was completely inhibited by low concentrations of cyanide.

The respiratory control of the complex as shown in the systems de-

scribed in Fig. 2 was obscured or abolished by exogenous cytochrome c, prior incubation of the complex with 2% cholate, or addition of Triton X-100 as low as 0.005% or 1.2 μM bilirubin. Poly-L-lysine did not show any effect.

Respiratory control was also observed in the classical ADP system (3) and found to be 3 to 6 (cf. Curve 3, Fig. 2). This fact led us to believe that the complex might also possess phosphorylating capabilities. In a system containing the complex and ascorbate, ADP stimulated oxidation and concurrently induced inorganic phosphate uptake. Experiments from three lines of evidence confirmed the occurrence of phosphorylation or ATP formation (Table I). The formation of ATP in the reaction mixture was shown by the luciferase-luciferin method. Although, the luciferase preparation may contain adenylate kinase, the initial light response of the reaction was greatly different in the complete system than in the system without the complex. Actually our determination with luciferase underestimated the amount of ATP formed in the oxidation because a correction was always made. Phosphorylation was also demonstrated by a system in which ATP was trapped as glucose-6-phosphate by hexokinase and the extent of TPN reduction then determined with glucose-6-phosphate dehydrogenase by the conventional method. Phosphate uptake was further confirmed by $^{32}P_i$ incorporation with or without the trapping agent of hexokinase.

The variation of the P/O ratios as summarized in Table I may not be due completely to the different methods employed for the determination of ATP but rather mainly to the different preparations of the complex used. It may be pointed out that these P/O ratios are still small for so-called Site III phosphorylation. However, our conditions were certainly not optimal. Moreover, the complex in the reaction system was found to be fragile as witnessed by loss of respiratory control after a prolonged oxidation. Recently Racker and Kandrach (12) reported the reconstitution of oxidative phosphorylation at Site III with a P/O ratio of 0.13 from five isolated proteins and one phospholipid ("hydrophobic protein,"

Table I. Demonstration of oxidative phosphorylation in the cytochrome c-cytochrome oxidase complex by various methods*

Method	P/O (or ADP/O)**
1. ADP/O	0.4-0.6
2. Light emission	0.08
3. TPNH formation	0.2
4. $^{32}P_i$ incorporation	0.4

*The reaction medium at pH 7.4 contained 5.4 mM Tris-maleate, 0.01% Emasol-1130, 97 mM sucrose, 2.7 mM $MgCl_2$, 5.4 mM inorganic phosphate (with 5×10^5 c.p.m. of $^{32}P_i$ in Method 4), 0.14 M glucose, 32 mM ascorbate, 0.21 mM EDTA and 0.05 mg/ml of hexokinase. At appropriate times, an aliquot of the reaction mixture was removed, deproteinized with 35% perchloric acid as usual and assayed for ATP formed according to the following methods: light emission by the luciferin-luciferase method (8), TPNH formation by the hexokinase and glucose-6-phosphate dehydrogenase system (9), and $^{32}P_i$ incorporation into ATP by the isotopic distribution or absolute counting (10).

**The difference in P/O ratios is partly due to the different batches of the cytochrome c-cytochrome oxidase complex used and the luciferase method evidently underestimated the ATP formed, see Text.

cytochrome oxidase, cytochrome c, oligomycin sensitivity conferral protein, F_1 (coupling factor 1), and purified soybean phospholipid). They did not observe any respiratory control in their system. Very recently Hinkel, Kim and Racker (13) claimed respiratory control but without oxidative phosphorylating activity in their system of cytochrome oxidase plus an exogenous phospholipid. In contrast, our system is much simpler but both respiratory control and oxidative phosphorylation have been reconstituted. The simplicity and clear definition of every component in our system may give a new impetus in pursuing the mechanisms of oxidative phosphorylation.

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