Biochimica et Biophysica Acta, 503 (1978) 67—77

BBA 47516

THE EFFECT OF MITOCHONDRIAL ENERGIZATION ON CYTOCHROME *c* OXIDASE KINETICS AS MEASURED AT LOW TEMPERATURES

II. THE BINDING AND REDUCTION OF DIOXYGEN

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(Received October 17th, 1977)

Summary

The effect of pre-energization of isolated mitochondria by ATP at room temperature upon the kinetics of oxygen intermediates (measured at very low temperatures) of cytochrome *c* oxidase has been studied. It was found that "energization" of mitochondria at room temperature had dramatic effects on several partial reactions of cytochrome aa_3. Thus, in the "energized" frozen state, the rate of O_2 binding to ferrous cytochrome a_3 and the subsequent formation of the "peroxy" compound B are accelerated, while oxidation of cytochromes *c* and c_1 is inhibited. These effects of ATP are abolished by oligomycin and uncoupling agents and may, therefore, be reflections of the coupling of the mitochondrial ATP synthetase to the respiratory chain at the level of cytochrome *c* oxidase, which is the basis of the mechanism of coupling respiration to ATP synthesis and respiratory control.

Introduction

Mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) is composed of four oxidation-reduction components: two hemes (cytochromes *a* and a_3), and two protein-bound copper ions. Low temperature studies on the mechanism of oxygen reduction indicate the existence of at least two intermediates (termed compounds A and B) involved in the physiological function of the enzyme

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Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

[1,2]. As previously demonstrated in low-temperature studies, the reaction of reduced cytochrome aa_3 with dioxygen results first in formation of "oxy-cytochrome oxidase" (compound A) with the probable structure $a^{2+}\ a_3^{2+} \cdot O_2$. The next detectable intermediate is "peroxycytochrome oxidase" (compound B), in which the bound dioxygen has apparently received electrons from the heme and copper redox centers. The next observable step (at warmer temperatures) is electron donation to cytochrome oxidase by cytochromes c and c_1 with concomitant oxidation of the latter [1,2].

Mitochondrial cytochrome c oxidase is also involved in energy transduction via oxidative phosphorylation. Subsequent to the observation of crossover points in the respiratory chain induced by changes in the concentrations of ADP, P_i and ATP [3,4], intermediates of the redox reactions in the respiratory chain capable of coupling to the phosphorylation mechanism have been sought. Wilson and Dutton [5,6] have described ATP-dependent shifts in the midpoint redox potentials of cytochromes a and a_3 (see also ref. 7), while Wikström et al. [8–11] and Wilson et al. [12,13] have shown that the conformation of heme aa_3 is sensitive to the energy state of the mitochondrion. Wikström has recently demonstrated that cytochrome c oxidase may function as a conformationally coupled redox-linked proton pump [14,29].

The development of low temperature kinetic techniques, by which the partial reactions of cytochrome c oxidase may be studied due to markedly slowed kinetics [1,2,15], affords an opportunity to determine effects of membrane energization on the intermediate reactions of cytochrome aa_3. Previous work [15,16] could not detect a difference in the rate of cytochrome a and a_3 oxidation after energization with ATP, although differences in extent of reaction were observed. In this communication we wish to report the effects of mitochondrial energization by ATP on the rates of formation of known oxygen intermediates of cytochrome c oxidase and the kinetics of electron donation to cytochrome oxidase by ferrocytochrome c. Preliminary accounts of this data have been published previously [7,17,18].

Materials and Methods

Pigeon heart mitochondira were prepared by the method of Chance and Hagihara [19] and supplemented with CO-saturated medium/ethylene glycol (40% v/v) mixture. The final preparation contained 15–20 mg/ml of mitochondrial protein in 75 mM mannitol/10 mM succinate/10 mM glutamate/30 mM Tris · HCl buffer, pH 7.2. The samples were loaded into quartz EPR tubes, incubated for 5 min at room temperature, and kept at dry ice temperature in the dark until used.

Energization of the membrane was achieved by the addition of ATP at room temperature to a final concentration of 8 mM, followed by a 30 s incubation period. The mitochondria were uncoupled by the addition of 20 μM final concentration carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) at room temperature. Oxygen was added to the samples by vigorous stirring with a tight fitting stainless steel spiral stirring rod at −30° C. The oxygenated samples were then trapped by quickly chilling to −78° C, as previously described [1,2].

At the time of use, the oxygenated samples were placed in the chamber of a multichannel time-sharing spectrophotometer [1,2]. The sample was cooled by a stream of cold nitrogen gas around it. Temperature control was provided by a regulated electric heater in the gas line; temperatures could be maintained within 2° of the desired value. The reaction was initiated by photolysis of the CO-liganded oxidase using a single 585 nm laser flash [1,2].

Results

As shown in the preceeding paper [20], flash illumination of the fully reduced CO-liganded cytochrome *c* oxidase results in dissociation of the $a_3^{2+} \cdot$ CO complex (see also refs. 21—23). Recombination of CO was demonstrated to occur much more rapidly in mitochondria that had been pretreated with ATP at room temperature ("energized mitochondria"). This effect of pre-energization upon CO recombination was also demonstrated to be abolished by uncouplers of oxidative phosphorylation, identifying the effect of ATP as occurring via a mechanism related to the coupling of mitochondrial ATP synthetase to the respiratory chain required in oxidative phosphorylation.

In the presence of oxygen, photodissociation of the CO · enzyme complex is followed by the binding of dioxygen to the enzyme (instead of CO), causing spectral changes nearly identical to those of CO recombination [1,2]. As seen in Fig. 1A, pre-energization of the mitochondria at room temperature with ATP accelerates the formation of the "oxy" compound A (increased rates of increase in absorption in channel a and decrease in absorption in channel c following the flash-induced transient, Fig. 1A). At −119°C the half-time for formation of compound A was approx. 7.5 s in the presence of ATP, but 62 s in the presence of both ATP and the uncoupling agent FCCP. Untreated preparations showed rates of compound A formation similar to those of samples treated with FCCP. In agreement with previous results [1,2], compound A is not photodissociated by subsequent flash illumination, and is hence clearly distinguished from the CO-compound. Concomitant with formation of compound A, no change in the absorption at 830 nm attributed to copper (channel g, Fig. 1) was observed (cf. ref. 2).

From these data it is clear that the binding of dioxygen to the ferrous enzyme is accelerated considerably by pre-energization of the mitochondria at room temperature, in good analogy to the findings made with the reaction of CO with the ferrous enzyme in the preceeding paper [20]. As shown in Table I, a marked difference between the rates of oxygen binding in energized and uncoupled samples is seen only at the higher oxygen concentrations, again in analogy with the data on CO binding [20]. It may be added that at the applied temperature (−119°C) the half-time of CO recombination with the enzyme is more than 10^3 s, which, together with the insensitivity to photolysis, is indicative of oxygen binding rather than CO binding.

Following the formation of compound A, electron transport from the reduced cytochrome oxidase to the bound oxygen occurs, resulting in the disappearance of compound A (decrease in absorption in channel a). In Fig. 1B (same experiment as in Fig. 1A, but on a different time scale) the accelerating

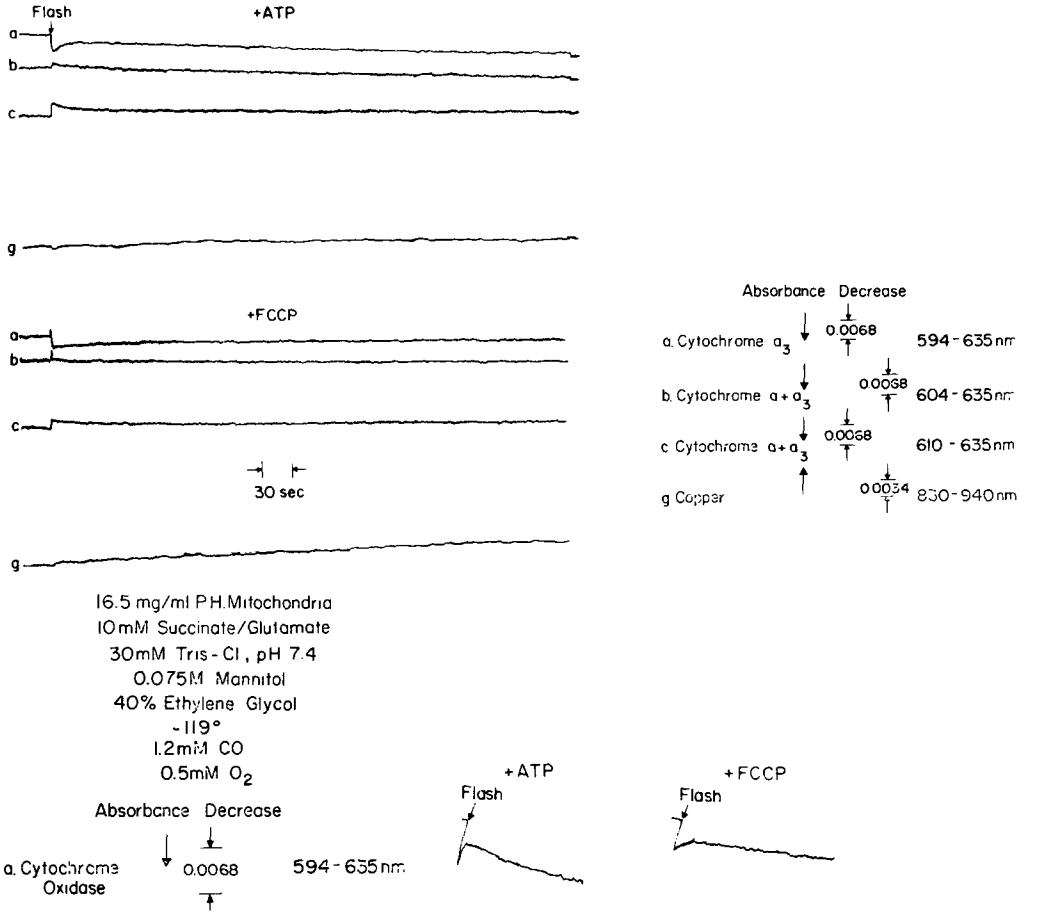

Fig. 1. A. Effect of energization/deenergization on compound A formation at −119°C. The samples were prepared as described in Materials and Methods. The protein concentration was 16.5 mg/ml. The reaction medium contained (final concentration) 40% (v/v) ethylene glycol/30 mM Tris/Cl (pH 7.4)/10 mM glutamate/10 mM succinate/0.5 mM O_2. The samples were energized by the addition of 8 mM ATP and deenergized by the addition of 4 μM FCCP (final concentrations). B. Effect of energization/deenergization on compound A formation and its disappearance at −119°C measured with the 594—635 nm wavelength pair. The experiment is the same as in A, but on a longer time scale. The experimental conditions are as in A.

TABLE I

EFFECT OF INCREASING O_2 CONCENTRATION ON COMPOUND A FORMATION IN ENERGIZED AND DEENERGIZED PIGEON HEART MITOCHONDRIA AT −119°C *

Approximate O_2 concentration (μM)	Half-time of compound A formation	
	+8 mM ATP	+20 μM FCCP
200	15 s	15 s
350	5.5 s	15 s
500	4.5 s	15 s

* As determined using 594—635 nm wavelength pair.

effect of ATP upon both appearance and subsequent disappearance of compound A is clearly shown.

The next event, most clearly observable at temperatures of −90 to −110°C, is a net decrease of the α-band absorption centered at approx. 609 nm, which has been attributed to formation of "peroxy" compound B [1,2]. This step, which follows the decrease of absorption at 594 nm due to disappearance of compound A, is nevertheless clearly not occurring simultaneously with the disappearance of compound A. This suggests the probable presence of an additional intermediate between compounds A and B, the nature of which is not yet understood. As shown in Fig. 2, the formation of compound B (decrease in absorption at channels b and c) is accelerated in mitochondria pretreated with ATP concomitant with an increased extent of the reaction. In Fig. 2 the increase of absorption at 830 nm (channel g), indicative of oxidation of copper, is also observed and is considerably accelerated by pre-energization of the mitochondria. As determined from linear Arrhenius plots (not shown) the energies of activation for formation of compound B are 11.2 and 10.9 kcal/mol in energized and non-energized mitochondria, respectively. These values compare favorably with the value of 12.5 kcal/mol reported by Chance et al. [1,2] for compound B formation in non-energized mitochondria.

At warmer temperatures the oxidation of cytochromes *c* and c_1 can be readily observed following flash photolysis of the CO-liganded enzyme (−55°C, Fig. 3), without significant oxidation of *b*-type cytochromes. As seen in Fig. 3

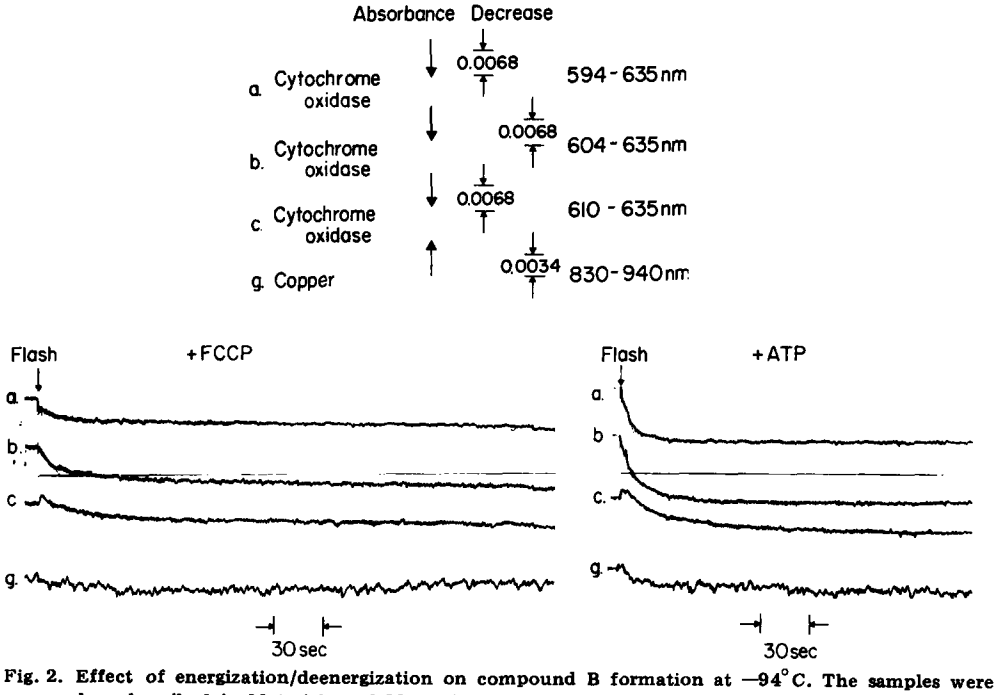

Fig. 2. Effect of energization/deenergization on compound B formation at −94°C. The samples were prepared as described in Materials and Methods. 8 mM ATP or 4 μM FCCP were added to energize or uncouple, respectively. Protein concentration is 15 mg/ml. The half-time of cytochrome *a* oxidation is 25 s in the presence of ATP and 62 s in the presence of FCCP.

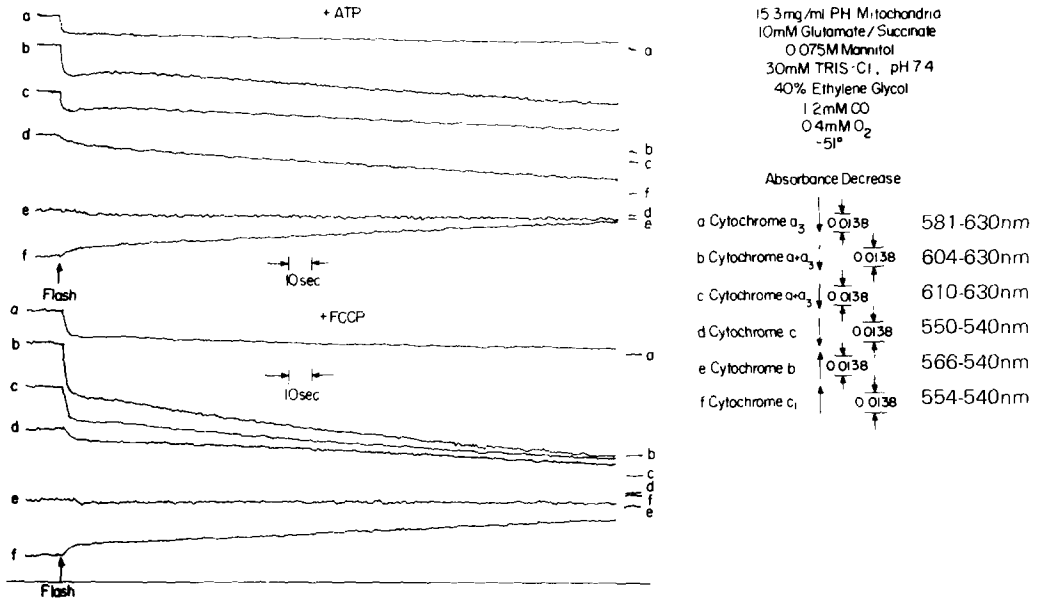

Fig. 3. Effect of ATP and FCCP addition of cytochromes aa_3, b, and $c + c_1$ at -51°C. Protein concentration is 15.3 mg/ml.

(channels d and f), the half-time of oxidation of cytochromes c and c_1 is approximately 25 s in the presence of uncoupler (FCCP) and 60 s in the presence of ATP. Thus, in contrast to partial reactions of cytochrome oxidase described above, the oxidation of cytochromes c and c_1 by cytochrome aa_3 is slowed by pre-energization of the mitochondria. Also, the effect of ATP was not observed in the presence of oligomycin or an uncoupler of oxidative phosphorylation.

The previously observed energy-dependent shifts in midpoint redox potential and spectral properties of cytochrome aa_3 have been shown to be dependent on the ratio ATP/ADP · P_i rather than on the concentration of ATP alone, consistent with a quasi-equilibrium between the respiratory chain event and the phosphorylating system under the experimental conditions [5,6,8,10]. The effect of varying the ratio ATP/ADP · P_i over a range of 10^5 M^{-1} between phosphate potentials of 8.4 and 1.37 kcal/mol upon the rate of formation of compound B at -96°C (cf. Fig. 2) is shown in Fig. 4. Apart from a slight increase in $t_{1/2}$ of this reaction at the lowest applied phosphate potentials, the rate of formation of compound B was relatively independent of phosphate potential in the measured range, in which the $t_{1/2}$ was found to be of the same magnitude as in the presence of 8 mM ATP alone (cf. Fig. 2). On the other hand, as shown in Fig. 5, the rate of formation of compound B appeared to be dependent on the concentration of ATP added at room temperature. At the measuring temperature (-96°C) the $t_{1/2}$ of formation of compound B was in excess of 100 s in the absence of ATP, in the presence of ADP alone, or in the presence of an uncoupling agent. The addition of 8 mM sodium chloride also had no effect on the rate of formation of compound B. We therefore conclude that ATP acceler-

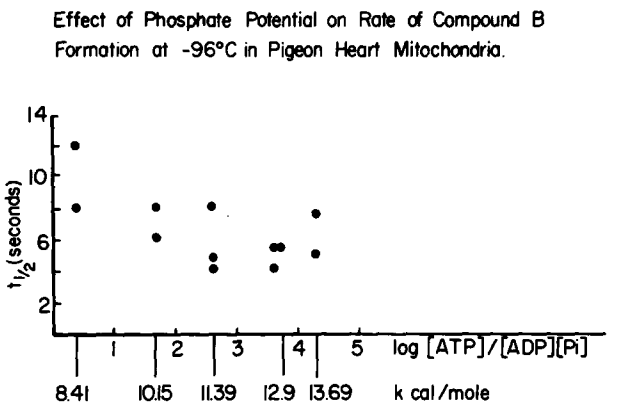

Fig. 4. Effect of phosphate potential on the rate of compound B formation in pigeon heart mitochondria at —96°C. Pigeon heart mitochondria were suspended in ethylene glycol mixture as described in Materials and Methods Nucleotides and phosphate were added at room temperature and the mixture allowed to incubate 45 s before being oxygenated at —35°C. Compound B formation was measured as the decrease in 612—635 nm signal intensity.

ates formation of compound B by a mechanism involving the energy-coupling pathway between the mitochondrial ATP synthetase and the respiratory chain. The dependence upon concentration of ATP rather than upon phosphate potential is discussed further below.

The quality of the isolated mitochondria, judged from the respiratory control value (in the absence of ethylene glycol) had a notable influence on the ATP-induced kinetic changes measured at low temperatures. Thus, pigeon heart mitochondria of acceptable quality (respiratory control ratios of 3—4 with glutamate-malate as substrate) showed the responses reported here, while completely "uncoupled" preparations or preparations not treated with ATP were unresponsive. In some preparations with excellent respiratory control values (in excess of 8), the addition of ATP at room temperatures caused a relatively large oxidation of cytochromes *a* and *c* (cf. refs. 20 and 24). Flash photolysis of such

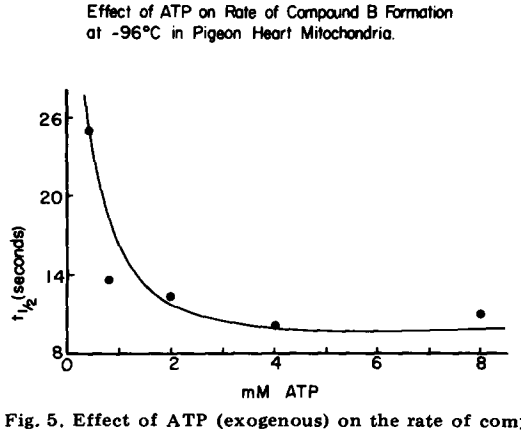

Fig. 5. Effect of ATP (exogenous) on the rate of compound B formation at —96°C in pigeon heart mitochondria. The conditions were the same as those listed in Fig. 4 except that ADP and inorganic phosphate were not added.

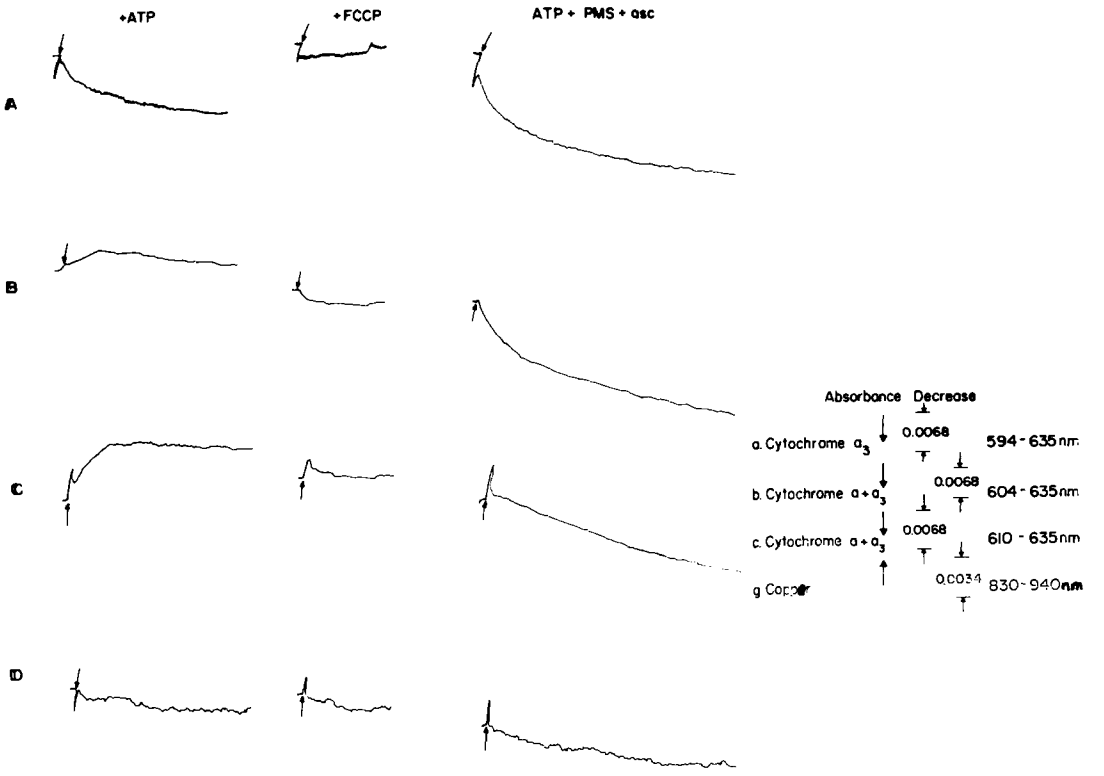

Fig. 6. Abolishment of ATP-induced compound C formation by the addition of FCCP or ascorbate plus phenazine methosulfate. 8 mM ATP, 4 μM FCCP, or 8 mM ascorbate (asc) plus 4 μM phenazine methosulfate (PMS) were added to sample tubes as indicated at 0°C. The samples were then incubated at 17°C for 45 s. The tubes were then oxygenated at −30°C as described in the text. Kinetic measurements were made at −113°C with 13 mg/ml protein.

preparations in the presence of oxygen resulted in an entirely different behaviour (seen in Fig. 6), viz., an increase in absorption of the α-band similar to the formation of "compound C" reported earlier [1,2]. This behaviour is observed (without ATP present) if cytochromes *a* and *c* are oxidized by ferricyanide prior to the flash and is, therefore, unexpected in the cases where ATP induced oxidation. However, in these preparations the reversed electron transfer could be counteracted by the addition of ascorbate plus phenazine methosulfate. The abolition of ATP-linked cytochrome *a* and *c* oxidation by phenazine methosulfate plus ascorbate resulted in responses comparable to those shown in Fig. 6 and are similar to those seen in Figs. 1B, 2, and 3. Hence, it may be concluded that the acceleration of the intramolecular electron transfer and ligand binding (O_2 and CO) by mitochondrial energization is not merely a result of different initial oxidation-reduction state of the enzyme caused by ATP hydrolysis.

Discussion

Pre-energization of pigeon heart mitochondria at room temperature with ATP causes a change in the state of cytochrome *c* oxidase which is at least in

part "remembered" in the frozen state. This is indicated by changes in the rates of binding of CO [20,21] and O_2 as well as the rates of intra- and intermolecular electron transfer in cytochrome *c* oxidase. Since the ATP-induced changes are blocked or abolished by oligomycin and uncoupling agents, they most probably reflect an effect of ATP hydrolysis communicated via reversal of the partial reactions of oxidative phosphorylation. It appears plausible to suggest that the kinetic changes are due to a change in conformation of the enzyme (probably in the redox centers and their vicinities) induced by ATP hydrolysis.

There are at least four spectrophotometrically-discernible steps of the reaction of cytochrome *c* oxidase with oxygen. Table II summarizes these steps and the observed effect of mitochondrial energization upon them. Pre-energization with ATP appears to accelerate all determined steps except electron donation from ferrocytochrome *c* to cytochrome aa_3. This electron transfer step is slowed considerably in the presence of ATP.

Mitochondrial energization has been observed to induce a number of changes in cytochrome *c* oxidase at room temperature, such as (i) an apparent decrease in the midpoint oxidation-reduction potentials of cytochromes *a* and a_3 [5,6]; (ii) spectral shifts indicative of changes in heme structure both in ferrous [13] and ferric [8—11] cytochrome oxidase; (iii) a decrease in affinity of cytochromes aa_3 for cyanide [25]; (iv) a change in ligand occupancy of the region near the heme a_3 site [20]; and (v) an increase in affinity of cytochrome oxidase for CO [26]. It is of interest that effects (i) and (ii) are dependent upon the ATP/ADP · P_i ratio, while effect (iii) had been reported to depend upon the ATP concentration alone [25]. As shown in Figs. 4 and 5, the effect of mitochondrial energization upon the rate of formation of compound B is a function of ATP concentration, but is relatively independent of ATP/ADP · P_i in the measured range. However, it should be noted that the presence of 40% (v/v) ethylene glycol is known to inhibit phosphorylating (state 3, ref. 15) respiration, while ATP-induced reversed electron flow is still observable (cf. Fig. 4 of ref. 20). Thus, the observed independence of the rate of compound B

TABLE II

SUMMARY OF EFFECTS OF ENERGIZATION ON OXYGEN INTERMEDIATE FORMATION

Step	Maximum absorption change (nm)	Direction of change	Proposed * reaction	Effect of ATP	Intermediate affected
1	591 612	Increase Decrease	$a^{2+}a_3^{2+} + O_2 \rightarrow a^{2+}a_3^{2+} \cdot O_2$	+	Compound A formation
2	591	Decrease	$a^{2+}a^{2+} \cdot O_2 \rightarrow a^{2+}a_3^{3+}O_2^{n-}$	+	Compound A loss
3	606	Decrease	$a^{2+}a_3^{3+} \cdot O_2^{n-} \rightarrow a^{3+}a_3^{3+} \cdot O_2^{m-}$	+	Compound B formation
4	550, 554	Decrease	$c^{2+} + c_1^{2+} \rightarrow c^{3+} + c_1^{3+}$	—	

* These structures are tentative, particularly those of steps 2 and 3. It is likely that $n = 2$ and $m = 4$, in which case the compound formed in step 2 is the peroxy species whereas compound B is actually the fully oxidized enzyme.

+, Acceleration of the reaction; —, slowing of reaction.

formation on the concentrations of ADP and P_i may be due to be the presence of ethylene glycol.

Table I shows that O_2 binding to cytochrome a_3 in energized and deenergized mitochondria at −119°C follows a pattern much like that of CO binding; i.e., only in the presence of ATP is the rate of O_2 binding dependent on O_2 concentration. Furthermore, Chance et al. [27] have shown that at a higher temperature (−105°C) the rates become O_2 concentration-dependent even in the deenergized or non-energized state. This parallels the behaviour of CO binding, although in the case of oxygen lower temperatures seem to be needed to "freeze out" the concentration dependence in the deenergized mitochondria (cf. ref. 20).

Although changes in midpoint potentials do not necessarily correlate with changes in kinetics, such a correlation is expected theoretically in uncomplicated cases. The difference in E_m (ΔE_m) between two reactive redox centers is related to the forward and reverse rate constants by the general equation

$$\Delta E_m = \frac{RT}{nF} \ln \frac{k_1}{k_{-1}} \quad (1)$$

Since the apparent E_m values of cytochromes *a* and a_3 are decreased by mitochondrial energization [5,6], while the E_m of cytochrome *c* is unaffected, the rate of electron transfer is expected to be inhibited between cytochromes *c* and *a*, and accelerated between cytochromes aa_3 and O_2. It is interesting that the observed effects of energization fit these expectations.

The total effect of energization upon cytochrome *c* oxidase kinetics is an increased rate of oxidation of cytochromes aa_3 by O_2 (and an increased rate of O_2 binding), and a decreased rate of electron donation to cytochromes aa_3 by ferrocytochrome *c*. This patterns of effects if expected to result in a decreased concentration of cytochrome a_3^{2+} in the energized steady state as compared to the non-energized steady state. Since the rate of respiration is dependent upon the concentration of a_3^{2+}, the observed pattern is consistent with the theory of respiratory control proposed by Wilson et al. [28], of which energy-dependent kinetic control of the $a_3^{2+} \rightarrow O_2$ reaction is a central feature.

Acknowledgements

This research was supported by USPHS grants GM 12202-13 and GM 019977-01. We wish to express our gratitude to Drs. B. Chance and M. Sharrock for helpful discussions.

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