ATP INDUCED OXIDATION OF EXOGENOUS CYTOCHROME C IN TERMINALLY INHIBITED PHOSPHORYLATING PARTICLES

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In previous experiments, an ATP-induced oxidation of mitochondrial cytochrome (Chance,B. 1961 a,b) has been shown to accompany an energy-linked reduction of pyridine nucleotide in mitochondria (Chance and Hollunger, 1960) which have been rendered anaerobic by treatment with dithionite or which are terminally inhibited by cyanide or hydrosulphide. This reaction has been observed in several types of mitochondria: rat liver, rat brain, and pigeon heart. This type of experiment identifies reversed electron transfer in the cytochrome chain and provides a measure of the kinetics and stoichiometry of the ATP-to-electron transfer reaction. The intact mitochondrial preparations studied are impermeable to added cytochrome c and thus are not suitable for a demonstration of the oxidation of "substrate" amounts of reduced cytochrome by addition of ATP. In more recent experiments submitochondrial particles have been obtained from digitonin treated preparations of pigeon heart mitochondria (U. Fugmann, unpublished observations); these particles react readily with added cytochrome c. Observations of the oxidation of added cytochrome c on addition of ATP to cyanide or sulfide-inhibited digitonin particles are reported here.

Materials and Methods'

A double-beam spectrophotometer (Chance,1954) with attached recording fluorometer (Chance, Conrad, and Legallais, 1958) was used for recording the oxidation of endogenous or added cytochrome c using 550 mμ as a measuring wave length and 540 mμ as a reference wave length (550-540 mμ).

Reduction of pyridine nucleotide was measured fluorometrically with 365 mμ excitation and 450 mμ measurement.

Preparations

Pigeon heart mitochondria prepared according to the method of Hagihara (Chance and Hagihara, 1960) were treated with digitonin according to the general procedure described by Devlin and Lehninger (1958) but modified specifically by one of us (U.F., unpublished observations). The particles obtained show a high phosphorylation efficiency and values of respiratory control 2 in the presence of glutamate and malate. In addition, they retain considerable pyridine nucleotide. The reaction medium consisted of 0.27 M mannitol, 0.03 M sucrose, 0.02 M "tris" (pH 7.4) and 1.7 mM versene. The temperature of the experiments was 26°.

Highly purified cytochrome c prepared from pigeon breast muscle was used (courtesy Dr. B. Hagihara).

Experimental Procedure

The oxidation of cytochrome c was studied as follows (cf Fig 1). The digitonin particles were suspended in the reaction medium and treated with 330 μM sulfide. Traces of endogenous substrate caused reduction of cytochrome c to be largely complete in about 40 seconds. In this way the contributions of mitochondrial and added cytochrome were separately evaluated. 670 μM ATP was then rapidly stirred into the suspension and the extent and rate of oxidation of ferrocytochrome c was recorded with the double beam spectrophotometer. The extent and rate of reduction of pyridine nucleotide was simultaneously recorded fluorometrically.

Experimental Results

Figure 1 illustrates a typical experiment. Addition of sulfide causes a reduction of 0.8 μM endogenous cytochrome c. Addition of ferricytochrome c leads to its reduction and the optical calibrations indicate

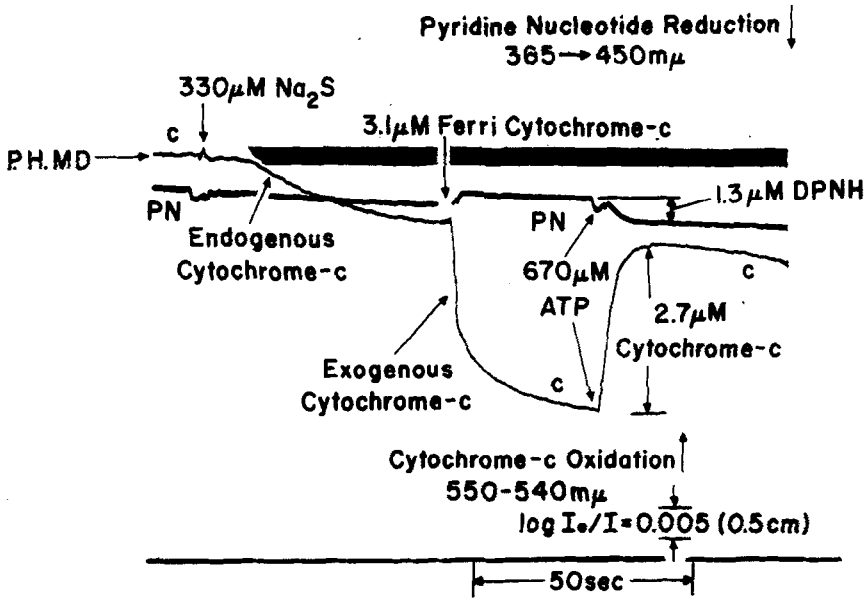

Fig. 1. Illustrating the reduction of endogenous cytochrome c and of exogenous cytochrome c in a sulfide-inhibited preparation. Addition of ATP causes oxidation of exogenous cytochrome c and reduction of endogenous pyridine nucleotide. Digitonin particles of pigeon heart mitochondria (0.8 μM cytochrome c). 26° C. (Expt. 276b).

the formation of 3.1 μM ferrocytochrome c. Addition of ATP causes an oxidation of 2.7 μM cytochrome c, 3 times the amount of the endogenous material. Thus at least 1.9 μM exogenous cytochrome c is oxidized. The fluorometer records the simultaneous reduction of mitochondrial pyridine nucleotide.

The record indicates the oxidation to be stable for a considerable interval; this is due to the low "ATPase" activity of the preparation.

Since mitochondrial cytochrome c was approximately 25 per cent of the total in the experiment of Figure 1, it was considered desirable to repeat the experiments over a range of mitochondrial concentrations so that the oxidation of exogenous cytochrome c could be demonstrated without any question of the contribution of the endogenous material. The results of such experiments are shown in the graph of Figure 2 where we have plotted the micromolar concentration of cytochrome observed to be

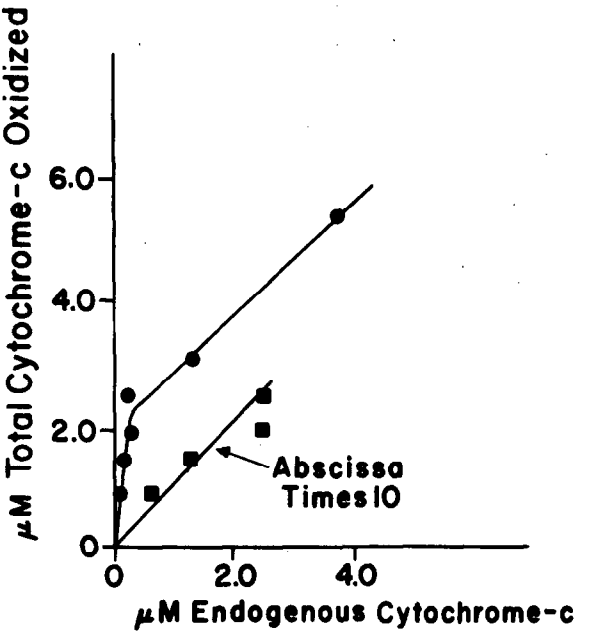

Fig. 2. Effect of variation in the concentration of particles (as evaluated by the micromolar concentration of cytochrome c (abscissa)) on the ATP-induced oxidation of cytochrome. In all experiments, the concentration of exogenous cytochrome c was 3-4 μM. Other conditions as in Fig. 1. (Expt. 276b).

oxidized by 670 μM ATP against the micromolar concentration of mitochondrial cytochrome c. This graph clearly shows that the oxidation of reduced cytochrome c by the particles in the presence of ATP does not involve an unlimited turnover of cytochrome c, and that presumably only a stoichiometric amount can be oxidized. At low mitochondrial concentrations, the curve rises abruptly, the initial slope corresponding to about 10 molecules of exogenous cytochrome c oxidized per molecule of endogenous cytochrome c. At higher concentrations of mitochondrial cytochrome c, the curve takes on a flatter slope. The reaction is completely inhibited by 3 γ/ml oligomycin and is more than 50 per cent inhibited by 1 γ/ml hydroxyquinoline N-oxide.

Discussion

The oxidation of exogenous cytochrome c in phosphorylating particles to which the material is accessible proceeds rapidly and to a high degree

of completion. The amount of reduced cytochrome c which can be oxidized by such preparations appears to reach a limiting ratio of roughly 10 molecules of cytochrome c oxidized to 1 molecule of cytochrome c present in the particles. While this ratio closely approximates the ratio of pyridine nucleotide (1 electron equivalents) to cytochrome c characteristic of these particles, only a portion of the total pyridine nucleotide is reduced. Thus, other substances may also act as electron acceptors for oxidation of cytochrome c under these conditions. Figure 1 clearly shows that the rate of the reaction is rapid. Thus, at low mitochondrial concentrations the "turnover number" of endogenous cytochrome c in oxidation of exogenous cytochrome c exceeds 1 sec^{-1}. This rate is no less than the rate of oxidation of endogenous cytochrome c.

The nature of the oxidant for exogenous cytochrome c requires consideration. It has been observed spectroscopically that ATP causes the oxidation of not only endogenous cytochrome c but also of cytochrome a (Chance, 1961 a, b). Since cytochrome a is an effective oxidant for cytochrome c, we are inclined to believe that it is the oxidant for cytochrome c under these conditions. In this case we would be observing that the cyanide- or sulfide-inhibited cytochrome oxidase can oxidize ferrocytochrome c by reversed electron transfer.

This interesting reaction suggests new approaches to the mechanism of phosphorylation and the process of electron transfer.

Summary

The oxidation of exogenous cytochrome c on addition of ATP to a suspension of sulfide-inhibited digitonin particles from pigeon heart muscle has been demonstrated. The reaction is rapid and reaches a degree of completion depending upon stoichiometric considerations; roughly 10 molecules of exogenous cytochrome c can be oxidized per molecule of endogenous cytochrome c.

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