AN EFFECT OF INORGANIC PHOSPHATE AND AMP AT THE THIRD PHOSPHORYLATIVE SITE OF THE RESPIRATORY CHAIN OF MYCOBACTERIUM PHLEI*

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An important feature of systems capable of coupling phosphorylation to oxidation is their ability to regulate electron transport. Although bacterial systems are capable of coupling phosphorylation to oxidation, they appear to lack the type of respiratory control exhibited by mammalian mitochondria (Lardy and Wellman, 1952; Chance and Williams, 1956). Oxidation by the main respiratory chains in bacterial systems occurs in the absence of the adenine nucleotides or inorganic phosphate; however, the rate of respiration is affected by the presence of components of the phosphate acceptor system (Worcel, et. al., 1965; Scocca and Pinchot, 1965; Ishikawa and Lehninger, 1962). The stimulation of respiration by inorganic phosphate in the phosphorylating systems from Mycobacterium phlei (Revsin and Brodie, 1967) and Alcaligenes faecalis (Scocca and Pinchot, 1967) requires the presence of coupling factors. These results suggest that the coupled electron transport chains in bacterial systems may be regulated by components of the phosphate acceptor system but that the mechanism differs from that of mammalian systems. This communication will report the effects of inorganic phosphate (P_i) and the adenine nucleotides on the rate of oxidation of the electron transport chain and rate of reduction of cytochromes c, b and a + a_3 of the electron transport particles from M. phlei.

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MATERIALS AND METHODS

Particles from Mycobacterium phlei, ATCC 354, were prepared by procedures described previously (Brodie and Gray, 1956; Brodie, 1959). The resulting particles were suspended in water (4 ml containing 100-200 mg protein), applied to a 25 x 1.5 cm column of Sephadex (G-25, coarse grade), and eluted with water. These particles were used directly without the further addition of Mg^{++} or KCl and are referred to as depleted particles. The supernatant fraction obtained by centrifugation of the cell free extract at 144,000 x g for 90 minutes was irradiated (360 mμ) for 5 hours in the cold (Brodie and Ballantine, 1960) and dialyzed for 48 hours at 2° with several changes of water. This fraction was used since it was known to contain a protein component(s) necessary for restoration of phosphorylation with the particles (Brodie, 1959).

The rate of oxidation was followed using a Clark Oxygen electrode. The simultaneous determination of cytochrome reduction and oxygen uptake was accomplished using a double beam spectrophotometer equipped with a vibrating platinum oxygen electrode.

RESULTS

The addition of P_i to a system containing nondepleted particles resulted in an increase in the rate of oxygen uptake, while the addition of the adenine nucleotides generally resulted in an inhibition of oxidation (Kurup and Brodie, 1967). However, these results were not consistent from preparation to preparation. By using the depleted particulate preparation as described above, the effects of the adenine nucleotides and P_i on the rate of oxidation were consistently observed (Table 1). In the absence of P_i, the addition of ADP or ATP to the depleted particulate system was found to inhibit the rate of oxidation, whereas the addition of AMP was found to exert a slight stimulatory effect on the rate of respiration. In contrast to the effects of the nucleotides, the addition of P_i to the reaction mixture resulted in a 38% increase in the rate of oxidation (Table 1). The maximum stimulation by P_i was found to occur at pH 7.5.

The stimulatory effect of inorganic phosphate on respiration was found to be

dependent upon the order of addition. When P_i was present in the reaction system, the rate of oxidation upon the addition of ADP was greater than the rate observed with ADP alone, while with ATP plus P_i, the rate of oxidation was slightly inhibited (Table 1). In the presence of P_i and AMP, the rate of oxidation was greatly increased, and even exceeded the rate with P_i alone. However, the addition of P_i to a reaction mixture which already contained ADP or ATP failed to reverse the inhibition caused by these nucleotides. Although the addition of AMP to a system containing inorganic phosphate results in an accentuation of the phosphate effect, the addition of P_i to a system containing AMP failed to result in an increase over that observed with AMP alone.

Table 1. The Effect of Adenine Nucleotides and Inorganic Phosphate on NAD^+-linked Oxidation in M. phlei

Nucleotide Addition	Oxygen Uptake	
	None	Phosphate
	µl/min/mg particle protein	
None	2.6	3.6
AMP	2.8	4.9
ADP	1.1	2.8
ATP	2.0	1.9

The reaction mixture contained 100 µmoles buffer, pH 7.5 [N-TRIS-(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid (Good, et.al., 1966)] 15 µmoles KF, 0.75 µmole NAD^+, 0.18 mg yeast alcohol dehydrogenase, 15 µmoles ethanol, 1.70 mg depleted particle protein and 1.60 mg irradiated, dialyzed supernatant protein. Water was added to a final volume of 2.25 ml. The P_i and the adenine nucleotides were added at a concentration of 1.0 µmoles/ml. In the experiments containing phosphate and nucleotide, the phosphate was preincubated with the reaction mixture for 2 minutes before the addition of the nucleotide.

In order to determine whether this increased rate of oxidation in the presence of P_i was due to a direct effect on the electron transport chain, the rate of reduction of cytochrome b, c and $a + a_3$ was measured. The rate of reduction of cytochromes b and c was affected little or not at all by the presence of inorganic phosphate. Since the stimulation of oxidation by P_i could not be accounted for by cytochromes b and/or c, the rate of reduction of cytochromes $a + a_3$ was examined. Figure 1 shows

the simultaneous determination of the rate of oxygen uptake and the rate of cytochromes a + a_3 reduction with and without P_i. The addition of P_i was found to stimulate both the rate and the extent of cytochromes a + a_3 reduction as well as the rate of oxygen uptake. The rate of reduction was stimulated by approximately 35% by P_i and 100% of the total amount of cytochromes a + a_3 was reduced enzymatically as determined by $Na_2S_2O_4$ addition at the end of the reaction. In the absence of P_i, 64% of the cytochromes a + a_3 was reduced enzymatically.

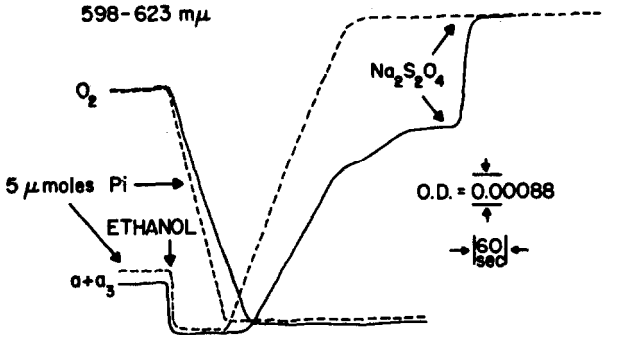

Figure 1. Effect of P_i on the Rate of Oxygen Uptake and the Rate of Cytochromes a + a_3 Reduction. The components of the reaction mixture were the same as described in Table 1 with water being added to a final volume of 3.0 ml. The system contained 7.5 mg particle protein and 2.3 mg supernatant protein. The solid curves were the controls (no P_i) and the dotted curves contained 5.0 μmoles P_i.

The conditions required for the stimulation of cytochromes a + a_3 reduction were similar to those necessary for the stimulation in oxygen uptake. The maximum stimulation of cytochromes a + a_3 reduction was found to occur at pH 7.5. The stimulation of reduction of cytochromes a + a_3 by P_i was found to require the presence of soluble coupling proteins (Fig. 2, A). On deletion of the coupling proteins from the reaction mixture, the stimulation of cytochromes a + a_3 reduction by P_i was no longer observed (Fig. 2, B) while coupling protein(s), in the absence of P_i, had no effect on the rate of reduction of the endogenous cytochromes a + a_3 of the particles (Fig. 2, C). Thus the stimulation in the rate of reduction of cytochromes a + a_3

required both phosphate and coupling protein(s). A stimulation in the rate of oxygen uptake and cytochromes $a + a_3$ reduction was also found when arsenate was used instead of P_i. The stimulation with arsenate was also found to require the coupling proteins.

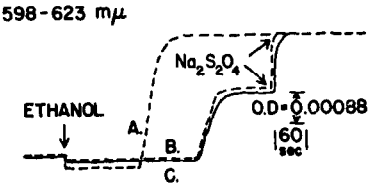

Figure 2. Effect of Coupling Protein(s) on the Rate of Cytochromes $a + a_3$ Reduction. The components of the reaction mixture were the same as described in Table 1 with water being added to a final volume of 3.0 ml. The system contained 6.4 mg particle protein and 1.9 mg supernatant protein. Curve A contained 5.0 μmoles P_i and supernatant, Curve B contained 5.0 μmoles P_i and no supernatant, and Curve C was the control with no P_i. Curve C was obtained with or without coupling protein(s).

The effects of the adenine nucleotides on the rate of reduction of cytochromes $a + a_3$ was also measured. AMP increased the rate of cytochromes $a + a_3$ reduction over that of the control with no P_i, but the rate was less than that of P_i alone. In the presence of P_i, the addition of AMP to the reaction mixture resulted in a further stimulation of the rate of cytochromes $a + a_3$ reduction that exceeded the rate obtained with P_i alone. These results are in agreement with the data shown in Table 1. Furthermore, in the presence of ADP or ATP the rate and extent of cytochromes $a + a_3$ reduction was inhibited. Although the inhibition with ADP (but not ATP) was partially abolished by the presence of P_i, the rate was always less than that of P_i alone.

DISCUSSION

The stimulation of respiration by P_i or P_i and AMP of the coupled electron transport chains of M. phlei can be explained by an effect of these components on a respiratory carrier. At least one site has been located at the cytochromes $a + a_3$ region of the chain; however, it is possible that P_i may in addition exert an effect at one or more sites prior to the cytochrome region. It appears that P_i is the prime activating factor with AMP assuming a secondary role since the P_i stimulated rate of

cytochrome a + a_3 reduction and rate of oxygen uptake was further enhanced by AMP whereas the AMP stimulation was not further enhanced by P_i.

A mechanism for the regulation of electron transport in Mycobacteria has been suggested by the work of Worcel, et. al., 1965, which indicated that the NADH dehydrogenase was subject to activation by AMP. The mechanism of the P_i or the P_i-AMP stimulation of oxygen uptake and cytochromes a + a_3 reduction remains unknown. However, it is of interest that the P_i or P_i-AMP effect was observed at one of the established phosphorylative sties in this bacterial system (Asano and Brodie, 1965; Kurup and Brodie, 1966) and was dependent upon the presence of coupling protein.

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