KINETIC EVIDENCE FOR A COMPLEX BETWEEN THE ATP-ADP EXCHANGE ENZYME OF OXIDATIVE PHOSPHORYLATION AND CYTOCHROME C[1]

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Previous reports from this laboratory (Wadkins and Lehninger, 1963a and b) have provided evidence that an ATP-ADP exchange reaction of rat liver mitochondria and phosphorylating submitochondrial particles is catalyzed by the enzyme responsible for the terminal step of oxidative phosphorylation. The exchange reaction in tightly coupled mitochondria is inhibited by uncoupling agents such as 2,4-dinitrophenol and arsenate and by coupling inhibitors such as oligomycin and azide and by induction of the reduced state of the electron transport chain.

An enzyme which catalyzes the exchange reaction has been extracted from intact mitochondria and submitochondrial particles and has been extensively purified by ammonium sulfate fractionation, chromatography on DEAE-cellulose, CM-cellulose, and hydroxyapatite to a state approaching homogeneity. The exchange activity of the purified enzyme can be rendered sensitive to 2,4-dinitrophenol when mixed with fresh submitochondrial particles (Wadkins and Lehninger, 1960). This recombination to form a

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dinitrophenol-sensitive exchange reaction would seem to require interaction of the soluble exchange enzyme with components of the coupling reactions because the dinitrophenol sensitivity is prevented by coupling inhibitors such as oligomycin and azide and also by enzymatic induction of the reduced state of the respiratory chain of the particles whereas the exchange activity of the purified enzyme is insensitive to these agents. That the enzyme is directly concerned in oxidative phosphorylation was shown by the recent finding that it will augment the P/O ratio of rat liver mitochondria following depletion of the exchange enzyme by salt extraction (Wadkins and Lehninger, 1963b). It was pointed out that the ATP-ADP exchange enzyme may be identical with a highly purified protein of beef heart mitochondria which Webster (1962) has found to restore phosphorylation at Site 3 and to catalyze an ATP-ADP exchange reaction.

Although these studies indicate that the exchange enzyme is a coupling factor whose catalytic properties can be altered by interaction with particulate-bound components of the coupling mechanism they do not reveal the identity of those components nor the nature of the interaction. Webster (1963) has recently reported that a soluble complex that contains cytochrome c is formed when the Site 3 coupling factor and reduced cytochrome c are added to respiring beef heart submitochondrial particles. The isolated soluble complex formed ATP when incubated with ADP and P_i and oxidized cytochrome c was released. In this paper we wish to report that the purified ATP-ADP exchange enzyme forms a kinetically detectable complex with reduced cytochrome c when incubated with ATP and that this interaction may be the primary site of action of uncoupling agents such as oligomycin and dinitrophenol.

The data presented in Figure 1 demonstrate that the initial reaction rate catalyzed by the ATP-ADP exchange enzyme is markedly dependent on the concentration of ADP, when the concentration of ATP and Mg^{++} are held constant. The initial exchange rate increases to a maximum at approximately 1.0 mM ADP and then falls to about half the maximum velocity at about 5.0

mM ADP. Identical results were obtained when the ATP:ADP ratio was maintained constant at 2.0:1.0, and the ADP concentration varied from 2.0 to 8 mM or when the Mg^{++} concentration was varied in proportion to the total ATP:ADP concentration. This behavior is characteristic of substrate inhibition as reported in the cases of malate dehydrogenase (Vestling, et al., 1960), acetylcholinesterase (Wilson and Bergmann, 1963) and triosephosphate dehydrogenase (Velick and Furfine, 1963). Such data have been interpreted in terms of two independent substrate binding sites on the enzyme having unequal substrate association constants.

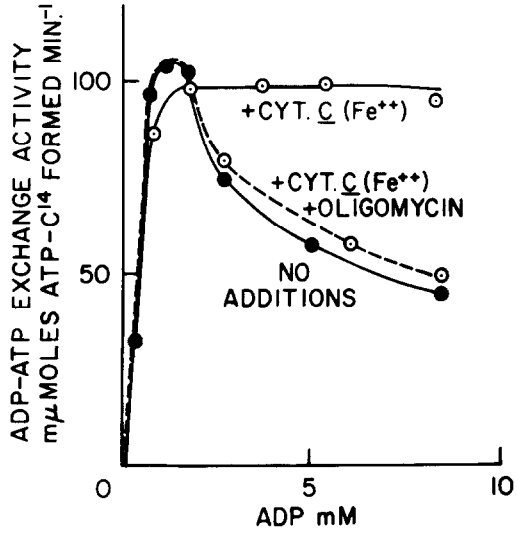

Figure 1. Substrate Inhibition of the ATP-ADP Exchange Reaction

Each reaction system contained 9.5 mM ATP, 4.2 mM $MgCl_2$, 4.2 mM imidazole buffer, pH 6.8 and 4.0 μgm exchange enzyme with the indicated concentration of ADP and 18,000 cpm 8-C^{14}-ADP in a final volume of 0.65 ml. Cytochrome c (Sigma Type III) was present at 7.6×10^{-6}M where indicated and was kept in the reduced state by addition of 7 mM ascorbate. Oligomycin was present at 0.8 μgm/ml. The initial rate of the ATP-ADP exchange reaction was determined exactly as described previously (Wadkins and Lehninger, 1963a).

The inhibition of the exchange reaction by ADP does not occur if reduced cytochrome c is added to the system. The results presented in Figure 1 show that in the presence of 7.5 μM reduced cytochrome c, increasing the concentration of ADP from 1.0 to 5.0 mM does not cause

inhibition of the exchange but rather yields a classical Michaelis-Menten curve, showing both first and zero order phases. The presence of reduced cytochrome c does not alter the apparent K_m of ADP of the first binding site which was derived from the first order phase and found to be approximately 0.5 mM.

These results indicate that reduced cytochrome c prevents the inhibition of the ATP-ADP exchange rate by high concentrations of ADP, presumably by displacing ADP from the second binding site. This conclusion is supported by the finding that increasing the concentration of ADP above 12 mM in the presence of 7.6 μM reduced cytochrome c will again produce inhibition of the exchange reaction. Oxidized cytochrome c does not replace reduced cytochrome c in preventing inhibition of the exchange by ADP. Experiments in which the basic protein salmine, or the hemoproteins hemoglobin, myoglobin, and catalase (Boehringer-crystalline) were substituted for cytochrome c (Table I) showed no stimulation of the exchange rate at concentrations substantially higher than those found to be optimal for the cytochrome c effect. The concentration of reduced cytochrome c that elicits half-maximal response in this system is approximately 3×10^{-6}M.

The cytochrome c-induced reversal of ADP inhibition of the exchange rate is markedly affected by certain inhibitors of oxidative phosphorylation. The results presented in Figure 1 and Table I show that oligomycin completely prevents the acceleration in exchange rate caused by reduced cytochrome c. A similar effect is given by sodium azide, which in some respects acts like oligomycin on the ATP-ADP exchange reaction of intact mitochondria (Wadkins and Lehninger, 1963a). Inorganic phosphate, and arsenate were found not to interfere with the reversal of the ADP inhibition by reduced cytochrome c.

The apparent binding of reduced cytochrome c by the ATP-ADP exchange enzyme can be potentiated by 2,4-dinitrophenol. The results shown in Figure 2 demonstrate that half-maximal stimulation of the exchange rate

Table I

Reversal of Substrate Inhibition of the ATP-ADP Exchange Reaction

The reaction conditions and analytical details are the same as described for Figure 1. The concentration of ADP is 7.1 mM in Experiments 1 and 2 and 4.5 mM in Experiment 3. 4.0 μgm exchange enzyme used in Experiment 1 and 3; 8.0 μgm used in Experiment 2.

Exp.	Additions	ATP-ADP Exchange mμmoles ATP-C^{14} min^{-}
1	None	45
	Cyto.c(1.5×10^{-5}M)	46
	Cyto.c(1×10^{-4}M)	46
	Cyto.c(1.5×10^{-5}M)+ ascorbate(7mM)	70
	Ascorbate(7mM)	45
	Salmine(56μg/ml)	45
2	None	100
	Ascorbate(7mM)+ Cyto.c(1μM)	110
	" " " (3μM)	125
	" " " (6μM)	145
	" " " " + oligomycin(0.8μg/ml)	100
	" " " " + Na azide(1mM)	100
3	None	40
	Cyto.c(6μM)+ ascorbate(7mM)	80
	Catalase(20μM)+ " "	40
	Oxyhemoglobin(40μM)	40
	" " + 0.3 mM DNP	41
	Oxymyoglobin(33μM)	41
	" " + 0.3 mM DNP	43

at 5 mM ADP in the presence of 0.3 mM dinitrophenol is obtained at approximately 2×10^{-7}M cytochrome c. The effect of dinitrophenol can be prevented by low concentrations of oligomycin or azide and in this respect resembles the inhibitory effect of oligomycin and azide on the dinitrophenol-stimulated ATPase activity of mitochondria and submitochondrial particles (Wadkins and Lehninger, 1963a).

Dinitrophenol had no significant effect on the exchange rate when catalase, hemoglobin, or myoglobin were substituted for reduced cytochrome

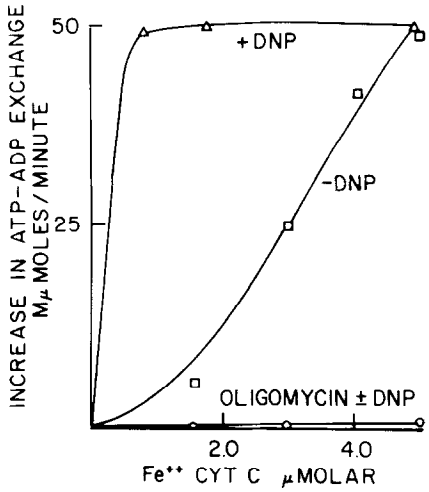

Figure 2. Potentiation of Cytochrome C Interaction with the ATP-ADP Exchange by Dinitrophenol

Each reaction system contained 9.5 mM ATP, 5.0 mM ADP-C^{14}(18,000 cpm), 4.2 mM $MgCl_2$, 4.2 mM imidazole buffer, pH 6.8, 7 mM ascorbate, 9 μgm exchange enzyme, and the indicated concentration of cytochrome c. Dinitrophenol was present at 0.3 mM and oligomycin at 1.0 μg/ml where indicated. The results are expressed as the net increase of exchange activity by reduced cytochrome c over the activity measured in the absence of cytochrome c.

c in the reaction system. These results are therefore consistent with those shown in Table I demonstrating the specificity for reduced cytochrome c in stimulating the ATP-ADP exchange rate of the soluble system.

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