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AN INHIBITORY HIGH AFFINITY BINDING SITE FOR ADP IN THE OLIGOMYCIN-SENSITIVE ATPase OF BEEF HEART SUBMITOCHONDRIAL PARTICLES

A.F. Fitin, E.A. Vasilyeva and A.D. Vinogradov+

Department of Biochemistry, School of Biology, Moscow State University, Moscow 117234, U.S.S.R.

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Summary: Kinetic evidence are presented for the existence of a high affinity inhibitory site for ADP /$K_i < 10^{-7}$ M/ in the oligomycin-sensitive ATPase of beef heart submitochondrial particles. The ATPase·ADP complex is completely inactive in the ATPase reaction; it can be converted into active ATPase in a slow ATP-dependent reaction. The dependence of a first order rate constant for activation of the enzyme·ADP complex on concentration of ATP gives a K_m value equal to that for ATP in the ATPase reaction. The data obtained suggest that the membrane-bound ATPase complex contains two kinetically distinct nucleotide-binding centers, i.e. center 1 binds ATP or ADP with a formation of enzyme-substrate or enzyme-competitive inhibitor complexes: center 2 binds ADP with a formation of a complex which is able to bind ATP in center 1 and unable to hydrolyze the bound ATP. The binding of ATP or ADP in center 1 changes the reactivity of center 2 towards ADP.

As is evidenced from its nucleotide specific hydrolytic activity and competitive inhibition of ATPase by ADP /1,2/, the membrane-bound and soluble coupling factor F_1 are able to bind ATP and ADP /free or magnesium complexes/ in a catalytically active center of the enzyme. The properties of this center have been characterized both in kinetic studies /1-5/ and direct binding experiments /2,3,6, for the recent review see ref. 7/. In addition up to 5 moles of tightly bound, slowly exchangeable nucleotides /presumably 3 ATP and 2 ADP/ per mole of F_1 are present in purified preparations of the mitochondrial ATPase /8/. Besides, a high affinity binding site, which is rather specific for ADP has been described for beef heart F_1 /2,6/, rat liver F_1 /3/ and coupling factor from chloroplasts /9,10/. It is uncertain

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+ To whom all the inquiries should be addressed.

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whether this site is present in the complete oligomycin-sensitive ATPase of submitochondrial particles, and the participation of the nucleotide binding sites in a catalytic cycle during ATPase reaction or oxidative phosphorylation remains obscure.

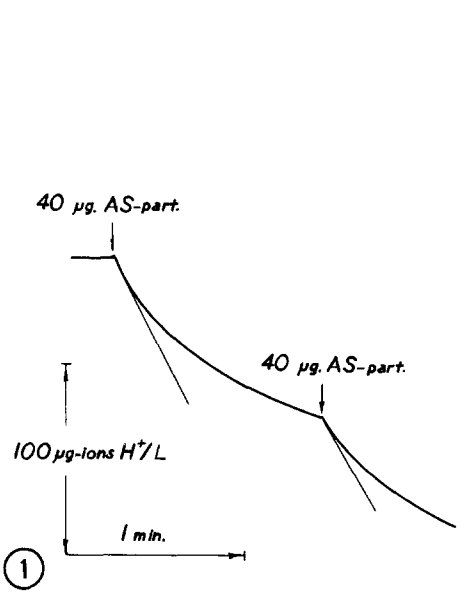
The experiments to be described in this report show that the high affinity binding site for ADP is present in the complete ATPase of submitochondrial particles and that ADP inhibits the oligomycin-sensitive ATPase in a way, which is clearly different from the simple competition between ATP and ADP for the single binding site.

METHODS

Beef heart submitochondrial particles essentially free of the ATPase inhibitor /AS-particles/ were prepared according to the method of Racker and Horstman /11/. The particles were suspended in small aliquots of 0.25 M sucrose - 100 μM EDTA and stored in liquid nitrogen. The protein content was determined by the biuret method /12/. The ATPase activity was measured spectrophotometrically as a decrease of absorption of NADH at 340 nm in a reaction mixture, which contained: 0.1 M KCl, 10 mM tris/Cl^-, 2 mM $MgCl_2$, 2 mM KCN, 1 mM potassium phosphoenol pyruvate, 0.3 mM NADH, 8 units of lactate dehydrogenase, 12 units of pyruvate kinase and various concentrations of ATP, pH 8.0, or potentiometrically as a hydrogen ion release during ATP hydrolysis /13/ in a mixture containing:0.1 M KCl, 2 mM Tris/Cl^-, 2 mM $MgCl_2$ and various concentrations of ATP, pH 8.0. All the activities were measured at 25°C and expressed as μmoles of ATP hydrolyzed per min.per mg of protein. The K_m values were calculated on the assumption, that the ATP·Mg complex is the species reacting with the enzyme, using dissociation constants obtained by the method of Burton /14/ for the conditions employed. The nucleotide and the enzymes of the ATP-regenerating system were obtained from "Reanal" /Hungary/, phosphoenol pyruvate and bovine serum albumin - from "Sigma"; other chemicals were purest commercially available.

RESULTS

Fig. 1 demonstrates the kinetics of hydrogen ion release during hydrolysis of 200 μM ATP under the conditions when the products of the reaction /ADP and P_i/ are accumulated in the medium. If the decrease of the rate is due to a decrease of the substrate /ATP/ and an increase of the products /ADP and P_i/ and a rapid equilibrium between the substrate and products and the active site of the enzyme exists, the rate after the second addition of the particles would be expected to be two times higher than those observed at the moment of the second addition. As is



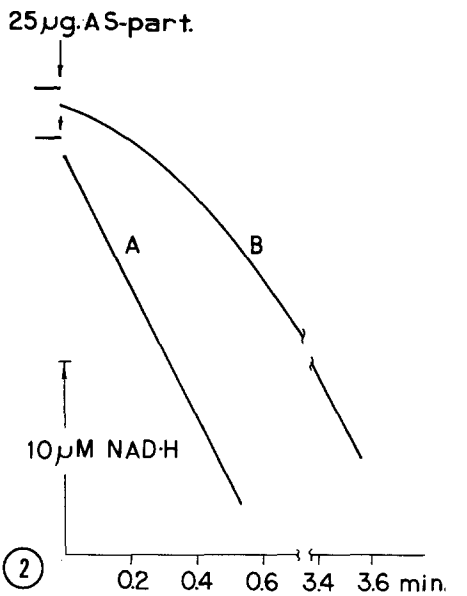
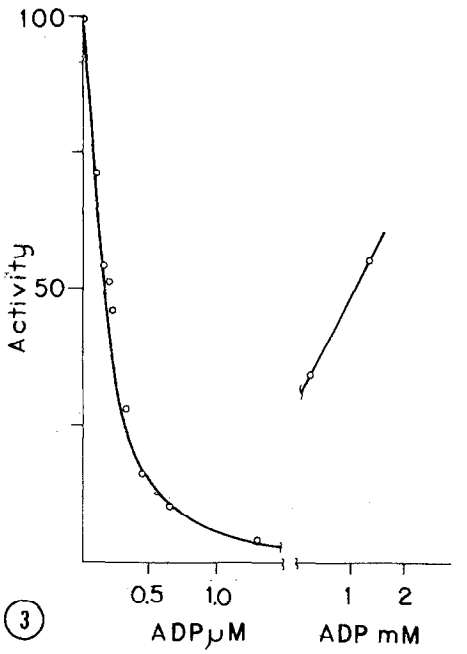


Figure 1. The kinetics of hydrogen ion release during ATPase reaction catalyzed by AS-particles. The composition of the reaction mixture is indicated in "Methods", the initial concentration of ATP was 200 μM. The initial rate of hydrolysis after the first addition of particles was 2.4 μmoles of ATP per min·per mg. All the hydrogen ion concentrations change were completely sensitive to oligomycin.

Figure 2. The kinetics of ATPase reaction catalysed by AS-particles in the presence of ATP-regenerating system. The condition for measurement of ATPase see "Methods", ATP concentration was 1 mM. The reaction was started by the particles preincubated for 3 min at 25°C in a mixture containing: 0.1 KCl, 10 mM Tris/Cl^-, pH 8.0, 2 mM $MgCl_2$, 25 μM EDTA, 0.25 per cent bovine serum albumin, 66 μg of AS-particles without /A/ and in the presence of 3.2 μM ADP /B/.

seen from Fig. 1, this is evidently not the case, i.e. the hydrolysis occurs at almost the same rate as that when the first portion of the enzyme was added.

It was shown in the separate experiments that no irreversible inactivation of the enzyme occurs during the time of the experiment and that the kinetics of hydrogen release are not changed, when Tris/Cl^- buffer was substituted by 5 mM phosphate. This suggests that ADP, which is formed decreases the ATPase activity of the particles in a time-dependent way. To test this the enzyme preparations were preincubated with ADP and the activity was measured in the presence of ATP-regenerating system.



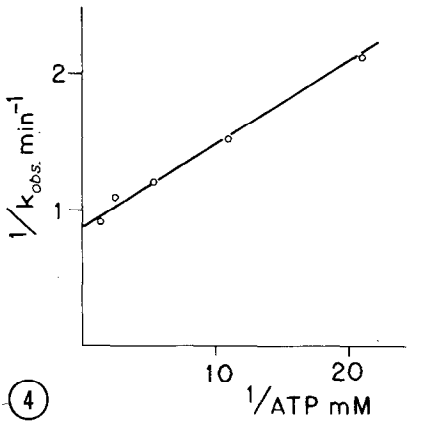


Figure 3. The dependence of the initial rate of ATPase on the concentration of ADP in preincubation mixture. The conditions for measurement of ATPase and preincubation were as in Fig. 2 except for 50 μg of particles were added in the preincubation mixture. 100 per cent of the activity corresponds to 4.15 μmoles of ATP hydrolyzed per min.per mg of protein.

Figure 4. The dependence of the apparent first order rate constant for activation of ADP-inhibited ATPase on concentration of ATP. The condition of measurement and preincubation see Fig. 2.

The rate of the ATPase reaction in the presence of the ATP-regenerating system is perfectly constant /Fig. 2A/; however, a considerable lag is observed when the reaction was started by the particles preincubated with ADP /Fig. 2B/. The dependence of the initial rate of ATPase on concentration of ADP in the preincubation mixture is shown in Fig. 3. The K_i value calculated from the hyperbolic part of the curve is equal to 2×10^{-8} M.

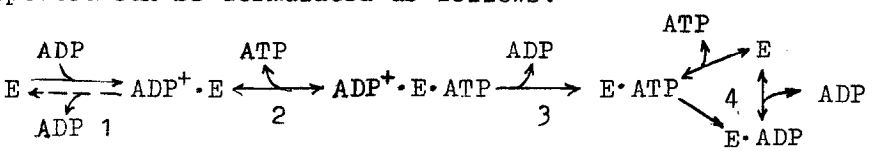
As seen from Fig. 2B the ATPase activity of the particles preincubated with ADP reaches after a certain time the same level as the controls. Thus, the inhibition by ADP is completely reversible under the conditions when free ADP is rapidly converted into ATP by the regenerating system. This reactivation occurs as a first order reaction. It seemed interesting to find out whe-

ther or not the apparent first order rate constant for the reactivation process depends on ATP concentration. Two alternatives are possible: i/ dissociation of the enzyme·inhibitor complex proceeds independently of ATP and ii/ the formation of the ternary enzyme·inhibitor·ATP complex occurs before the dissociation of the inhibitor.

As seen from Fig. 4 the apparent first order rate constant for the reactivation is strongly dependent on the concentration of ATP having the maximal value at saturating concentrations of ATP equal to 1.2 min^{-1}. The K_m^{ATP} for the reactivation of the ADP-inhibited enzyme /1.4 x 10^{-4}M/ is in perfect agreement with the K_m^{ATP} value for the ATPase reaction itself measured under the same conditions /1.4 x 10^{-4}/.

DISCUSSION

The simplest model compatible with the experimental data reported can be formulated as follows:



where the cyclic reaction 4 represents the ATPase reaction in a very simplified way, and ADP^+ stands for ADP bound at the site different from that involved in the ATPase reaction. The participation of the ternary complex ADP^+·E·ATP in the reactions 1-4 rests on the observation that the rate of reactions 1-3 strongly depends on concentration of ATP. Since the K_m^{ATP} values for the cyclic reaction 4 and the sequence of the reactions 2 and 3 are found to be the same, it seems likely that ATP is bound in the ternary complex in the same site as in the E·ATP complex. An additional support for this follows from the observation that high concentrations of ADP prevent the inhibition induced by low concentrations of ADP /Fig. 3/. Relevant to the present discussion it should be emphasized that the basic question as to whether the ATPase reaction is the precise reversal of the events during the respiration-dependent phosphorylation has not been answered yet. Regarding the question on the role of a high affinity inhibitory site for ADP in the ATPase complex of mitochondrial membrane two possibilities are worthwhile to consider. The tightly bound ADP may play a regulatory role as it

has been proposed for CF_1 /9/. Indeed, the rate of dissociation of ADP in reaction 3 even at the saturating concentration of ATP is too small to be directly involved in a catalytic cycle during ATPase reaction /1.2 min^{-1} as compared to the turnover number of ATPase $\sim$ 15 000 min^{-1} under the same conditions/. Another possibility is that the complex $ADP^+ \cdot E$ is the part of a catalytic cycle during oxidative phosphorylation. It is important to note that no information is available so far on the effect of the respiration-driven proton pump on the kinetic properties of the reactions 1-3. The possibility of the effect of $\Delta_\mu H^+$ on the properties of the ADP-specific site involved in respiration-driven phosphorylation is consistent with the recent finding /15/ that K_m^{ADP} for oxidative phosphorylation is strongly dependent on the presence of uncoupler.

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