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THE EFFECTS OF ADP ON REVERSE ELECTRON FLOW AND THE OXYGEN EXCHANGE REACTIONS CATALYZED BY BOVINE HEART MUSCLE SUBMITOCHONDRIAL PARTICLES

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1,N^6-Ethenoadenosine diphosphate (ϵ-ADP) inhibits reverse electron flow (succinate → NAD^+ driven by ATP) by competing with ATP, in contrast to ADP which we have shown previously to be a noncompetitive inhibitor. From these and other data it is concluded that the noncompetitive inhibition noted with ADP results from a combination of competitive inhibition plus non- or uncompetitive inhibition, the former occurring at a relatively nonspecific catalytic site and the latter at an extracatalytic site apparently quite specific for ADP.

ADP, which stimulates ATP ⇌ H_2O and P_i ⇌ H_2O exchanges appears to be necessary for inhibition by arsenate of these exchanges. It is suggested that the ATP-supported P_i ⇌ H_2O exchange may be predominantly of the medium or intermediate type, depending on the concentrations of the Mg^{2+} complexes of ADP and P_i. Thus only exchanges involving medium ADP and P_i would be expected to show arsenate sensitivity.

Inhibition of reverse electron flow (succinate → NAD^+, ATP) by ADP and P_i (or its analog, As_i[1]) is complex, insofar as one ATP hydrolysis product, ADP, acts as a weak noncompetitive inhibitor with respect to ATP, whereas the other product (P_i) actually stimulates the reaction in the absence of ADP by lowering the apparent K_m for ATP. However, in the presence of both ADP and P_i (or As_i) strong noncompetitive inhibition by ADP is observed. These results can be explained both qualitatively and quantitatively by assuming that the enzyme complex can exist in various inhibitory and stimulatory forms, and that the effects of ADP and P_i arise from alterations in the equilibrium levels of the various forms (1). Such data do not, however, indicate if noncompetitive inhibition by ADP arose because ADP acted primarily at an extracatalytic site, or from a superimposition of competitive inhibition at a catalytic site plus non- or uncompetitive inhibition at an extracatalytic site. If inhibition by ADP occurs by the latter process, then it might be possible to discriminate between the two sites of inhibition by using an ADP analog which possesses a differential affinity for one of the sites. As reported herein, ϵ-ADP

[1] Abbreviations: As_i, inorganic arsenate; ϵ-ADP, 1, N^6-ethenoadenoadenosine diphosphate.

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appears to have such a property, since it acts to inhibit ATP-driven reverse electron flow simply by competing with ATP. As will be reported in more detail elsewhere, ϵ-ATP and $\beta\gamma$-methylene phosphonate ATP also are accessible to the catalytic site and act as alternative substrate and competitive inhibitor, respectively.

A second purpose of the work reported in the present paper was to examine the relationship between the ADP-stimulated oxygen exchange reactions and the As_i sensitivity of the exchanges. In the presence of an ATP-regenerating system the ATP $\rightleftharpoons$ H_2O exchange is nearly, if not completely abolished, and the $P_i \rightleftharpoons H_2O$ reaction is suppressed (2, 3), and moreover, it is not markedly inhibited by high concentrations of As_i (4).[2] If the stimulation of oxygen exchanges by ADP occurs as a consequence of the overall reaction, $\sim E + ADP + P_i \rightleftharpoons E + ATP + H_2O$, then the ADP-stimulated reactions might be expected to be inhibited by As_i, in contrast to the residual $P_i \rightleftharpoons H_2O$ exchange found in the presence of an ATP-regenerating system.

METHODS

Submitochondrial particles from bovine heart mitochondria were prepared as described previously except that they were prepared just prior to use from frozen mitochondria by using a Bronson sonifier with special microtip attachment. ϵ-ADP was prepared as described by Secrist et al. (5). Energy-linked reduction assays and ^{18}O analysis procedures were essentially the same as described previously (1, 6). Kinetic data were analyzed by a program which employs the methods of Wilkinson (7) and Cleland (8). Values of kinetic constants obtained from fitting each data set to the hyperbolic form of the Michaelis-Menten equation were used to generate CalComp plots in double reciprocal form, and on the basis of analysis of slope and intercept dependence on inhibitor concentration, groups of data sets were fitted to an appropriate inhibition equation (in these cases linear competitive or linear noncompetitive sufficed). Velocities in the text are expressed per milligram of mitochondrial protein, as determined by the Lowry method with bovine serum albumin as standard.

RESULTS

Figure 1 shows the typical noncompetitive type of inhibition obtained with ADP. In this experiment, as in Fig. 2, 10 mM P_i was present. Since P_i in the absence of ADP acts to stimulate reverse electron flow by lowering the apparent K_m for ATP (1, 9), the data obtained for zero ADP concentration were not included in the analysis. Fitting the data to the equation for S-linear I-linear inhibition gave the values, $K_{ATP} = 0.62 \pm 0.06$ mM, $V_{ATP} = 150.6 \pm 7.8$ nmole min^{-1} mg^{-1}, $K_{is} = 0.59 \pm 0.06$ mM, $K_{ii} = 0.95 \pm 0.13$ mM. K_{ii} and K_{is} are the inhibitor constants which modify the intercept and slope respectively of the double reciprocal plot. They should be regarded as apparent inhibitor

[2]We have observed recently that 0.5 mM dinitrophenol does not inhibit the residual $P_i \rightleftharpoons H_2O$ exchange obtained in the presence of regenerating system, if the extra exchange is expressed as extra atoms of oxygen from water incorporated per P_i released. If the uncoupler-stimulated ATPase is considered, then there is more oxygen in the P_i pool when uncoupler is present since the pool size is larger.

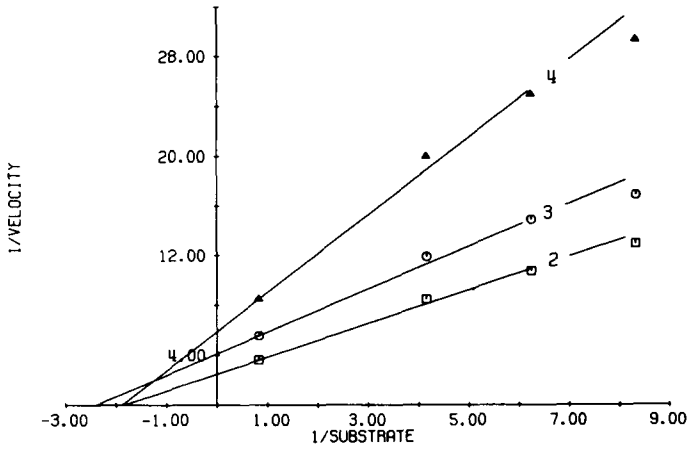

Fig. 1. Noncompetitive inhibition (with respect to ATP) of reverse electron flow by ADP. Submitochondrial particles (0.34 mg) were incubated at 25°C, pH 7.5, for 5 min in medium containing 0.25 M sucrose, 50 mM Tris sulfate, 20 mM $MgSO_4$, 10 mM K^+ succinate, 10 mM K^+ phosphate, 1 mM NAD^+ and 1 mM KCN. The reaction was started by addition of ATP and ADP simultaneously, and NADH formation at 340 nm was measured using a 1 cm lightpath cuvette, final volume 1 ml. Velocities are expressed as increases in absorbance per 2 min and were measured before adenylate kinase activity had effected large changes in relative pool sizes. Plots 2, 3, and 4 correspond to 0.36, 0.9, and 1.8 mM ADP respectively.

constants, since in this system the slope and intercept terms are modified by groups of inhibitory and stimulatory terms although the former predominate in this system and at fixed P_i, ADP behaves overall as a noncompetitive inhibitor (1).

Figure 2 shows the results from a similar experiment in which ϵ-ADP replaced ADP. The analog was considerably less effective as an inhibitor than ADP and correspondingly higher concentrations of ϵ-ADP were needed to cause appreciable inhibition. Unlike ADP, inhibition by ϵ-ADP was competitive with respect to ATP. Fitting the data to the equation for linear competitive inhibition gave $K_{ATP} = 0.31 \pm 0.02$ mM; $V_{ATP} = 81.8 \pm 1.4$ nmole min^{-1} mg^{-1}, $K_i = 1.35 \pm 0.12$ mM.

Table I shows that the inhibitory effect of As_i on certain energy-linked reactions is strongly dependent on the amount of ADP present. In this experiment, insufficient pyruvate kinase was present to eliminate ADP effects completely, since a slight inhibition of both $P_i \rightleftharpoons H_2O$ and reverse electron flow was noted. But these inhibitory effects are quite small in comparison with the effect of As_i in the presence of 0.5 mM added ADP. The effects of ADP and As_i, either singly or in combination, on ATP hydrolysis and reverse electron flow are in close accord with earlier reports (1). In the presence of ADP the $P_i \rightleftharpoons H_2O$ exchange was inhibited 38% whereas in the absence of ADP only 9% inhibition was obtained. ATP $\rightleftharpoons H_2O$ exchange was inhibited about 50% by As_i.

DISCUSSION

Purified mitochondrial ATPase from bovine heart mitochondria contains tightly

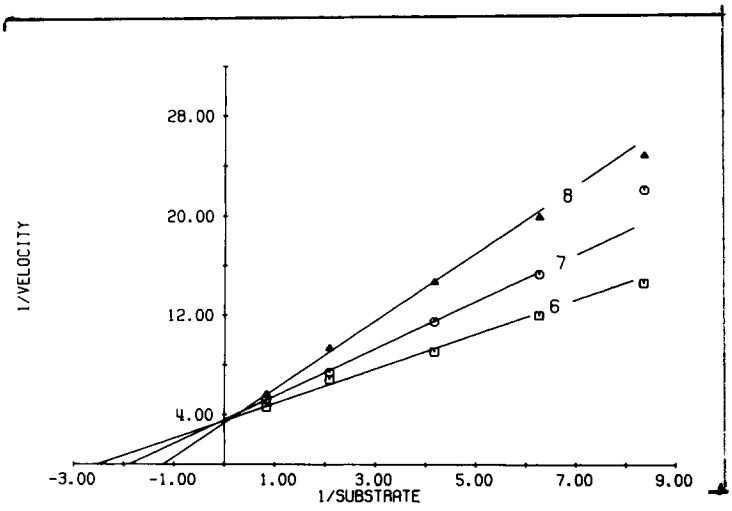

Fig. 2. Competitive inhibition (with respect to ATP) of reverse electron flow by ϵ-ADP. The conditions used were similar to those given in Fig. 1.except that 0.28 mg of particles were used and the reaction was started by addition of ATP and ϵ-ADP. Plots 6, 7, and 8 correspond to 0.37, 1.12, and 1.87 mM ϵ-ADP.

TABLE I. Inhibitory Effect of Arsenate on Mitochondrial Reactions in the Presence of Low and High ADP Concentrations

Additions	P_i released	NADH formed	Atom % excess ^{18}O in P_i	Atom % excess ^{18}O in Nucl. P
None	3.32[1]	0.325[1]	0.223[2]	0.002
+As_i	2.78	0.256	0.204	0.002
+ADP	1.03	0.288	0.456	0.023
+ADP +As_i	0.88	0.007	0.281	0.012

[1] Expressed as μmoles min^{-1}.

[2] Oxygen incorporated due to hydrolysis (0.2 atom % excess) has been corrected for. Submitochondrial particles (0.62 mg ml^{-1} final concentration) were incubated with 0.25 M sucrose, 10 mM potassium succinate, 50 mM Tris sulfate, 25 mM MgSO4, 2 mM KCN, 1 mM NAD^+, and where indicated, 20 mM potassium arsenate or 2 mM ADP. 2 mM ATP was used and in experiments with no added ADP, a regenerating system (0.05 mg ml^{-1} pyruvate kinase plus 2 mM phosphoenolpyruvate) was included. Reactions were carried out at 25°C, pH 7.5, in a final volume of 20 ml, for 14 min for samples containing ADP, and 7 min for samples minus ADP.

bound nucleotides which do not readily exchange with externally added nucleotides (10). Hilborn and Hammes (11) have reported the presence of a tight and loose nucleoside diphosphate binding site on the isolated protein, the latter appearing to correspond to the catalytic site. The function of the tightly bound nucleotides and their possible relationship to the role of the tight binding site of Hilborn and Hammes is currently unknown. This paper contains a description of further studies using substrate or product analogs to

attempt to discriminate between effects of products or reactants at catalytic as opposed to possibly regulatory sites. For example, an analog which has a differential effect at a catalytic as opposed to a regulatory site may help to discriminate between superimposition of effects by a normal substrate at both sites. Competitive inhibition by ϵ-ADP of reverse electron flow is ascribed to the inability of this analog to mimic effects of ADP at an extracatalytic site, either because it fails to bind at that site, or if it binds, is ineffective in inducing inhibition. Alternatively, an analog which binds at the catalytic site and also exerts an influence similar to the normal metabolite at a regulatory site may inhibit reactions requiring the normal metabolite at the catalytic site while stimulating or inhibiting at the regulatory. The inhibition of oxygen exchange reactions by As_i in the presence of ADP is attributed to competition between As_i and P_i at a catalytic site responsible for medium $P_i \leftrightharpoons H_2O$ exchange and ATP $\leftrightharpoons H_2O$ exchange, and is in sharp contrast, for example, to the ability of As_i to duplicate inhibitory or stimulatory effects of P_i on reverse electron flow (1, 9).

The observation that conditions necessary for conferring resistance of the $P_i \rightleftharpoons H_2O$ exchange to As_i (the presence of an ATP-regenerating system) also served to confer resistance of this exchange to 2, 4-dinitrophenol (Lamos and Mitchell, unpublished results) could be indicative of readily reversible pyrophosphate formation and hydrolysis on the enzyme surface, as has been suggested by Boyer et al. (12). Other explanations are, of course, possible, and the relationship between the inhibitor-resistant exchange and medium P_i activation requires further study.

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