Kinetics of Interaction of Adenosine Diphosphate and Adenosine Triphosphate with Adenosine Triphosphatase of Bovine Heart Submitochondrial Particles

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The short preincubation of submitochondrial particles with low concentrations of ADP in the presence of Mg^{2+} results in a complete loss of their ATPase and inosine triphosphatase activities. Other nucleoside diphosphates (IDP or GDP) do not affect the ATPase activity. The ADP-inhibited ATPase can be activated in a time-dependent manner by treatment of submitochondrial particles with the enzyme converting ADP into ATP (phosphoenolpyruvate plus pyruvate kinase). The activation is a first-order reaction with rate constant 0.2min^{-1} at 25°C . The rate constant of activation is increased in the presence of ATP up to 2min^{-1} , and this increase shows saturation kinetics with K_m value equal to that for ATPase reaction itself (10^{-4}M at 25°C at pH 8.0). The experimental results obtained are consistent with the model where two alternative pathways of ADP dissociation from the inhibitory site of ATPase exist: one is spontaneous dissociation and the second is ATP-dependent dissociation through the formation of the ternary complex between ADP, the enzyme and ATP. ADP-induced inactivation and ATP-dependent activation of ATPase activity of submitochondrial particles is accompanied by the same directed change of their ability to catalyse the ATP-dependent reverse electron transport from succinate to NAD^{+} . The possible implication of the model suggested is discussed in terms of functional role of the inhibitory high-affinity binding site for ADP in the mitochondrial ATPase.

Although the fundamental question whether the oligomycin-sensitive ATPase reaction catalysed by submitochondrial particles is a precise reverse sequence of the events taking place during oxidative phosphorylation has not been answered yet, this reaction seems to be most helpful for the elucidation of the individual steps of the energy-accumulation mechanisms in mitochondria. Much evidence has accrued establishing that the interrelations between ATP synthetase and ATPase reactions in mitochondria are not simple, and the rates of these reactions and their apparent equilibrium are under the control of several factors, including concentrations of adenine nucleotides (see, e.g., Sorgato *et al.*, 1978; Gomez-Fernandez & Harris, 1978; and references cited therein). The membrane-bound mitochondrial ATPase (coupling factor F_1) (Penefsky *et al.*, 1960; Pullman *et al.*, 1960) serves as the main part of the energy-accumulating machinery, being able to catalyse ATP hydrolysis or synthesis depending on the experimental conditions. How this enzyme interacts with the substrates and

products of oxidative phosphorylation is of obvious importance. Information on such interaction has been obtained from both kinetic (Selwyn, 1967; Hammes & Hilborn, 1971; Schuster *et al.*, 1975; Moyle & Mitchell, 1975) and binding (Zalkin *et al.*, 1965; Klingenberg, 1967; Catterall & Pederson, 1972; Harris *et al.*, 1973; Hilborn & Hammes, 1973; Garret & Penefsky, 1975) experiments. Factor F_1 is able to bind up to 5 mol of nucleotides/mol of the enzyme (Garret & Penefsky, 1975). One of these binding sites is apparently involved in the activity of the enzyme during its catalytic turnover, as evidenced from its high ATPase activity and from the competitive inhibition of ATPase by ADP (Hammes & Hilborn, 1971). The functional importance of the others sites is not clear. Two sites for ADP binding differing in their affinity are observed in bovine heart submitochondrial particles or in isolated factor F_1 (Hilborn & Hammes, 1973). The presence of adenine nucleotides that are not easily dissociated from ATPase was earlier demonstrated by Zalkin *et al.* (1965) and Klingenberg (1967). In recent years the presence of

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ADP and ATP (operationally termed 'tightly bound' nucleotides) in isolated and membrane-bound factor F_1 have attracted considerable attention (Harris *et al.*, 1973, 1977; Leimgruber & Senior, 1978; Garrett & Penefsky, 1975). The function of these tightly bound nucleotides in the operation of coupling ATPase is not known, and several hypotheses on the subject have been reviewed (Harris, 1978).

Little information is available on the correlation between the nucleotide-binding pattern for isolated or membrane-bound factor F_1 and their catalytic activities in ATPase or ATP synthetase reactions. Rosing *et al.* (1975) have claimed that factor F_1 containing only ADP as tightly bound nucleotide is inactive as ATPase. It has been reported that incubation of factor F_1 in the presence of ADP (without Mg^{2+}) leads to a partial inactivation of ATPase (Harris *et al.*, 1978). The activity can be restored by ATP. This phenomenon was not observed when oligomycin-sensitive ATPase of submitochondrial particles was studied under the same conditions (Harris *et al.*, 1977).

Recently we reported a new type of the interaction between ADP and membrane-bound (Fitin *et al.*, 1979) or isolated (Minkov *et al.*, 1979) factor F_1 . An important feature of this interaction is that inactivation of ATPase by ADP can be reversed by ATP. It has been suggested that the ternary complex ADP-ATPase-ATP exists in which binding of ATP influences the properties of the ADP-binding site (Fitin *et al.*, 1979).

In the present paper we provide further kinetic evidence for the existence of such a complex and give some new data on the effect of ADP on the functional state of mitochondrial ATPase.

Materials and Methods

Bovine heart submitochondrial particles free of protein ATPase inhibitor (AS-particles) were prepared essentially as described by Racker & Horstman (1967). The particles were suspended in small volumes of 0.25 M-sucrose containing 100 μ M-EDTA, pH 7.4, and stored in liquid N_2 . The samples were rapidly thawed before use and stored during the experiments at 25°C. The protein content was determined by the biuret method (Gornall *et al.*, 1949). The ATPase activity was measured spectrophotometrically as a decrease of absorption of NADH at 340 nm in a reaction mixture containing (final concentrations) 0.1 M-KCl, 10 mM-Tris/HCl buffer pH 8.0, 2 mM- $MgCl_2$, 2 mM-KCN, 1 mM-potassium phosphoenolpyruvate, 0.3 mM-NADH, 10 units of pyruvate kinase/ml, 12 units of lactate dehydrogenase/ml and ATP (concentrations indicated in legends to the Figures and Tables). The inosine triphosphatase activity was measured under the same conditions except for the amount of

pyruvate kinase, which was increased 6-fold. Both activities were measured at 25°C and expressed as μ mol of the substrate hydrolysed/min per mg of protein. To measure the ATP-dependent reduction of NAD^+ by succinate, the particles were 'coupled' by oligomycin (Lee & Ernster, 1965). For this purpose a portion of the suspension was treated for 20 min at 25°C with oligomycin (0.15 μ g/mg of protein) in a reaction mixture containing (final concentrations) 0.25 M-sucrose, 0.1 mM-EDTA and 20 mM-potassium succinate, pH 8.0. After the incubation the suspension was placed in ice, where it was stored during the experiments. The reaction was measured as an increase of absorption of NADH at 340 nm in a reaction mixture containing (final concentrations) 0.25 M-sucrose, 10 mM-KCl, 20 mM-Tris/HCl buffer, pH 8.0, 2 mM- $MgCl_2$, 2 mM-KCN, 50 μ M-EDTA, 1 mM-ATP, 10 mM-potassium succinate, 1 mM- NAD^+ , 1 mg of bovine serum albumin/ml, 30 units of pyruvate kinase/ml and 1 mM-phosphoenolpyruvate.

The K_m values given were calculated on the assumption that ATP- Mg^{2+} complex is the species reacting with the enzyme, from dissociation constants obtained by the method of Burton (1959) in 0.1 M-KCl/10 mM-Tris/HCl buffer, pH 8.0.

The nucleotides (potassium salts), pyruvate kinase and lactate dehydrogenase were obtained from Reanal (Budapest, Hungary). The enzymes were extensively dialysed before use. Phosphoenolpyruvate and defatted bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of the purest grade commercially available.

Results

Dissociation of ADP from ADP-inhibited ATPase

It has been shown (Fitin *et al.*, 1979) that incubation of AS-particles with very low (approx. 1 μ M) concentrations of ADP causes complete inhibition of their ATPase activity. This inhibition can be seen experimentally as a lag in ATPase activity measured in the presence of an ATP-regenerating system. Since measurement of ATPase activity is possible only in the presence of ATP, an ambiguity exists in the interpretation of the activation phenomenon during the reaction. The increase of the reaction rate may be due either to practically irreversible spontaneous dissociation of ADP from the ADP-ATPase complex or to activation occurring as an ATP-dependent process. It is also possible that both spontaneous and ATP-dependent activations occur when ADP-inhibited ATPase is placed in a reaction mixture containing ATP and an ATP-regenerating system. Two types of experiments were carried out to discriminate between these possibilities. In the first type

submitochondrial particles were preincubated with ADP, then phosphoenolpyruvate and pyruvate kinase were added, and the initial rate of ATP hydrolysis was measured as a function of time. The results of these experiments are presented in Fig. 1, where the actual tracing of ATP hydrolysis (*a*) and the initial rate measured (*b*) are plotted as a function of time. An activation of the enzyme measured as in Fig. 1 can be considered as an ATP-independent process, since the total concentration of nucleotides in the mixture used for activation is negligible. The time-dependence of activation gives a straight line when the results are plotted with semi-logarithmic co-ordinates (Fig. 2), with the first-order rate constant k_{-1} for the reaction:

equal to 0.2 min^{-1} .

The same rate of ATP-independent activation was observed when phosphocreatine and creatine kinase instead of phosphoenolpyruvate and pyruvate kinase were used as an ADP-utilizing system (not shown) or the concentration of ADP-inhibited enzyme was decreased 5.6-fold. No activation was observed when any component of the ADP-utilizing systems was omitted from the preincubation mixture.

In another type of experiment the rate of activation was measured directly during the course of the ATPase reaction, i.e. in the presence of ATP.

Fig. 2. Semi-logarithmic plot of ATP activation by the ADP-utilizing system

The conditions of the experiment were as given in Fig. 1. Line 1, complete system: ●, content of ADP-treated particles as in Fig. 1; O, content of ADP-treated particles decreased 5.6-fold. Line 2, phosphoenolpyruvate omitted. Line 3, pyruvate kinase omitted. Line 1 does not pass exactly through the zero, apparently because the precision of the initial-rate determinations during the beginning of activation is not very high, and the first points correspond to rate values that are evidently over-estimated.

Fig. 1. Activation of ADP-inhibited ATPase by phosphoenolpyruvate and pyruvate kinase

AS-particles ($240 \mu\text{g}$) were preincubated for 3 min at 25°C in 0.12 ml of a mixture containing (final concentrations) 0.1 M-KCl , 2 mM-MgCl_2 , $10 \mu\text{M-EDTA}$, 10 mM-Tris/HCl buffer, pH 8.0, and $3 \mu\text{M-ADP}$; $2 \text{ mM-phosphoenolpyruvate}$ and 5 units of pyruvate kinase were then added at zero time and incubation was continued for the time indicated. The registration of ATPase activity was started by the addition of $15 \mu\text{l}$ of the mixture to 3 ml of the assay medium (see the Materials and Methods section) containing 1 mM-ATP . (a) Actual tracing of ATPase activity recorded; the numbers on the curves represent the time interval (in min) after addition of phosphoenolpyruvate and pyruvate kinase. (b) Dependence of the initial rate of ATP hydrolysis on time after addition of the ADP-utilizing system.

Fig. 3. Activation of ADP-inhibited ATPase in the presence of ATP

(a) The particles were preincubated with ADP as described in Fig. 1 and small portions were withdrawn to start the reaction in the assay medium containing 1 mM-ATP. (b) Semilogarithmic plot of the result presented in (a).

The results are shown in Fig. 3. The apparent first-order rate constant for activation process is 1.9 min^{-1} , which is about 10 times that measured without ATP. Thus ATP increases the apparent rate of interconversion of the ADP-inactivated ATPase into its active form.

Model for interaction of ATPase with ADP and ATP during the inactivation-activation transition

The simplest model for the qualitative description of the activation phenomenon is presented in Scheme 1, where K_1 and K_2 are the equilibrium constants for the nucleotide interaction with different forms of ATPase and k_{+1} , k_{-1} etc. are the individual rate constants for association and dissociation processes; k_{cat} is the first-order rate constant of the decomposition of enzyme-substrate complex in the catalytic turnover of ATPase. Assuming that interaction of ATP with the enzyme is in equilibrium during the interconversion of inactive E-ADP complex into active ATP-E complex, and that the concentration of free ADP is negligible owing to high activity of the regenerating system, the system of differential equations for the kinetics of the interconversion can be written as follows:

$$\dot{x} = k_{-2} \cdot y - k_{+2} \cdot x \cdot [\text{ATP}] - k_{-1} \cdot x \quad (2)$$

$$\dot{y} = k_{+2} \cdot x \cdot [\text{ATP}] - k_{-2} \cdot y - k_{-3} \cdot y \quad (3)$$

where x and y are the concentrations of the inactive complexes E-ADP and ATP-E-ADP respectively and $\dot{x}$ and $\dot{y}$ are the differentials of x and y . The sum of eqns. (2) and (3) is:

$$\dot{x} + \dot{y} = -k_{-1} \cdot x - k_{-3} \cdot y \quad (4)$$

Scheme 1. Scheme of ADP and ATP interactions with mitochondrial ATPase
See the text for details. Signs + and - stand for the association and dissociation direction respectively.

From the equilibrium assumption $x \cdot [\text{ATP}]/y = K_2$ eqn. (4) can be written as:

$$x + \frac{[\text{ATP}]}{K_2} \cdot x = -k_{-1} \cdot x - \frac{k_{-3} \cdot [\text{ATP}]}{K_2} \cdot x \quad (5)$$

The solution of eqn. (5) is:

$$x_t = x_0 \cdot \exp\left(\frac{k_{-3} \cdot [\text{ATP}] + k_{-1} \cdot K_2}{K_2 + [\text{ATP}]}\right) \quad (6)$$

Fig. 4. Dependence of activation rate on ATP as compared with that predicted from the model

The continuous line shows the dependence of $1/k_{\text{obs}}$ on $1/[\text{ATP}]$ according to eqn. (8) with the values of parameters k_{-3} , k_{-1} and K_2 2.0 min^{-1} , 0.2 min^{-1} and 10^{-4} M respectively. The points represent the experimental results obtained as in Fig. 3.

where x_0 is the concentration of the inactive enzyme at zero time when the process of activation is started by the addition of completely inactive ATPase into reaction mixture containing regenerating system and ATP. In other words the process of activation according to the model presented in Scheme 1 can be described as a first-order reaction with the apparent rate constant k_{obs} :

where:

$$k_{\text{obs}} = \frac{k_{-3} \cdot [\text{ATP}] + k_{-1} \cdot K_2}{K_2 + [\text{ATP}]} \quad (8)$$

The value of k_{-1} was determined to be 0.2 min^{-1} (data of Fig. 2). The values of k_{-3} and K_2 can be obtained according to eqn. (8) from the plot of $1/k_{\text{obs}}$ versus $1/[\text{ATP}]$ for the experimental conditions given in Fig. 3. The results of such determination are shown in Fig. 4, where the straight line represents the calculated dependence of $1/k_{\text{obs}}$ on $1/[\text{ATP}]$ according to eqn. (8) with the values k_{-3} , k_{-1} and K_2 equal to 2.0 min^{-1} , 0.2 min^{-1} and 10^{-4} M respectively, and the points are the experimental values obtained as shown in Fig. 3. It should be pointed out that, according to eqn. (8), the plot of $1/k_{\text{obs}}$ versus $1/[\text{ATP}]$ is linear only in a limited range of ATP concentration. If $[\text{ATP}] \sim 0$, the value of k_{obs} becomes equal to k_{-1} . This is apparently the case, since k_{obs} indeed does not depend on the

Fig. 5. Kinetics of the ATPase reaction catalysed by AS-particles

(a) Direct plot for low concentrations of the substrate; (b) double-reciprocal plot. For conditions of the assay see the Materials and Methods section.

Table 1. Kinetic constants for ADP and ATP interactions with mitochondrial ATPase at 25°C at pH 8.0
For definitions of the constants see Scheme 1 and the text.

Constant	Value obtained	Method of determination
K_1	$2 \times 10^{-8} \text{ M}$	K_1 for ADP (Fitin <i>et al.</i> , 1979)
k_{+1}	$10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$	Calculated from K_1 and k_{-1} values
K_2	10^{-4} M	Dependence of activation rate on concentration of ATP (the present paper)
K_4	10^{-4} M	Double-reciprocal plot for ATPase reaction (the present paper)
k_{-3}	2.0 min^{-1}	Extrapolation of k_{obs} to infinite concentration of ATP (the present paper)
k_{cat}	10^4 min^{-1}	From value of V for ATPase and titre for oligomycin (the present paper)

dilution of the system containing very low concentration of nucleotides (Fig. 2).

It was of especial interest to compare the values of K_2 and K_4 . According to the model of Scheme 1 the latter represents the K_m value for the ATPase reaction. Since the data in the literature on K_m^{ATP} for ATPase vary, depending on the preparations and the conditions used by different authors, we measured the standard kinetic parameters of this reaction under exactly the same conditions as for the activation process. As shown in Fig. 5, the ATPase reaction catalysed by AS-particles obeys simple Michaelis–Menten kinetics, with K_m 10^{-4} M and turnover number 10^4 min^{-1} . The latter was calculated from the enzyme content in AS-particles determined from the oligomycin titre, which was 0.36 nmol/mg of protein (A. F. Fitin, unpublished work). The straight line in Fig. 5 does not pass through the origin because the preparations of NADH used were slightly contaminated with ADP (about 0.5% on a molar basis). No change in K_m was observed when the particles that had not been inhibited by ADP or had been inhibited and then re-activated were used as ATPase in experiments similar to that presented in Fig. 5.

The results presented above show that the experimental results on the activation of ADP-inhibited ATPase are consistent with the model formulated in Scheme 1 with the equilibrium and rate constants summarized in Table 1.

Specificity of the inhibition–activation phenomenon for nucleotides

As documented in Table 2, nucleoside diphosphates other than ADP apparently have no inhibitory effect on the ATPase activity of the particles. On the other hand ADP has the same effect on the initial rate of the ITPase reaction catalysed by the particles as was found for ATPase. No dependence of activation rate for the inosine triphosphatase reaction on ITP concentration was found.

Effect of Mg^{2+} on the inhibition of ATPase by ADP

The inhibition of the initial rate of ATP hydrolysis catalysed by AS-particles after preincubation

Table 2. Specificity of ADP for inhibition of nucleoside triphosphatase reactions catalysed by AS-particles
AS-particles were preincubated with nucleoside diphosphates under the conditions indicated in Fig. 1, and ATPase or ITPase were assayed as described in the Materials and Methods section with 150 μM -ATP or 550 μM -ITP.

Nucleoside diphosphate in preincubation mixture	Initial rate of nucleoside triphosphate hydrolysis ($\mu\text{mol/min per mg}$)	
	ATP	ITP
None	2.0	0.46
ADP (5 μM)	0.0	0.0
GDP (25 μM)	1.7	
GDP (100 μM)	2.1	
GDP (250 μM)	2.1	
IDP (100 μM)	2.2

Fig. 6. Effect of Mg^{2+} on inhibition of ATPase by ADP
AS-particles were preincubated for 3 min in the mixture indicated in Fig. 1 with (●) and without (O) 3 μM -ADP and different concentrations of MgCl_2 . The activity was assayed as described in the Materials and Methods section with 200 μM -ATP.

with low concentrations of ADP requires Mg^{2+} . As shown in Fig. 6, at $3\text{ }\mu\text{M}$ -ADP half-maximal inhibition is observed at $30\text{ }\mu\text{M}$ - MgCl_2 . Assuming the dissociation constant for ADP- Mg^{2+} complex to be $2 \times 10^{-3}\text{ M}$, it seems unlikely that ADP- Mg^{2+} complex is the species that binds to ATPase.

Effect of ADP on ATP-dependent reverse electron transport

As pointed out in the introduction, the basic question whether the ATPase reaction catalysed by different mitochondrial preparations is the true reversal of the events during respiration-driven phosphorylation is not answered. In view of the data presented here and in our previous reports (Fitin *et al.*, 1979; Minkov *et al.*, 1979), it was decided to compare the inhibitory effect of ADP on ATPase with that on some other ATP-dependent reactions catalysed by submitochondrial particles, which might be considered as a functional ATPase. ATP-dependent reduction of NAD^+ by succinate (L6w & Vallin, 1963) may serve as an example of such an ATPase. AS-particles used in the present studies are unable to catalyse this reaction, presumably because their coupling membrane is leaky for protons. This leakage can be significantly diminished by oligomycin (Lee & Ernster, 1965; Hinkle & Horstman, 1971). Indeed, when AS-particles used in the present studies were treated with a substoichiometric amount of oligomycin ($0.15\text{ }\mu\text{g}/\text{mg}$ of protein) a high rate ($60\text{--}100\text{ nmol}$ of NAD^+ reduced/min per mg) of the ATP-dependent reverse electron transport was observed. The effect of ADP on this 'functional' ATPase is shown in Fig. 7. As with uncoupled ATPase, preincubation of oligomycin-treated AS-particles with ADP results in complete inhibition of the initial activity, which then

is activated. This lag in the activity can be eliminated by preincubation of ADP-inhibited particles with phosphoenolpyruvate and pyruvate kinase. An apparent first-order rate constant for activation is about 0.3 min^{-1} , which is in good agreement with that for uncoupled ATPase (see Fig. 4). This result suggests that ADP inhibits equally both uncoupled and coupled ATPase, and that both activities can be progressively regenerated in the presence of ATP.

Discussion

The basic purpose of the present studies was to obtain quantitative information on the kinetics of nucleotide interaction with mitochondrial ATPase. The results presented, together with those previously published (Fitin *et al.*, 1979; Minkov *et al.*, 1979), show that ATPase of submitochondrial particles interacts specifically with the substrate of oxidative phosphorylation, ADP, and this interaction leads to the dramatic change of its ATPase activity. The sluggish active-inactive interconversion of soluble and membrane-bound mitochondrial ATPase has been described by Moyle & Mitchell (1975). In their experiments inactivation was induced by Mg^{2+} and activation was achieved by EDTA or activating anions. We incline to the belief that the phenomenon described by Moyle & Mitchell (1975) and the ADP-dependent inhibition reported in the present paper are closely related. This can be supported by the following reasons. (i) The amount of ADP that is able to inhibit ATPase is extremely small; the K_i value was reported to be $2 \times 10^{-8}\text{ M}$ (Fitin *et al.*, 1979) and the submitochondrial particles usually contain some amount of endogenous adenine nucleotides (Klingenberg, 1967; Leimgruber & Senior, 1976). In fact, we have been

Fig. 7. ADP-induced inhibition of ATP-dependent NAD^+ reduction by succinate

Curve 1, AS-particles were treated with oligomycin as described in the Materials and Methods section; the 'coupled' particles were then treated with $3\text{ }\mu\text{M}$ -ADP as described in Fig. 1 and the reverse electron transport was assayed as described in the Materials and Methods section. Curve 2, the same as curve 1 except that pyruvate kinase and phosphoenolpyruvate were added and incubation was continued for 5 min before the assay.

able to demonstrate that the presence of tightly bound nucleotides in factor F_1 is sufficient to produce Mg^{2+} -dependent inactivation of ATPase (Minkov *et al.*, 1979). (ii) As shown in the present paper, the ADP-induced inactivation is strongly dependent on the presence of Mg^{2+} . (iii) We have observed that incubation of ADP-inhibited enzyme in the presence of ATP and sulphite results in a rapid restoration of ATPase activity (A. F. Fitin, unpublished work). A more detailed comparative analysis of the phenomenon described by Moyle & Mitchell (1975) and by us (Fitin *et al.*, 1979; Minkov *et al.*, 1979; the present paper) is not possible, since the two groups used different assays of ATPase activity (by release of protons and with an ATP-regenerating system respectively), and, as has been shown, an accumulation of ADP during the ATPase reaction can seriously affect the kinetics of ATP hydrolysis (Fitin *et al.*, 1979).

ADP- and ATP-dependent changes of factor F_1 ATPase have been described by Harris *et al.* (1978). According to their results ADP treatment without Mg^{2+} results in about 50% inhibition of factor- F_1 ATPase, and this inhibition can be reversed by incubation with ATP. This phenomenon was not observed with submitochondrial-particle ATPase. Unfortunately the authors gave no actual traces of the activities recorded before and after preincubation with ADP: in our experiments ADP preincubation results in inhibition of only the initial rate of ATP hydrolysis. The final conclusion on the relationship between the inactivation of mitochondrial ATPase described by Moyle & Mitchell (1975), by Harris *et al.* (1978) and by us should await further experiments under well-defined conditions.

In contrast with others (Schuster *et al.*, 1975; Alexandre *et al.*, 1975), we were unable to find any significant deviation from regular Michaelis-Menten kinetics for the ATPase reaction catalysed by submitochondrial particles (Fig. 5). The data in the present paper show that the final value for the ATPase activity is a complicated function of ATP, ADP and time of the reaction. It may be expected that, under the conditions where ADP is accumulated, the observed dependence of v on $[S]$ would not be the simple hyperbolic. It is noteworthy that the kinetic model we suggest is very similar to, if not identical with, the scheme proposed by Rabin (1967), for which apparent kinetic co-operativity was theoretically predicted.

The experimental results on activation of ADP-inhibited ATPase in the presence or absence of ATP are in good agreement with the model of Scheme 1. It follows from the model that binding of ADP at one site of the enzyme does not prevent binding of ATP at the other site (the ternary complex ADP-E-ATP), but it does prevent the hydrolysis of bound ATP. Moreover, since K_2 equals K_4 (Table 1), the

affinities of ATP for free and ADP-containing enzymes are the same. On the other hand binding of ATP to the enzyme results in change of ADP-specific site reactivity towards ADP. This follows from the distinct difference between k_{-1} and k_{-3} . Because of the obvious difficulties due to the interconversion of ATP into ADP by the enzyme, we are unable to discriminate between two possible reasons for the change of ADP-specific site reactivity caused by ATP: it may be a result of a decrease of affinity for ADP or due to the simultaneous increase of association and dissociation rate for ADP without change of equilibrium constant. The values of k_{cat} , k_{-3} and k_{-1} clearly demonstrate that neither E-ADP nor ATP-E-ADP can be intermediates of the uncoupled ATPase. The same is evidently true for the coupled ATPase (see the results shown in Fig. 7). On the other hand the rate constant for the formation of E-ADP calculated from the values of K_1 for ADP and k_{-3} is $10^7 M^{-1} \cdot min^{-1}$, which is high enough to consider the formation of E-ADP as a possible first step of oxidative phosphorylation. In other words one may consider the ADP-specific site as an active site of ATP synthetase.

The affinity of ADP for ATPase is high enough to consider ADP-inhibited enzyme as containing 'tightly bound' ADP. The possible involvement of tightly bound nucleotides in the realization of the inactivation-activation phenomenon has been suggested (Minkov *et al.*, 1979). According to a report by Boyer's group the tightly bound nucleotides do not participate in catalytic turnover of ATP synthetase, as was evidenced by rapid kinetics of their exchange with external ATP (Boyer *et al.*, 1977). The dependence of the exchange rate of tightly bound nucleotides on the energization of mitochondrial membrane has been reported by Harris *et al.* (1977). Here we emphasize the possible participation of tightly bound nucleotides in the catalytic turnover of ATPase or ATP synthetase without being rapidly exchanged with the surrounding medium. These nucleotides may serve as internal phosphoryl-group carriers in the enzyme molecule, participating in a step-by-step concerted transfer of phosphoryl group from ATP bound in one active site to the other molecule bound at the site of P_i release into the medium. The inhibitory effect of preincubation of the enzyme with ADP in the presence of Mg^{2+} can be explained in terms of this hypothesis as a formation of ATPase with a wrong set of tightly bound nucleotides, and activation of ATPase as a slow restoration of a correct pattern of tightly bound nucleotides.

Another possibility for the functional role of the ADP-specific inhibitory site (and perhaps tightly bound nucleotides) is the regulation of ATP formation in mitochondria. The relatively slow inter-

conversion of active and inactive ATPases is, as shown in the present paper, under the control of ADP and ATP. In other words ATPase has a locus that is sensitive to the ATP/ADP concentration ratio in the surrounding medium. It is noteworthy that the direction to which activity of the enzyme is changed by the nucleotides may not be the same for direct (ATPase) and reverse (ATP synthetase) reactions.

References

- Alexandre, A., Carignani, G. & Rossi, C. A. (1975) *FEBS Lett.* **52**, 111–115
- Boyer, P. D., Gresser, M., Vinkler, C., Hackney, D. & Choate, G. (1977) in *Structure and Function of Energy-Transducing Membranes* (van Dam, K. & van Gelder, B. F., eds.), pp. 261–274, Elsevier, Amsterdam
- Burton, K. (1959) *Biochem. J.* **71**, 388–395
- Catterall, W. A. & Pedersen, P. L. (1972) *J. Biol. Chem.* **247**, 7969–7976
- Fitin, A. F., Vasilyeva, E. A. & Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun.* **86**, 434–439
- Garret, N. E. & Penefsky, H. S. (1975) *J. Biol. Chem.* **250**, 6640–6647
- Gomez-Fernandez, J. C. & Harris, D. A. (1978) *Biochem. J.* **176**, 967–975
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Hammes, G. G. & Hilborn, D. A. (1971) *Biochim. Biophys. Acta* **233**, 580–590
- Harris, D. A. (1978) *Biochim. Biophys. Acta* **463**, 245–273
- Harris, D. A., Rosing, J., Van de Stadt, R. S. & Slater, E. C. (1973) *Biochim. Biophys. Acta* **314**, 149–153
- Harris, D. A., Radda, G. K. & Slater, E. C. (1977) *Biochim. Biophys. Acta* **459**, 560–572
- Harris, D. A., Gomez-Fernandez, J. C., Klungsøyr, L. & Radda, G. K. (1978) *Biochim. Biophys. Acta* **504**, 364–383
- Hilborn, D. A. & Hammes, G. G. (1973) *Biochemistry* **12**, 983–990
- Hinkle, P. C. & Horstman, L. (1971) *J. Biol. Chem.* **246**, 6024–6028
- Klingenberg, M. (1967) in *Mitochondrial Structure and Compartmentation* (Quagliariello, E., Papa, S., Slater, E. C. & Tager, S. M., eds.), pp. 320–324, Adriatica Editrice, Bari
- Lee, C. P. & Ernster, L. (1965) *Biochem. Biophys. Res. Commun.* **18**, 523–529
- Leimgruber, R. M. & Senior, A. E. (1976) *J. Biol. Chem.* **251**, 7110–7113
- Leimgruber, R. M. & Senior, A. E. (1978) *Biochem. Biophys. Res. Commun.* **83**, 837–842
- Löw, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374
- Minkov, I. B., Fitin, A. F., Vasilyeva, E. A. & Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun.* **89**, 1300–1306
- Moyle, J. & Mitchell, P. (1975) *FEBS Lett.* **56**, 55–61
- Penefsky, H. S., Pullman, M. E., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* **235**, 3330–3336
- Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* **235**, 3322–3329
- Rabin, B. R. (1967) *Biochem. J.* **102**, 22c–23c
- Racker, E. & Horstman, L. (1967) *J. Biol. Chem.* **242**, 2547–2556
- Rosing, J., Harris, D. A., Kemp, A. & Slater, E. C. (1975) *Biochim. Biophys. Acta* **376**, 13–26
- Schuster, S. M., Ebel, R. E. & Lardy, H. A. (1975) *J. Biol. Chem.* **250**, 7848–7853
- Selwyn, M. J. (1967) *Biochem. J.* **105**, 279–288
- Sorgato, M. C., Ferguson, S. J., Kell, D. B. & John, P. (1978) *Biochem. J.* **174**, 237–256
- Zalkin, H., Pullman, M. E. & Racker, E. (1965) *J. Biol. Chem.* **240**, 4011–4016