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Kinetic mechanism of mitochondrial adenosine triphosphatase

Inhibition by azide and activation by sulphite

Elena A. VASILYEVA, Ignat B. MINKOV,* Alexey F. FITIN and Andrei D. VINOGRADOV†
Department of Biochemistry, School of Biology, Moscow State University, Moscow 117234, U.S.S.R.

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1. The initial rapid phase of ATP hydrolysis by bovine heart submitochondrial particles or by soluble F_1-ATPase is insensitive to anion activation (sulphite) or inhibition (azide). 2. The second slow phase of ATP hydrolysis is hyperbolically inhibited by azide ($K_i \sim 10^{-5}$ M); the inosine triphosphatase activity of submitochondrial particles or F_1-ATPase is insensitive to azide or sulphite. 3. The rate of interconversion between rapid azide-insensitive and slow azide-sensitive phases of ATP hydrolysis does not depend on azide concentration, but strongly depends on ATP concentration. 4. Sulphite prevents the interconversion of the rapid initial phase of the reaction into the slower second phase, and also prevents and slowly reverses the inhibition by azide. 5. The presence of sulphite in the mixture when ADP reacts with ATPase of submitochondrial particles changes the pattern of the following activation process. 6. Azide blocks the activation of ATP-inhibited ATPase of submitochondrial particles by phosphoenolpyruvate and pyruvate kinase. 7. The results obtained suggest that the inhibiting effect of azide on mitochondrial ATPase is due to stabilization of inactive E*·ADP complex formed during ATP hydrolysis; the activation of ATPase by sulphite is also realized through the equilibrium between intermediate active E·ADP complex and inactive E*·ADP complex.

In the preceding paper we presented evidence that the hydrolysis of ATP by soluble (F_1-)ATPase or membrane-bound mitochondrial ATPase in the presence of the ATP-regenerating system is biphasic (Vasilyeva *et al.*, 1982). Apparently two forms of E·ADP complexes are present during the second, slower, steady-state ATPase reaction: one is an active intermediate of catalysis and the other is inactive E*·ADP complex formed as a result of isomerization of the former into the latter. The existence of these two complexes raises the question of the mechanism of action of some known inhibitors and activators of mitochondrial ATPase. Among those, azide and anion activators have attracted our attention because both of them were shown to affect ATPase in a time-dependent fashion (Mitchell & Moyle, 1971), which is indicative of the slow protein-conformational change involved into the inhibition and activation processes.

Azide is known to be a potent inhibitor of ATP hydrolysis catalysed by mitochondria (Novikoff *et al.*, 1952; Robertson & Boyer, 1955; Myers & Slater, 1957), oligomycin-sensitive ATPase (Hatefi *et al.*, 1974), some solubilized preparations of F_1-ATPase (Meyerhof & Ohlmeyer, 1952; Pullman *et al.*, 1960; Pedersen, 1975) and isolated subunits of the coupling factor from a thermophilic microorganism (TF_1-ATPase) (Yoshida *et al.*, 1977*b*). With bovine heart F_1-ATPase and rat liver submitochondrial particles Mitchell & Moyle (1971) have shown non-competitive inhibition of ATPase by azide. Also, like many other compounds, azide is able under certain conditions act as a nondirectional inhibitor of coupling between oxidation and phosphorylation (Robertson & Boyer, 1955; Kobayashi *et al.*, 1971; Burns & Midgley, 1976).

In contrast with the effect of azide, some oxyanions stimulate mitochondrial ATPase (Myers & Slater, 1957; Cereijo-Santalo, 1968; Stockdale & Selwyn, 1971; Lambeth & Lardy, 1971; Ebel & Lardy, 1975; Soper & Pedersen, 1976; Adolfson & Moudrianakis, 1978) and affect oxidative phosphorylation (Christiansen *et al.*, 1969), although the magnitude of the stimulating effect depends on several factors, including the source (rat

* Present address: Department of Animal and Human Physiology, Sofia State University, Sofia, Bulgaria.
† To whom all correspondence should be addressed.

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liver or bovine heart) and the state (membrane-bound or soluble) of the enzyme.

In the present paper we provide evidence that the inhibiting effect of azide and the activating effect of oxyanion (sulphite) are both connected with the isomerization of an active intermediate E·ADP complex into an inactive E*·ADP complex: i.e. sulphite prevents the isomerization and azide stabilizes the inactive form of the enzyme.

Experimental

The enzyme preparation and methods for measurement of the ATPase activity were described in the preceding paper (Vasilyeva *et al.*, 1982). The solutions of sodium salts of azide and bisulphite were freshly prepared just before the experiments. It was observed that sulphite has a slight inhibiting effect on the activity of pyruvate kinase, so that the amount of this enzyme when used in the presence of sulphite was increased to ensure that the activity of the ATP-regenerating system was always in great excess over that of ATPase. The experimental details are indicated in the legends to the Figures.

Results

Effect of azide on the fast and the slow phases of ATP hydrolysis

Fig. 1 demonstrates the time course of ATP hydrolysis by submitochondrial particles and F_1-ATPase in the presence and in the absence of azide. As shown in Fig. 1, azide has no effect on the initial rate of ATP hydrolysis, being a strong inhibitor of the second, slower, phase. Qualitatively the effect of azide is the same for both membrane-bound and soluble ATPases, although the rates of interconversion of the rapid phase into a partially inhibited (without azide) or an almost completely inhibited phase (with azide) are quite different. The K_i values for azide calculated from the inhibition of the second phase of the reaction are 15 and 25 μM for AS-particles and F_1-ATPase respectively. Some properties of the azide effect not shown in Fig. 1

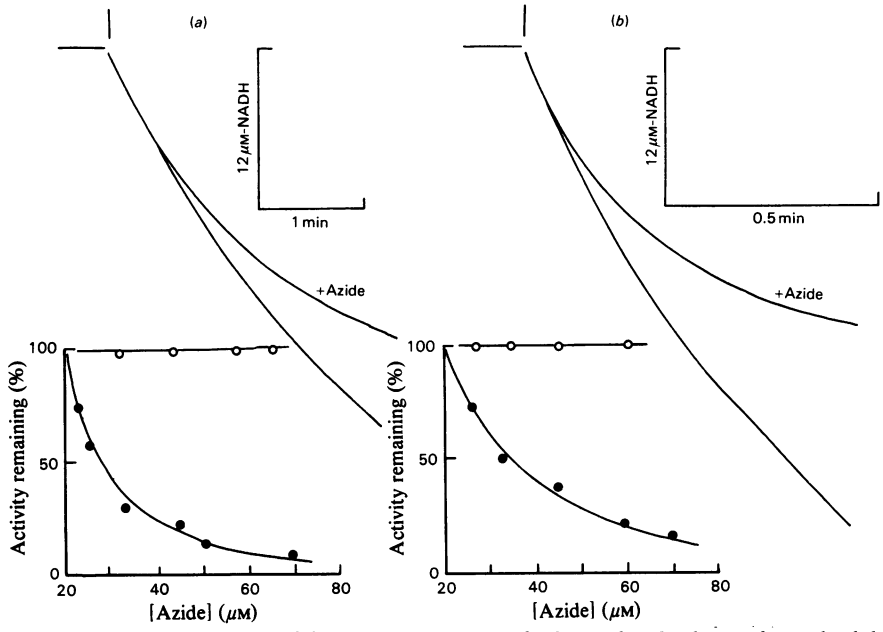


Fig. 1. *Effect of azide on the time course of the ATPase activities of submitochondrial particles and soluble F_1-ATPase*

(*a*) AS-particles (0.5 mg) were activated by incubation for 45 min at 25°C in 0.24 ml of a mixture containing (final concentrations) 0.1 M-KCl, 10 mM-Tris/HCl buffer, pH 8.0, 2 mM-$MgCl_2$, 50 μM-EDTA, 5 mM-phosphoenolpyruvate and 25 units of pyruvate kinase/ml. The reaction was started by the addition of 10 mg of activated particles to 3 ml of the reaction mixture containing (final concentrations) 0.1 M-KCl, 10 mM-Tris/HCl buffer, pH 8.0, 2 mM-$MgCl_2$, 50 μM-EDTA, 2 mM-KCN, 1 mM-phosphoenolpyruvate, 0.3 mM-NADH, 3.3 units each of pyruvate kinase and lactate dehydrogenase/ml and 1 mM-ATP–Mg^{2+} (1:1). Azide (100 μM) was added to the reaction mixture where indicated. (*b*) The ATPase reaction was started by 3 μg of F_1-ATPase to the reaction mixture containing the same components as in (*a*) except for the concentration of ATP, which was 0.1 mM, and the buffer (4-morpholinepropanesulphonic acid/NaOH, pH 7.0). The insets in (*a*) and (*b*) show the dependence of the initial (○) and final (●) rates of ATP hydrolysis on the concentration of azide.

are considered to be important for further discussion. The rates of ITP hydrolysis by the particles activated by preincubation with phosphoenolpyruvate and pyruvate kinase or by F_1-ATPase are constant with respect to time and completely insensitive to azide. Preincubation of the enzyme with azide for the time corresponding to that of the assay does not cause any change in the reaction pattern shown in Fig. 1. This result indicates that no time-dependent interaction of the enzyme with the inhibitor occurs unless the catalytic turnover is commenced.

When the concentration of azide in the assay mixture was high enough to inhibit practically completely the second phase of ATP hydrolysis, it was quite possible to measure the rate of inhibition as a function of several parameters. An example of a semi-logarithmic plot for the time-dependent inhibition of ATPase by azide is given in Fig. 2, where the straight lines correspond to the first-order rate constants of the inhibition process $0.3\,min^{-1}$ and $2.6\,min^{-1}$ for AS-particles and F_1-ATPase respectively.

It was shown that these first-order rate constants were independent of azide concentration provided that the inhibitor concentrations were high enough to inhibit the initial rate by 90%. This result would be expected if the formation of the enzyme–azide complex is not rate-limiting in the overall process of inhibition.

On the other hand the rate of inhibition was found to be strongly dependent on the initial rate of ATP hydrolysis. Fig. 3 shows the linear correlation between the initial rate of ATP hydrolysis by AS-particles and the first-order rate constant for the inhibition process (the latter was measured as indicated in Fig. 2). The dependence of the initial rate of hydrolysis on ATP concentration (Fig. 3) corresponds to a K_m value for ATP equal to 10^{-4} M, which is in a good agreement with the value found in our previous work (10^{-4} M) for the same conditions (Vasilyeva *et al.*, 1980). The results of Fig. 3 would be expected if the rate-limiting step in the process of inhibition of ATPase by azide is a slow isomerization of one of the catalytic intermediates into the form that then rapidly interacts with the inhibitor. Such a mechanism of azide inhibition is also supported by the data in Fig. 4, showing that azide changes the apparent $V_{max.}$ of ATP hydrolysis (for the second, slower, phase of the reaction) without having any effect on K_m for ATP.

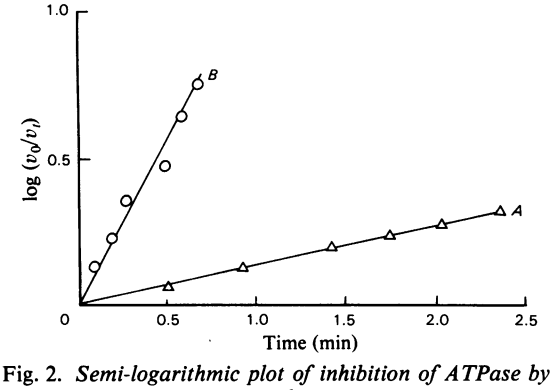


Fig. 2. *Semi-logarithmic plot of inhibition of ATPase by azide*

The conditions of the experiment were as given in the legend to Fig. 1; azide concentration was 100 μM. Curve *A*, AS-particles; curve *B*, F_1-ATPase.

Effect of sulphite on the fast and the slow phases of ATP hydrolysis

The opposite effects of azide and some activating anions on mitochondrial ATPase have been reported (Mitchell & Moyle, 1971; Moyle & Mitchell, 1975; Ebel & Lardy, 1975). As was shown above, azide does not decrease the initial rate of ATPase reaction. Because of this fact and the results reported in the preceding paper (Vasilyeva *et al.*, 1982), it was decided to find out whether the effect of the activating anion depends on the phase of the

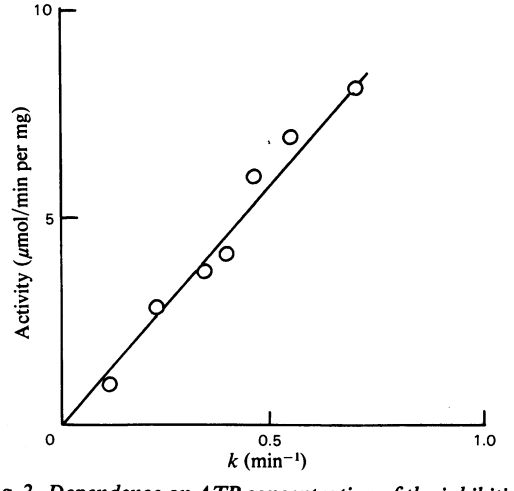


Fig. 3. *Dependence on ATP concentration of the inhibiting effect of azide*

AS-particles were activated and assayed in the presence of 150 μM-azide as indicated in the legend to Fig. 1 except for concentrations of ATP–Mg^{2+}, which were varied in the range 0.02–1 mM. The initial rates of the ATP hydrolysis plotted on the ordinate correspond to the K_m^{ATP} and $V_{max.}$ values of 10^{-4} M and 9 μmol of ATP hydrolysed/min per mg of protein at 25°C. First-order rate constants for the inhibition process determined as in Fig. 2 are plotted on the abscissa.

ATPase reaction. Sulphite was chosen as the anion, since it activates both membrane-bound and soluble ATPases. The results given in Table 1 clearly demonstrate that, similarly to azide, sulphite significantly influences the ATP hydrolysis only in the second phase of the reaction. Also similarly to azide, sulphite increases the apparent $V_{max.}$ at the second phase of ATP hydrolysis without any effect on the K_m value for ATP (results not shown). In agreement with the results reported by Ebel & Lardy (1975), we found no activation of ITP hydrolysis by sulphite provided that the particles were preincubated with phosphoenolpyruvate and pyruvate kinase, and the hydrolysis rate of ITP was constant during assay. It seems noteworthy to compare this result with that on the specificity of biphasic hydrolysis to ATP (Vasilyeva *et al.*, 1982) and the specificity of the high-affinity inhibitory site of ATPase for ADP (Vasilyeva *et al.*, 1980).

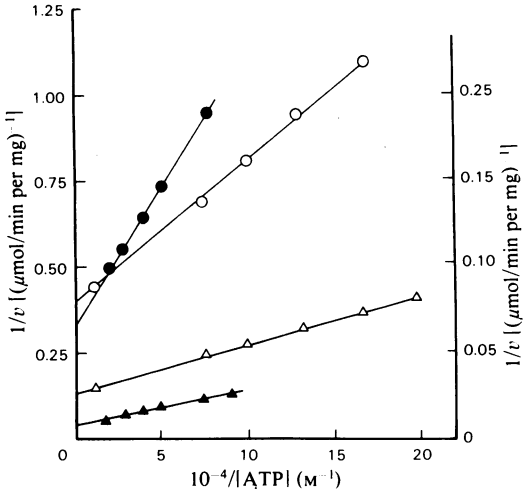


Fig. 4. *Non-competitive inhibition of the ATPase activities of AS-particles and F_1-ATPase by azide*

AS-particles were activated as indicated in the legend to Fig. 1. The initial rates of ATPase activity of the particles (○ and △, left ordinate) and F_1-ATPase (● and ▲, right ordinate) in the absence of azide (△ and ▲) and the delayed constant rates in the presence of 50μM-azide (○ and ●) were measured as given in Fig. 1.

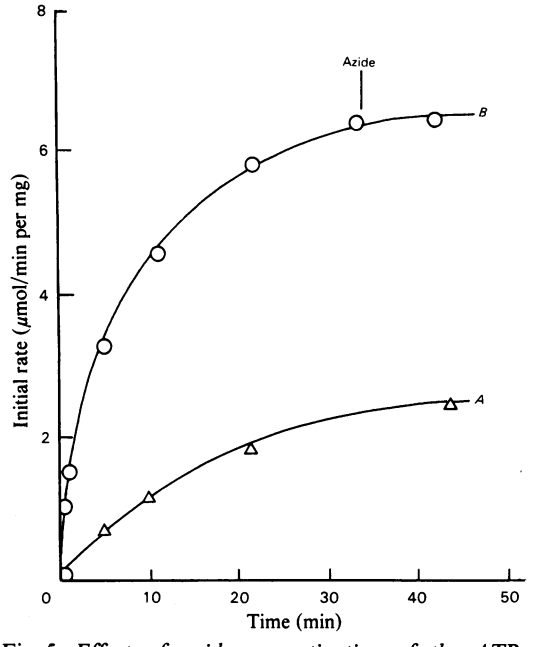


Fig. 5. *Effect of azide on activation of the ATPase activity of AS-particles inhibited by ADP in the presence of an ADP-utilizing system*

AS-particles (0.6mg) were incubated for 3min at 25°C in 0.3ml of a mixture containing (final concentrations) 0.1M-KCl, 10mM-Tris/HCl buffer, pH8.0, 2mM-$MgCl_2$, 50μM-EDTA and 1.5μM-ADP. At zero time 5mM-phosphoenolpyruvate and pyruvate kinase (30units/ml) were added, and the initial rates of 1mM-ATP hydrolysis were determined as indicated in the legend to Fig. 1. Curve *A*, 100μM-azide was added before activation was started; curve *B*, 100μM-azide was added where indicated after the activation had reached maximum.

Table 1. *Effect of sulphite on the initial and delayed rates of ATP hydrolysis catalysed by AS-particles and F_1-ATPase*

AS-particles were activated as indicated in the legend to Fig. 1. Sulphite (5mM) was added to the assay mixture given in Fig. 1(*a*) where indicated. Values in parentheses indicate percentage activities.

	Activity (μmol of ATP hydrolysed/min per mg of protein)			
	Initial rates		Delayed rates (3min after the reaction was started)	
Preparation	No sulphite	Plus sulphite	No sulphite	Plus sulphite
AS-particles	7.5 (100)	7.5 (100)	4 (53)	7.4 (98)
F_1-ATPase	60.4 (100)	63.5 (105)	25 (41)	42 (70)

Effects of azide and sulphite on high-affinity ATP-reversible inhibition of ATPase by ADP

All the results presented above indicate that the component that is in slow equilibrium with the active intermediate of the ATP-hydrolytic reaction is the target of azide inhibition and sulphite activation. Although the results reported in the preceding paper (Vasilyeva *et al.*, 1982) and the results presented in Fig. 3 strongly suggest that this component is an inactive E*·ADP complex described previously (Fitin *et al.*, 1979; Minkov *et al.*, 1979; Vasilyeva *et al.*, 1980), it was impossible to rule out conclusively the possibility of the interaction between azide (or sulphite) and the E·ATP complex which should be the intermediate of ATP hydrolysis. Thus it was desirable to study directly the influence of azide and sulphite on the formation and dissociation of the E*·ADP complex, which is formed when the submitochondrial particles or F_1-ATPase are preincubated with low concentrations of ADP (Vasilyeva *et al.*, 1980).

The results of such an experiment are given in Fig. 5. When submitochondrial particles were preincubated with ADP, their ATPase activity measured as the initial rate of ATP hydrolysis was completely lost; the slow activation of ATPase occurred after phosphoenolpyruvate and pyruvate kinase were added at zero time. If azide was added just before phosphoenolpyruvate and pyruvate kinase, inhibition of activation was observed. If azide was added after activation had been completed no change in ATPase activity was found. These results clearly indicate that azide stabilizes the E*·ADP complex, thus making ADP inaccessible for pyruvate kinase.

It has been shown that ADP can be released from the specific binding site of the enzyme in ATP-dependent and ATP-independent reactions characterized by the different first-order rate constants (Vasilyeva *et al.*, 1980). As shown in Fig. 6, azide prevents both types of dissociation. In the presence of azide progressive inhibition of ATPase reaction is observed (curve *a*); the slow activation is seen when the reaction was started by the particles preincubated with ADP (curve *b*, no azide present); the enzyme is kept inhibited when the particles were first preincubated with ADP and then transferred to the

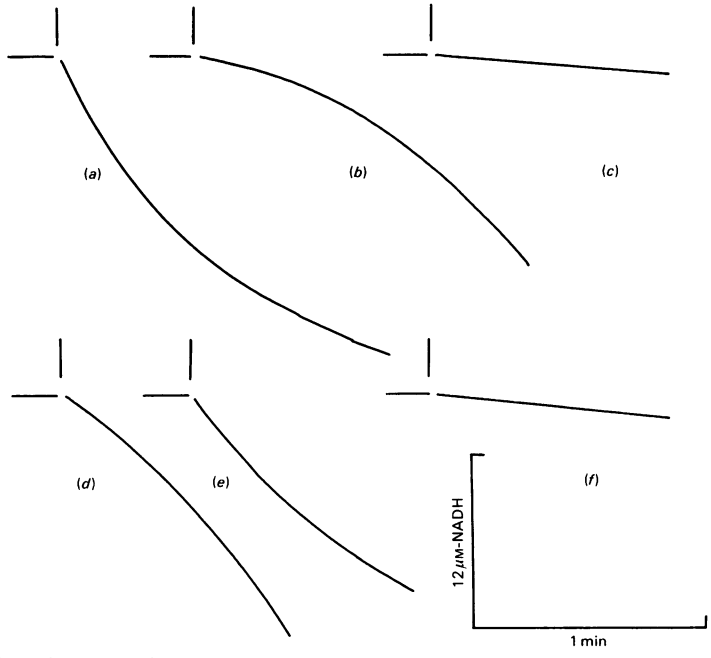


Fig. 6. *Effect of azide on the slow active–inactive transition of ATPase induced by ATP and ADP*

(*a*)–(*c*) AS-particles (120 μg) were incubated for 3 min at 25°C in 60 μl of a mixture containing (final concentrations) 0.1 M-KCl, 10 mM-Tris/HCl buffer, pH 8.0, 2 mM-$MgCl_2$ and 50 μM-EDTA; 1.5 μM-ADP was added to samples (*b*) and (*c*). The ATPase reaction was started by the addition of 8 μl of the mixture to the assay medium (as indicated in the legend to Fig. 1*a*) containing 100 μM-azide [(*a*) and (*c*)]. (*d*)–(*f*) The particles were incubated as described for sample (*b*), and 8 μl of the mixture was transferred to the assay cuvette (250-fold dilution) containing all the components except for ATP and ATP-regenerating system. Incubation was continued for 7 min, and the reaction was started by the addition of a mixture containing 1 mM-ATP and ATP-regenerating system. Azide (100 μM) was added to samples (*e*) and (*f*) before the particles (*f*) or together with the ATP-regenerating system (*e*).

assay mixture containing azide and ATP. Thus the ATP-dependent release of ADP from the specific inhibitory site is prevented by azide. The inhibition of the ATP-independent release of ADP is illustrated by Fig. 6, curves (*d*)–(*f*). As shown in Fig. 6, a substantial initial rate of ATP hydrolysis is observed when the particles preincubated with ADP were diluted so that the ADP concentration became negligible (6 nM) before the incubation for 7 min (curve *d*). This rate is slowly decreased by azide under the conditions of the enzyme turnover (curve *e*); when, however, the particles were diluted and then incubated for 7 min in the presence of azide, no activation occurs, indicating the inhibition of the spontaneous ATP-independent release of ADP.

Sulphite also has a pronounced effect on interaction between ADP and ATPase. As shown in Fig. 7, addition of sulphite to the preincubation mixture where ADP reacts with ATPase increases the activation rate when the reaction is started by the addition of ADP-inhibited enzyme to the assay medium. Interestingly, the initial rate of the ATP hydrolysis in this case is still zero, although the rate of activation becomes significantly higher. Thus sulphite apparently does not prevent the interaction between ADP and ATPase, but changes the conformational state of the enzyme induced by ADP. As a result sulphite has an apparent preventive effect on the inhibition of ATPase by azide and is also capable of slow activation of azide-inhibited enzyme (Fig. 8).

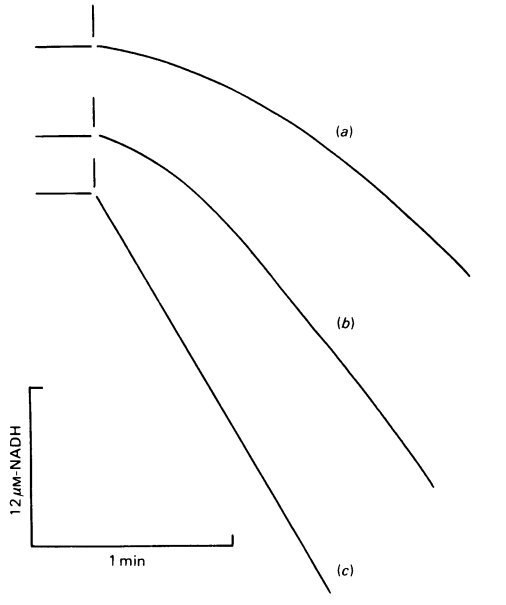


Fig. 7. *Effect of sulphite on ADP-induced inhibition of ATPase*

AS-particles were preincubated as indicated for samples (*a*)–(*c*) in the legend to Fig. 6. 1.5 μM-ADP (*a*), 1.5 μM-ADP and 2.5 mM-sulphite (*b*) and 2.5 mM-sulphite (*c*) were added to the preincubation medium. The ATPase reaction was then started as indicated.

Discussion

In the preceding paper (Vasilyeva *et al.*, 1982) we presented evidence that during the steady-state hydrolysis of ATP the mitochondrial ATPase exists as a slowly (compared with turnover) equilibrating mixture of catalytically active intermediates and an inactive complex of the enzyme with ADP (E*·ADP). The study of inhibition and activation of ATP hydrolysis by azide and sulphite allowed us to confirm and extend the kinetic scheme of the reaction as shown in Scheme 1. Before discussion of some implications of the scheme, a brief summary of the supporting data may be useful. The existence of route (1)–(3) is evident from the high nucleoside triphosphatase activity of mitochondria, submitochondrial particles and soluble F_1-ATPase (Cooper & Lehninger, 1957; Pullman *et al.*, 1960; Hammes & Hilborn, 1971). The ADP release is apparently the rate-limiting step in the sequence (1)–(3) depicted in Scheme 1 in the light of the results obtained by Gresser *et al.* (1980), who have shown the presence of 1 mol of pyruvate kinase-inaccessible ADP/mol of F_1-ATPase during hydrolysis of a very low con-

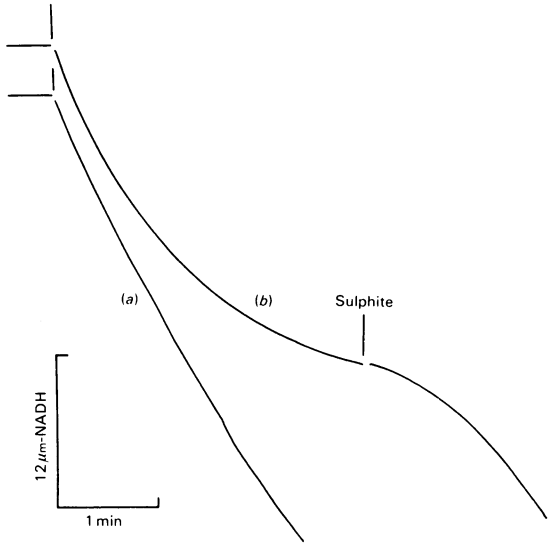
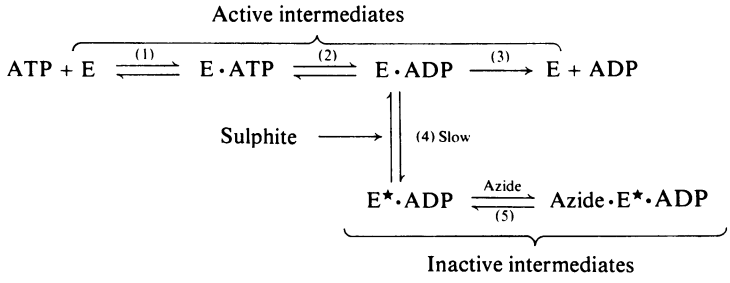


Fig. 8. *Time-dependent effect of azide and sulphite on the ATPase activity*

AS-particles were activated and assayed under the conditions indicated in the legend to Fig. 1(*a*) in the presence of 100 μM-azide. (*a*) 2.5 mM-Sulphite was present in the assay mixture; 2.5 mM-sulphite was added where indicated.



Scheme 1.

centration of ATP. The evidence for the existence of slow equilibrium between two enzyme·ADP complexes (step 4) has been presented in the present and the preceding papers (Vasilyeva *et al.*, 1982). Neither azide nor sulphite was shown to be an effector of the nucleoside triphosphatase reaction occurring through the route (1)–(3) (initial rate of ATP hydrolysis or hydrolysis of ITP). Both become effectors when formation of the E·ADP complex is possible. The non-competitive time-dependent inhibition of ATPase by azide can readily be explained on the assumption that the inhibitor stabilizes the E·ADP complex in a rapid reaction in step (5). This stabilizing effect of azide was directly demonstrated with submitochondrial particles preincubated with ADP (Figs. 5 and 6). In contrast, sulphite prevents the formation of the slowly dissociated E*·ADP complex in step (4) (Fig. 7), thus acting as a non-competitive activator of steady-state ATP hydrolysis without any effect on hydrolysis of ITP.

The results presented in the preceding paper (Vasilyeva *et al.*, 1982) show that the formation of the E·ADP complex is responsible both for ATPase inactivation during preincubation of the enzyme with ADP (Fitin *et al.*, 1979; Vasilyeva *et al.*, 1980) and for the decrease in ATP hydrolysis rate during the steady-state turnover of the enzyme. Apparently, in the former case E*·ADP is formed through routes (1)–(4), where first ADP binds to the active site operating as the ATP-binding site during the ATPase reaction, and then isomerization of the E·ADP complex into the E*·ADP complex occurs (step 4). Indeed, sulphite increases the rate of activation of ADP-inhibited enzyme (Fig. 7). If the mechanism depicted in the kinetic scheme is correct, it may be expected that the simple competitive inhibition of ATPase by ADP in the presence of anion activators would not be changed. Mitchell & Moyle (1971) and Moyle & Mitchell (1975) found a decrease of K_i for ADP in the presence of phosphate, arsenate and sulphate, a fact not easily reconciled with the mechanism of Scheme 1. It should be noted, however, that the K_i values reported by Mitchell & Moyle (1971) are time-dependent and apparently differ from those for simple competitive inhibition (Hammes & Hilborn, 1971).

An interesting problem for the present discussion is the structural basis for the slow isomerization step (4). Since the kinetic behaviour of the membrane-bound and soluble ATPases is qualitatively the same, the F_1-ATPase molecule must be considered as the object of the interconversions presented in Scheme 1. Two reversible binding sites for ADP have been found per molecule of F_1-ATPase (Hilborn & Hammes, 1973; Wielders *et al.*, 1980) among the total of five binding sites (Garret & Penefsky, 1975). The slow isomerization step (4) may thus be considered as a dislocation of ADP from one site to the other. The kinetic evidence for the existence of a ternary ATP·E·ADP complex (Fitin *et al.*, 1979; Vasilyeva *et al.*, 1980) is in agreement with this proposal. Both β- and α-subunits of F_1-ATPase can bind nucleotides (Ohta *et al.*, 1980; Kozlov & Milgrom, 1980, and references cited therein). The most striking observation in view of the kinetic scheme under discussion is that made by Yoshida *et al.* (1977*b*), who have shown that hydrolysis of ATP catalysed by the combination of purified β- and γ-subunits from TF_1-ATPase is stimulated by sulphite and inhibited by azide. In the light of the findings reported in the present paper, these results suggest that either β–β-subunit interaction occurs during ATP hydrolysis or the γ-subunit, which apparently serves as the gate for the proton channel of membrane-bound ATPase (Yoshida *et al.*, 1977*a*), is also able to bind ADP. It would be interesting to find out whether ATPase activity catalysed by the combinations of purified $\beta+\gamma$-subunits is sensitive to ADP in a manner similar to that described by Minkov *et al.* (1979).

The chemical basis for azide inhibition and sulphite activation of ATPase remains obscure. Taking into account the properties of azide as a ligand, one might suggest that the inhibitor substitutes the co-ordination sphere of tightly bound Mg^{2+}, which is, apparently, a natural constituent of

F_1-ATPase (Senior, 1979). This proposal still leaves unanswered the question why the E*·ADP complex then becomes stabilized, as shown in the present paper. The sulphite concentration needed to activate the slow phase of ATP hydrolysis is rather high, so that its activating effect, perhaps like that of other activating anions, may be due to an alteration of the hydration of the protein (Katz *et al.*, 1974). This phenomenon could result in alterations in the nucleotide-binding properties of F_1-ATPase. The effect of some salts on the release of tightly bound nucleotides from F_1-ATPase and submitochondrial particles has been demonstrated (Leimgruber & Senior, 1976).

The problem of interrelations between ATPase and ATP synthesis is of great importance for current studies of the mechanism of energy conservation in mitochondria. In this respect the detailed studies of unidirectional inhibitors of ATPase (true or apparent ones) seems useful. Azide has been shown to be such an inhibitor (Robertson & Boyer, 1955; Kobayashi *et al.*, 1971) and the strong inhibition of the ATPase activity of submitochondrial particles without significant effect on their phosphorylating activity has also been observed in our laboratory (I. B. Minkov & A. D. Vinogradov, unpublished work). Azide was shown to stabilize the E·ADP complex, and ADP under certain conditions acts as an unidirectional inhibitor of ATPase (Minkov *et al.*, 1980). Taken together, these findings support our hypothesis that F_1-ATPase catalysis of the hydrolysis and synthesis of ATP involves two different slowly interconvertible states. In fact, during the last three decades several papers have appeared from different laboratories in which the possibility of different routes for ATP synthesis and hydrolysis has been suggested (Cooper, 1958; Lardy *et al.*, 1964; Vasington & Greenawalt, 1964; Mitchell *et al.*, 1967; Huang & Mitchell, 1972; Penefsky, 1974; Pedersen, 1975; Moudrianakis & Adolfson, 1975; Recktenwald & Hess, 1980). We believe that the principal difficulty in resolution of the problem is that, in the resolved mitochondrial ATPase systems such as submitochondrial particles, only a small part of the observed ATPase can be directly related to the energy-linked processes (Löw & Vallin, 1963; Huang & Mitchell, 1972; Harris *et al.*, 1978). This makes it practically impossible to analyse quantitatively the direct and reverse 'coupled' ATPase. Whether the qualitative comparison of these activities is basically correct is a problem that must await more conclusive evidence.

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