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CHARACTERISTICS OF ADENYLYL IMIDODIPHOSPHATE- AND ADP-BINDING SITES INSOLUBLE AND PARTICULATE MITOCHONDRIAL ATPase

STUDIES WITH METHANOL

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The characteristics of the binding sites for ADP and adenylyl imidodiphosphate have been studied in soluble and particulate F_1-ATPase from bovine heart mitochondria. ADP, but not electrochemical gradients, removes the inhibitory effect of adenylyl imidodiphosphate on ATPase activity in coupled submitochondrial particles. In soluble F_1-ATPase, methanol at 20% concentration diminishes the ability of ATP and adenylyl imidodiphosphate to inhibit ATP and ITP hydrolysis; these findings suggest that ADP and adenylyl imidodiphosphate inhibit hydrolysis by acting on the same site. Methanol at 20% stimulates the hydrolytic activity of soluble F_1-ATPase, but fails to stimulate significantly the activity of the particulate enzyme, even though in particulate F_1-ATPase methanol markedly diminishes the inhibiting action of added ADP and adenylyl imidodiphosphate on ATP and ITP hydrolysis. This is consistent with the idea that in the particulate system there are two inhibitory binding sites for ADP, one accessible to methanol, and another which is inaccessible to methanol; the latter is transitorily occupied by ADP arising from ATP hydrolysis. Indeed, experiments on the effect of ADP on ITP hydrolysis by submitochondrial particles show the existence of two ADP inhibitory sites.

Introduction

Coupling H^+-ATPases from a wide variety of organisms possess an as yet undetermined number of adenine nucleotides bound to the F_1 component [1–3]. Along this line, it has been reported that some of the nucleotides exist in catalytic [4,5] and regulatory [6–15] sites, and there is evidence indicating that adenine nucelotides may also be necessary for maintaining or stabilizing the structure of F_1-ATPase [1,16]. Therefore, it would appear that for a proper understanding of the mechanism of oxidative phosphorylation, it is necessary to determine not only the number of adenine nucleotide-binding sites and their positions among the various subunits of F_1-ATPases, but also their possible role in the catalytic process Moreover, it is necessary to explore whether the adenine nucleotide-binding sites exhibit similar characteristics and functions in the soluble and particulate F_1-ATPase. In this work, some of the characteristics of these binding sites have been investigated.

Ado*PP*[NH]*P* is a nonhydrolyzable analog of adenine nucleotides which strongly inhibits ATP hydrolysis, but does not affect synthesis [2,7,17–22]. To explain these peculiar characteristics of Ado*PP*[NH]*P*, it has been proposed that during oxidative phosphor-

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Abbreviation: Ado*PP*[NH]*P*, adenylyl imidodiphosphate.

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ylation, ADP [18,23] and/or electrochemical gradients [20,32] induce its release from F_1-ATPase. Also, it has been reported that ATP may compete with Ado*PP*[NH]*P* for a common site [7,18,20,24].

In agreement with the conclusions of other authors [23,25], it has been found that ADP and Ado*PP*[NH]*P* may bind to common sites inducing inhibition of hydrolysis, but it is also shown that in the soluble enzyme, methanol is able to modify these inhibitory adenine nucleotide-binding sites. On the basis of their sensitivity to methanol, it was found that these adenine nucleotide-binding sites exhibit different characteristics in the soluble and membrane-bound F_1-ATPase.

Material and Methods

Mitochondria from bovine heart were prepared as described by Löw and Vallin [26]. In the preparation of submitochondrial particles, 14–20 mg of mitochondrial protein were suspended in 1 ml of 0.25 M sucrose/6 mM ATP/6 mM $MgSO_4$/1 mg bovine serum albumin and adjusted to pH 7.0–7.1; about 10 ml of this mixture were sonicated for 35 s using the maximal output of an MSE machine. The sonicated mixture was centrifuged at 12 000 $\times g$ for 10 min; the supernatant was subsequently centrifuged at 105 000 $\times g$ for 60 min. The pellet was suspended in 0.25 M sucrose/3 mM $MgSO_4$ ('MgATP particles'). To free these particles from a portion of inhibitor protein, the particles were passed through a G-50 Sephadex (coarse) column previously equilibrated with 75 mM sucrose/25 mM KCl/30 mM Tris-sulfate, pH 8.0/2 mM EDTA. The column was eluted with the same solution. The pellet was suspended with 0.25 M sucrose and centrifuged. The final pellet was suspended in 0.25 M sucrose ('MgATP-Sephadex particles'). F_1-ATPase was prepared as described in Ref. 28. Protein was determined by the biuret method, or according to the method of Lowry et al. [29].

ATP hydrolysis was measured in the presence of an ATP-regenerating system [27], by recording NADH oxidation, or by the assay of P_i formed from ATP or ITP hydrolysis in mixtures of compositions which are detailed under Results. P_i was determined according to the method of Summer [30].

Phosphorylation of ADP was assayed in the presence of an ADP-regenerating system, 170 mM sucrose/50 mM Tris-acetate, pH 7.3/300 μM ADP/3 mM magnesium acetate/8 mM potassium phosphate/20 mM glucose/5 mM succinate/5 mM NADP/7 U hexokinase/7 U glucose-6-phosphate dehydrogenase/5 mM AMP. Reduction of NADP recorded at 340 nm was taken as an index of ATP formation. For the calculation of ATP formation by oxidative phosphorylation, ATP formed by myokinase activity was substracted.

Results

Chernyak and Kozlov [32] described experiments indicating that the inhibitory effect of Ado*PP*[NH]*P* on ATP hydrolysis by submitochondrial particles (which apparently possessed most of their ATPases in the inhibited state through action of the inhibitor protein) could be reversed by dilution, and that the time required for reversal was diminished by electron transport. This suggested that electrochemical gradients could induce the dissociation of Ado*PP*[NH]*P* from its binding site(s) in F_1-ATPase. Their conclusions have been reexamined under different experimental conditions.

MgATP submitochondrial particles, previously passed through a Sephadex column to eliminate possible interference with the assay by the natural ATPase inhibitor protein [33], were incubated with different concentrations of Ado*PP*[NH]*P* in the presence and absence of succinate and/or ADP. After 3 min of incubation, ATP hydrolysis was initiated and assayed by introducing ATP and pyruvate kinase into the system (added ADP was removed by pyruvate kinase in less than 5 s). Immediately after exhaustion of ADP, the rates of ATP hydrolysis remained constant for at least 1 min and were used for the calculation of the data shown in Fig. 1.

Under these conditions, it was observed that preincubation of the particles with succinate did not modify the action of Ado*PP*[NH]*P*. In contrast, preincubation with ADP did diminish the action of Ado*PP*[NH]*P* (Fig. 1); this action of ADP was not modified by including succinate in the preincubation mixture (data not shown). However, it is important to observe that ADP does not decrease the inhibiting action of 100 μM Ado*PP*[NH]*P* (Fig. 1) (a concentration of Ado*PP*[NH]*P* which does not inhibit oxidative phosphorylation with 300 μM ADP in the mixture; data not shown, and Ref. 23).

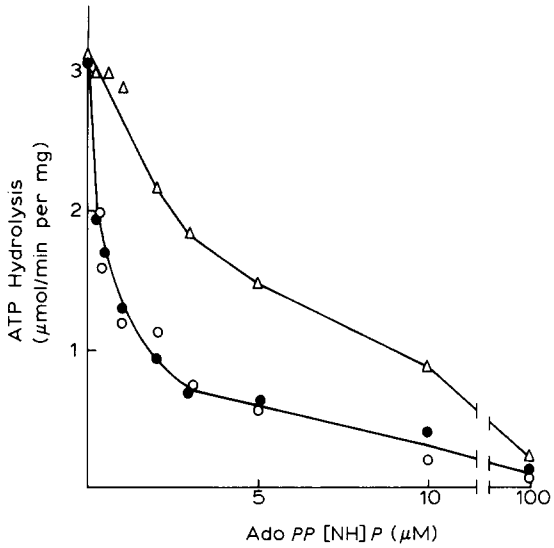

Fig. 1. Effect of ADP and succinate on the inhibitory action of Ado*PP*[NH]*P* on the hydrolytic activity of MgATP-Sephadex particles. MgATP-Sephadex particles (106 μg) were preincubated for 3 min at 33°C in 3 ml of a mixture containing 50 mM sucrose/50 mM Tris-acetate, pH 7.4/30 mM potassium acetate/1 mM magnesium acetate/3 μg rotenone/1 mM phospho*enol*pyruvate/3 mM NADH/10 U lactate dehydrogenase; the mixture also contained the indicated concentrations of Ado*PP*[NH]*P*, and 300 μM ADP or 5 mM succinate. After the preincubation, 20 U pyruvate kinase and 1 mM ATP were added and the oxidation of NADH was recorded. After exhaustion of ADP (less than 5 s), hydrolysis rates of ATP were linear for at least 1 min and were used for the calculations. •, control; △, ADP; ○, succinate.

The experiment of Fig. 1 indicates that ADP, but not electron transport, modifies the action of Ado*PP*[NH]*P*. Further evidence for this postulate is afforded by the results of the experiments in which MgATP-Sephadex particles were preloaded with Ado*PP*[NH]*P*, i.e., particles were incubated with Ado*PP*[NH]*P* and subsequently washed (twice with 0.25 M sucrose) to remove free Ado*PP*[NH]*P*. The ATPase activity of particles preloaded with Ado*PP*[NH]*P* was strikingly lower (50 nmol/min per mg) than that of particles which had not been exposed to Ado*PP*[NH]*P* (810 nmol/min per mg). The low activity of the particles treated with Ado*PP*[NH]*P* was increased to a value of 660 nmol/min per mg by preincubation with 300 μM ADP, but not when they were exposed to electron transport. It should be acknowledged that these particles still carried out oxidative phosphorylation (43 nmol ATP formed/min per mg).

Accordingly, the aforementioned data indicate that ADP overcomes much more effectively than electron transport the inhibiting action of Ado*PP*[NH]*P*. In addition, these experiments show that the ADP effect is observed only at low concentrations of Ado*PP*[NH]*P* (see Fig. 1), or when the assay is carried out at high ADP/Ado*PP*[NH]*P* ratios. These conclusions are in agreement with those of Penefsky [23] who suggested that ADP induced the release of bound Ado*PP*[NH]*P* from F_1-ATPase. However, they do not rule out the possibility that electrochemical gradients induce the release of bound Ado*PP*[NH]*P*, if the latter is bound on a noninhibitory site of ATP hydrolysis.

The following experiments were carried out to characterize some of the properties of the site(s) through which Ado*PP*[NH]*P* and ADP inhibit ATP hydrolysis in soluble and particulate F_1-ATPase. It is known that methanol enhances the hydrolytic activity of soluble F_1-ATPase [34,35], and it has been proposed that methanol modifies the regulatory site through which ADP inhibits hydrolysis [34]. Thus, it

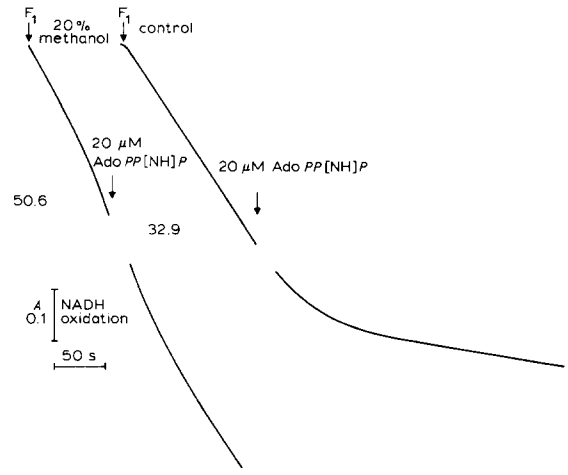

Fig. 2. Effect of Ado*PP*[NH]*P* on the hydrolytic activity of soluble F_1-ATPase incubated with and without methanol. ATP hydrolysis was followed under the same conditions as in Fig. 1, except that the reaction was started by adding F_1-ATPase to a mixture already containing pyruvate kinase. In the trace on the left, the mixture also contained 20% methanol. Where shown, 20 μM Ado*PP*[NH]*P* was added to the mixture. The nunber at the side of each trace is the rate of NADH oxidation (in μmol NADH oxidized/min per mg).

was decided to study whether methanol also modifies the inhibiting action of Ado*PP*[NH]*P*.

Fig. 2 shows that in soluble F_1-ATPase, methanol increases the time required for Ado*PP*[NH]*P* to inhibit ATP hydrolysis; it also diminishes the extent of the inhibition induced by Ado*PP*[NH]*P*. These experiments were carried out in the presence of phospho*enol*pyruvate and pyruvate kinase in which the concentration of ADP was kept at very low levels in order to avoid interference by ADP with the assay on the effect of Ado*PP*[NH]*P*. It should be noted (Fig. 2) that methanol increases the rate of ATP hydrolysis even in the presence of an ATP-regenerating system. This observation is of importance for the interpretation of the data obtained with methanol (see the Discussion). Methanol does not affect the behavior of pyruvate kinase and lactate dehydrogenase used in the assay of ATP hydrolysis.

A Dixon plot of the action of methanol on the inhibiting effect of various concentrations of Ado*PP*[NH]*P* (Fig. 3) shows that methanol diminishes significantly the affinity of the enzyme for Ado*PP*[NH]*P*.

Since it has been reported [34] that methanol affects the adenine nucleotide-binding site for ADP, the results of Fig. 3 would suggest that the binding site of Ado*PP*[NH]*P* corresponds to that of ADP. To study further this possibility, the action of methanol on the effect of Ado*PP*[NH]*P* and ADP on ITP hydrolysis by soluble F_1-ATPase was studied (Fig. 4). Along this line, it should be recalled that Penefsky [34] has shown that methanol overcomes the inhibiting action of ADP on ITP hydrolysis. Ado*PP*[NH]*P* inhibited ITP hydrolysis much more effectively than did ADP, notwithstanding the presence of methanol (Fig. 4). In addition, if the data of Figs. 3 and 4 (inset) are compared, it can be observed that Ado*PP*[NH]*P* is more effective in inhibition of ITP than is ATP hydrolysis. Apparently, ATP competes more strongly than ITP for an Ado*PP*[NH]*P*-binding site; the latter conclusion is in agreement with previous data [7,24,36]. However, the results in Fig. 4 also show that methanol diminishes the ability of both

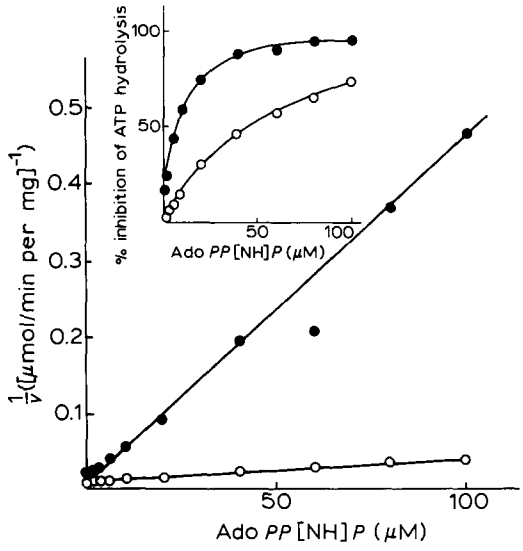

Fig. 3. Effect of methanol on the inhibiting action of Ado*PP*[NH]*P* on ATP hydrolysis by soluble F_1-ATPase. Soluble F_1-ATPase (10 μG) was added to a mixture indentical to that of Fig. 2, except that the indicated concentrations of Ado*PP*[NH]*P* were included. Where shown the mixture also contained 20% methanol. After 3 min incubation at 33°C, the reaction was stopped with trichloroacetic acid (6% final concentration) and P_i determined. The amount of ATP hydrolyzed was 102 and 186 μmol/min for the enzyme incubated with and without methanol, respectively. •, control; ○, methanol.

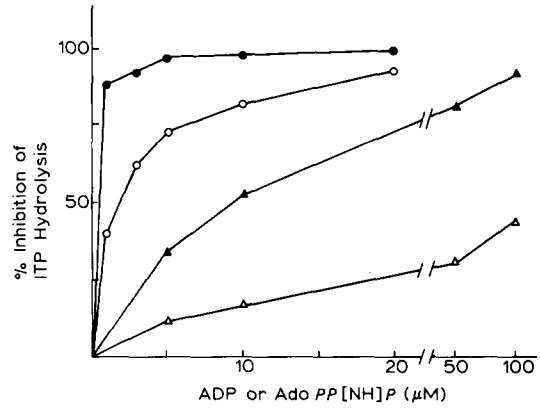

Fig. 4. Effect of methanol on the inhibiting action of ADP and Ado*PP*[NH]*P* on ITP hydrolysis by soluble F_1-ATPase. Hydrolysis was measured as in Fig. 3, except that pyruvate kinase and phospho*enol*pyruvate were omitted. Instead of ATP the mixture contained 1 mM ITP. As shown, 20% methanol and the indicated concentrations of ADP and Ado*PP*[NH]*P* were included. The hydrolysis rates in water and 20% methanol were considered as 100% to make the respective calculations. •, Ado*PP*[NH]*P*; ○, Ado*PP*[NH]*P* + methanol; ▲, ADP; △, ADP + methanol.

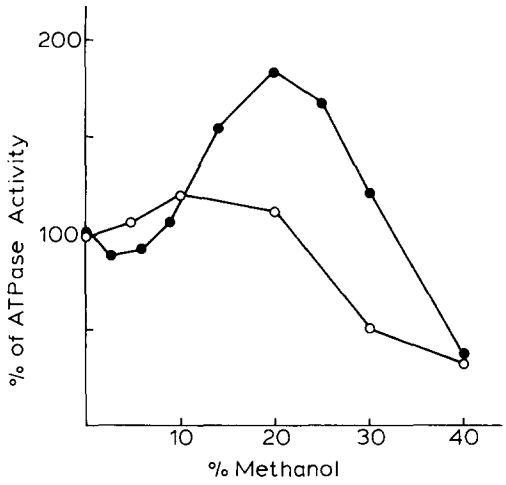

Fig. 5. Effect of different concentrations of methanol on the ATPase activity of soluble and particulate F_1-ATPase. The experimental conditions were the same as in Fig. 4, except that ATP was the substrate and the mixture contained the indicated concentrations of methanol. To the mixtures either MgATP-Sephadex particles (180 μg) or soluble F_1-ATPase (10 μg) were added. •, soluble; ○, particulate.

Ado*PP*[NH]*P* and ADP to inhibit ITP hydrolysis, which further confirms the suggestion that ADP and Ado*PP*[NH]*P* act on a common site(s).

It is known that in some respects the properties of soluble F_1-ATPase differ from those of the particulate system [11,39,40]. Therefore, the sensitivity of the membrane-bound enzyme to methanol was studied.

In agreement with other authors [34,35], it was found that methanol induces a marked enhancement of ATPase activity in soluble F_1-ATPase (Fig. 5). On the other hand, in the particulate system, methanol did not induce significant activation. The small stimulating effect of methanol on particulate F_1-ATPase shown in Fig. 5 is not reproducible, i.e., we have assayed the effect of methanol in MgATP, EDTA, and in A-S submitochondrial particles, and only with a few preparations have we observed a small stimulation. In six experiments which were carried out with MgATP submitochondrial particles, we observed stimulation ranging from 0 to 12% with an average of 5.0%. Thus, it appears that the attachment of F_1-ATPase to the membrane changes the characteristics of the enzyme with respect to methanol.

As the data of Figs. 2–4 and those of another author indicate that in the soluble enzyme methanol affects a site(s) which binds ADP [34] and Ado*PP*[NH]*P* as shown in this work, it was decided to study whether in the particulate system this methanol-sensitive inhibitory site is also present, even though no stimulation of ATP hydrolysis was observed.

Fig. 6A shows that the inhibiting action of Ado*PP*[NH]*P* on ATP hydrolysis by soluble and particulate F_1-ATPase is drastically diminished by methanol at concentrations which induce stimulation of ATPase activity (cf. data of Figs. 5 and 6). Therefore, it would appear that in the soluble and in the particulate system the inhibitory binding site for Ado*PP*[NH]*P* is still accessible to methanol. In addition, it was observed that methanol also affects the inhibitory binding site for added ADP in both the soluble and particulate enzyme (Fig. 6B).

A more detailed analysis of the effect of methanol on the inhibition of ITP hydrolysis by ADP in particulate ATPase is shown in Fig. 7, 50% inhibition being attained with approx. 50 μM ADP. Methanol at 20% inhibits ITP hydrolysis by about 50% (Fig. 7), and starting at this level of activity, ADP, up to a con-

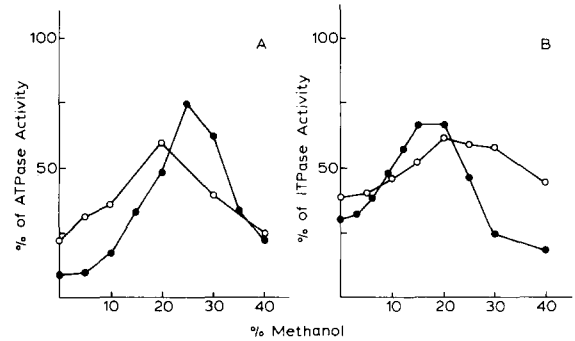

Fig. 6. Effect of different concentrations of methanol on the inhibiting effect of Ado*PP*[NH]*P* and ADP on ATP (A) and ITP (B) hydrolysis by soluble and particulate F_1-ATPase. The experimental conditions were the same as in Fig. 4, except that in A the substrate was 1 mM ATP and the inhibitor was 50 μM Ado*PP*[NH]*P*, while in B the substrate was 1 mM ITP and the inhibitor 100 μM ADP for the particulate and 50 μM ADP for the soluble ATPase. The mixtures to which either particulate or soluble F_1-ATPase were added (as indicated) also contained the indicated concentrations of methanol. •, soluble; ○, particulate.

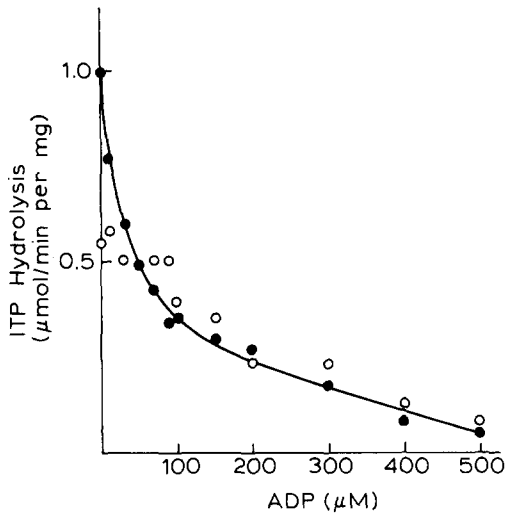

Fig. 7. Effect of different concentrations of ADP on ITP hydrolysis by particulate F_1-ATPase. The incubation conditions were as in Fig. 4, except that particulate F_1-ATPase was employed. The mixture contained 1 mM ITP and the indicated concentrations of ADP, and 20% methanol as shown. •, control; ○, methanol.

centration of 90 μM, fails to inhibit the activity. At higher ADP concentrations, the activities in the absence and presence of methanol are about the same. These findings suggest the existence of at least two ADP inhibitory binding sites. Indeed, a Dixon plot of the data of the effect of ADP in the absence of methanol showed two clearly distinguishable slopes (data not shown).

It should be noted in Fig. 7 that methanol diminishes the ability of the particulate enzyme to hydrolyze ITP. In the latter respect, both the soluble (see Ref. 7, and legend to Fig. 4) and the particulate enzymes show similar behavior in methanol.

Therefore, in the sense that methanol inhibits ITP hydrolysis and induces resistance to the inhibiting action of added ADP, the particulate and the soluble enzyme behave almost identically. It is with respect to the effect of methanol on ATPase activity in which the particulate and soluble enzyme behave markedly different.

Discussion

Several lines of evidence suggest that ADP and Ado*PP*[NH]*P* have a common site(s) in their inhibition of the hydrolytic activity of F_1-ATPase [25,34], and indeed some of the results of this work substantiate the idea: (1) ADP prevents the inhibiting action of low concentrations of Ado*PP*[NH]*P* on ATP hydrolysis in submitochondrial particles; (2) the ATPase activity of particulate F_1-ATPase previously loaded with Ado*PP*[NH]*P* is increased upon exposure of particles to ADP; (3) methanol diminishes to a very similar extent the inhibiting action of added Ado*PP*[NH]*P* and ADP in both the soluble and the particulate system.

In addition, it has been found that the effects of methanol on ATPase activity are largely due to decreases in the affinity of the binding sites for either ADP or Ado*PP*[NH]*P*. In soluble F_1-ATPase, half-maximal inhibition of the hydrolytic activity by Ado*PP*[NH]*P* was attained at 9 μM in an aqueous system, while in 20% methanol this value was 42 μM; for ADP, 250 and 750 μM were required to induce 50% inhibition in water and methanol, respectively, using 1 mM ATP as substrate (data not shown). With ITP as substrate, methanol also diminished in a similar manner the ability of Ado*PP*[NH]*P* and ADP to inhibit hydrolysis (see Fig. 4). Therefore, it would seem that the stimulatory effect of methanol on ATPase activity of soluble F_1-ATPase (which has been observed by others [34,35]) is due to modification of the ADP (or Ado*PP*[NH]*P*) inhibitory site(s).

The latter site(s) seem to have different characteristics in soluble and particulate F_1-ATPase, since in submitochondrial particles no stimulating action of methanol was observed. This implies that in particles this site is absent, inaccessible to methanol, or non-inhibitory. In this respect, it was observed that although in particles methanol does not stimulate hydrolysis, it diminishes or overcomes the inhibiting action of added ADP and Ado*PP*[NH]*P*.

The paradoxical situation in which methanol in the particulate system diminishes or overcomes the inhibitory effect of ADP without stimulating ATP hydrolysis may be explained by assuming that there are at least two ADP binding inhibitory sites, and that one of these is transitorily occupied by ADP arising from ATP hydrolysis. In the soluble enzyme, both would be accessible to and modified by methanol, while in particles only one site would be accessible to or capable of being modified by methanol. The latter site should correspond to that which becomes appar-

ent with added ADP (Figs. 4, 6A and 7). The other site should correspond to that which binds ADP arising from ATP hydrolysis, and which transitorily binds a site which in the particulate system is inaccessible to methanol. In other words, in the soluble enzyme the modification by methanol of the site occupied by ADP arising from ATP hydrolysis results in stimulation of catalytic activity. As in the particulate system, the site is inaccessible to or not capable of being modified by methanol; no stimulation of hydrolysis is obtained.

The existence of the regulatory action of ADP arising from ATP hydrolysis are further evidenced by the observation that methanol stimulates the hydrolytic activity of F_1-ATPase even in the presence of an ATP-regenerating system (Fig. 2). Since under these conditions free ADP is maintained at extremely low levels, it would be difficult to explain the stimulation of activity by methanol through a process which involves free ADP. More likely, methanol modifies the action of ADP arising from ATP hydrolysis and transitorily occupying a site which limits the rate of catalysis. In the particulate system, the site of this nascent ADP would seem to be inaccessible to methanol. However, it should be stressed that in this scheme both the particulate and the soluble enzyme possess a site which accepts added ADP (and Ado*PP*-[NH]*P*) and which is also capable of being modified by methanol.

The presently described effects of methanol on ATPase activity fit well with the mechanisms of ATP synthesis and hydrolysis proposed by Koslov and Skulachev [37]. In this mechanism, which has recently been further elaborated [38–40], it is proposed that some adenine nucleotide-binding sites in the α-subunits transfer substrate adenine nucleotide to the catalytic site, which subsequently moves to a region of different polarity in the membrane.

In principle, the proposed mechanism [37] implies the existence of adenine nucleotide-binding sites which control the catalytic process, and which in the particulate system exist in regions of different accessibility to various chemicals. The striking difference in the effect of methanol on a regulatory inhibiting site of hydrolysis in soluble and particulate F_1-ATPase is consistent with this idea.

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