Mixed Anhydrides of Nucleotides and Mesitylenecarboxylic Acid as New Specific Inhibitors of Mitochondrial Adenosine Triphosphatase

By I. A. KOZLOV, M. V. SHALAMBERIDZE, I. YU. NOVIKOVA, N. I. SOKOLOVA
 and Z. A. SHABAROVA

*Department of Bioenergetics, A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry,
 and the Department of the Chemistry of Natural Compounds, Moscow State University,
 Moscow 117234, U.S.S.R.*

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Mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid inhibit soluble mitochondrial ATPase (adenosine triphosphatase), but do not inhibit ATPase of submitochondrial particles. Inhibition of soluble mitochondrial ATPase by the mixed anhydride of ϵ -ATP and mesitylenecarboxylic acid is followed by the covalent binding of one nucleotide residue to a molecule of the protein. It is suggested that this covalent binding occurs in the catalytic site of the mitochondrial ATPase. The mixed anhydride of ADP and mesitylenecarboxylic acid inhibits the ATPase activity of submitochondrial particles and has no effect on the activity of soluble mitochondrial ATPase. After separation of the submitochondrial particles from the mixed anhydride of ADP and mesitylenecarboxylic acid, their ATPase activity is restored to its original value (half-time of reactivation 3–4 min). Incubation of submitochondrial particles or soluble mitochondrial ATPase with the mixed anhydride of ADP and mesitylenecarboxylic acid results in AMP formation.

In our previous research the interaction of mitochondrial ATPase with several specific inhibitors was studied (Kozlov, 1975; Kozlov & Chernyak, 1976, 1978; Kozlov & Skulachev, 1977; Budker *et al.*, 1977; Imedidze *et al.*, 1978). The most significant result obtained in these studies is the establishment of the location of the active (catalytic) site in the depths of the hydrophobic phase of the mitochondrial membrane.

In the present paper the reaction of mitochondrial ATPase with new inhibitors, mixed anhydrides of

nucleotides and mesitylenecarboxylic acid, is studied. The results obtained show that inhibition of soluble mitochondrial ATPase by these mixed anhydrides is accompanied by the covalent binding of one nucleotide residue to a molecule of the protein. It has also been established that soluble mitochondrial ATPase (factor F_1) and membrane-bound ATPase (submitochondrial particles) have different sensitivity to the mixed anhydrides of nucleotides and mesitylenecarboxylic acid. Some of the preliminary results of this study have been published (Kozlov *et al.*, 1977).

Methods

Submitochondrial particles were obtained by the method of Hansen & Smith (1964), and factor F_1 was prepared by the method of Horstman & Racker (1970).

Abbreviations used: ATPase, adenosine triphosphatase; GTPase, guanosine triphosphatase; ADP-MC, AMP-MC, CDP-MC, GDP-MC, mixed anhydrides of ADP, AMP, CDP, GDP and mesitylenecarboxylic acid; ATP-MC, CTP-MC, GTP-MC, mixed anhydrides of ATP, CTP, GTP and mesitylenecarboxylic acid; ϵ -ATP-MC, mixed anhydride of 1, N^6 -ethenoadenosine triphosphate and mesitylenecarboxylic acid.

Mixed anhydride of ATP and mesitylenecarboxylic acid

The initial rates of the ATPase reaction were measured by the 'pH-metric' method of Chance & Nishimura (1967). To measure the ATPase or GTPase activity of factor F_1 , factor F_1 (1–12 $\mu\text{g/ml}$) was added to 6 ml of the reaction mixture containing 3 mM-Tris/HCl, pH 8.3, 2 mM-MgSO₄ and 2 mM-ATP or 2 mM-GTP. The ATPase activity of the submitochondrial particles was measured similarly. Besides the above listed components, the reaction mixture contained 0.25 M-sucrose and 20 μM -2,4-dinitrophenol. The concentration of the submitochondrial particles was 20–100 $\mu\text{g/ml}$. The factor F_1 protein was determined by the method of Lowry *et al.* (1951). The submitochondrial-particle protein was determined by the method of Cleland & Slater (1953). The kinetic measurements were made at 30°C, and details of the methods are given by Kozlov & Kononenko (1975).

The method of synthesizing the mixed anhydrides of nucleotides and mesitylenecarboxylic acid and some properties of these compounds are given by Nosova *et al.* (1975) and Shumyantseva *et al.* (1976). ϵ -ATP-MC was prepared from ATP-MC by the method of Barrio *et al.* (1972). The purity of ϵ -ATP-MC was established by paper chromatography.

The fluorescence of factor F_1 treated with ϵ -ATP-MC was measured at 415 nm by using an Aminco-Bowman spectrofluorimeter. The wavelength used

for fluorescence excitation was 320 nm. The fluorescence intensity of ϵ -ATP-MC was proportional to its concentration at concentrations below 5 μM . Fluorescence measurements were carried out in a solution containing 0.1% sodium dodecyl sulphate and 10 mM-Tris/HCl, pH 7.5. The contribution of the background scattering of the protein solution to the fluorescence intensity did not exceed 10%. It was shown in a control experiment that removal of the fluorescent label from the protein (0.5 M-HClO₄; 48 h; 25°C) does not lead to changes in the fluorescence parameters of the label. This means that data on the intensity of the fluorescence of factor F_1 treated with ϵ -ATP-MC can be used in determining the stoichiometry of the reaction of ATPase with ϵ -ATP-MC.

Results and Discussion

In the first series of experiments the effects of mixed anhydrides of nucleotides and mesitylenecarboxylic acid on isolated factor F_1 were studied. Mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid (ϵ -ATP-MC, ATP-MC, CTP-MC, GTP-MC) were effective inhibitors of factor F_1 in water solution (Fig. 1). The inhibition of factor F_1 by 1 mM-ATP-MC (Fig. 1a, curve 1) complies with the kinetics of the pseudo-monomolecular reaction ($t_{1/2} \approx 4$ min).

Fig. 1. Inhibition of factor F_1 by mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid (a) and separation of factor F_1 modified by ϵ -ATP-MC from free ϵ -ATP-MC (b)

Curves 1, 2 and 3 show ATPase activity of factor F_1 plotted against the time of preincubation of factor F_1 with mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid. Curve 4 shows binding of the fluorescent label to factor F_1 during the reaction of the enzyme with ϵ -ATP-MC. Curve 1: 1 mM-ATP-MC (○), 1 mM- ϵ -ATP-MC (▲); curve 2: 2 mM-GTP-MC (△), 2 mM-CTP-MC (●), 2 mM-ATP-MC (■); curve 3: 1 mM-ATP-MC, in the presence of 10 mM-ATP; curve 4: 2 mM- ϵ -ATP-MC. ATPase activity of unmodified factor F_1 corresponds to 30–50 $\mu\text{mol/min}$ per mg of protein. Factor F_1 (1 mg/ml) was preincubated with 1–2 mM-mixed anhydride of nucleoside triphosphate and mesitylenecarboxylic acid in 10 mM-Tris/HCl/2 mM-EDTA, at pH 7.5. Small samples were taken from the reaction mixture to determine the ATPase activity (curves 1, 2 and 3). In determining the stoichiometry of the reaction of factor F_1 with ϵ -ATP-MC (curve 4), the reaction mixture was fractionated on Sephadex G-50. (b) The reaction mixture containing 2 mg of factor F_1 was fractionated on a column (15 cm $\times$ 1.5 cm) of Sephadex G-50 (fine grade). The elution rate was 0.8 ml/min. —, A_{254} ; ○, intensity of the fluorescence of the fractions. Peak I, modified factor F_1 ; peak II, free fluorescent label.

Using ϵ -ATP-MC as an inhibitor of factor F_1 , we attempted to determine the number of nucleotide residues that are bound by a molecule of the enzyme during inhibition of the ATPase reaction. The data obtained (Fig. 1) show that inhibition of factor F_1 by ϵ -ATP-MC is followed by binding of one molecule of the inhibitor to a molecule of the enzyme. Incubation of factor F_1 with ϵ -ATP-MC for 1 h in the presence of 10 mM-ATP results in the binding of 0.15 mol of the label to 1 mol of the enzyme (results not shown). Treatment of modified factor F_1 with ice-cold 5% (v/v) trichloroacetic acid does not lead to the removal of the fluorescent label from the enzyme. The fluorescent label also remains bound to factor F_1 after repeated dialysis against 8 M-urea or 0.1% sodium dodecyl sulphate. Thus, from the data obtained, it follows that the covalent binding of one nucleotide residue to a molecule of the enzyme occurs as a result of the reaction of factor F_1 with ϵ -ATP-MC.

As shown by Nosova *et al.* (1975), mixed anhydrides of nucleotides and mesitylenecarboxylic acid can serve as phosphorylating agents in reactions with various compounds containing amino, thio and other nucleophilic groups. These reactions, which are accompanied by the transfer of the nucleotide residue to the functional groups of amino acids, readily proceed in anhydrous organic solvents and are used to obtain various nucleotide derivatives. In aqueous media at neutral pH, mixed anhydrides of nucleotides and mesitylenecarboxylic acid are stable, their reactivity is weak and the reactions with nucleophilic reagents proceed slowly.

The rapid reaction of factor F_1 with mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid is apparently caused by the catalytic effect of the enzyme itself on this reaction. It seems probable that mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid modify the active (catalytic) site of factor F_1 . Thus, as noted above, ATP, which can be bound in the absence of ions of bivalent metals in the catalytic site of factor F_1 , forming a non-productive enzyme-substrate complex (Kozlov *et al.*, 1975), protects factor F_1 from modification by ϵ -ATP-MC. It also protects ATPase from the inhibitory effect of ATP-MC (Fig. 1a, curve 3). The similar effectiveness of ATP-MC, ϵ -ATP-MC, CTP-MC and GTP-MC as inhibitors of factor F_1 (Fig. 1) is apparently determined by the fact that the catalytic site of ATPase does not exhibit high specificity towards the type of heterocyclic base in the nucleoside triphosphates (Kozlov *et al.*, 1978). It is noteworthy that mixed anhydrides of nucleoside diphosphates and mesitylenecarboxylic acid (ADP-MC, GDP-MC and CDP-MC), as well as AMP-MC, proved ineffective as inhibitors of soluble mitochondrial ATPase. This result is understandable, since it has been established

that the presence of at least three negative charges in a molecule of the nucleotide is necessary for it to bind in the active site of factor F_1 (Kozlov & Skulachev, 1977; Kozlov *et al.*, 1978).

It appeared that binding one nucleotide residue with factor F_1 caused the inhibition not only of the ATPase, but also of the GTPase activity of the enzyme (Fig. 2). The inhibition of the ATPase and GTPase activities of factor F_1 by ATP-MC takes place at a similar rate (Fig. 2). Philo & Selwyn (1974) and Schuster *et al.* (1975, 1976) believe that the hydrolysis of ATP and GTP occurs in the different nucleotide-binding sites of factor F_1 . However, the results obtained by us (the inhibition of the ATPase and GTPase activities of factor F_1 as the result of binding by the enzyme of one nucleotide residue) are in better agreement with the suggestion that the hydrolysis of ATP and GTP occurs in one and the same site of factor F_1 .

Next, the effects of mixed anhydrides of nucleotides and mesitylenecarboxylic acid on the ATPase activity of submitochondrial particles was studied. The most effective inhibitor in those studies was ADP-MC. Incubation of submitochondrial particles for 10 min with ADP-MC decreased the ATPase activity to 30% of the initial rate (Fig. 3, curve 1). The reaction of submitochondrial particles with ADP-MC was not accompanied by the removal of factor F_1 from the mitochondrial membrane, since throughout the reaction (2 h) the sensitivity of mitochondrial ATPase to oligomycin (1 μ g/ml), which causes 95% inhibition of ATPase, did not change.

As noted above, ADP-MC does not inhibit the ATPase activity of the isolated factor F_1 in water solution. In this connection, it is supposed that the

Fig. 2. Inhibition of ATPase (□) and GTPase (●) activities of factor F_1 by ATP-MC (2 mM)

GTPase activity of unmodified factor F_1 is 10 μ mol/min per mg of protein, and ATPase activity 30 μ mol/min per mg of protein. For conditions see Fig. 1.

Fig. 3. Inhibition of ATPase of submitochondrial particles by mixed anhydrides of nucleotides and mesitylenecarboxylic acid

Initial activity of submitochondrial particles is 1 $\mu\text{mol/min}$ per mg of protein. Curve 1: ADP-MC; curve 2: ϵ -ADP-MC; curve 3: AMP-MC; curve 4: ATP-MC ($\square$), GDP-MC ($\blacktriangle$), CDP-MC ($\circ$). To the particles (5mg of protein) suspended in 0.2ml of 50mM-morpholinepropanesulphonate buffer, pH 7.5, containing 0.25M-sucrose, was added 3mM-inhibitor; at the given intervals samples of this reaction mixture were added to the medium to determine the ATPase activity. All these operations, from the moment of taking the sample to complete determination of activity, lasted 1 min.

reason for the inhibition of ATPase of submitochondrial particles by ADP-MC is the modification of the non-catalytic ATP-binding site of factor F_1 (Kozlov, 1975; Kozlov & Skulachev, 1977). This conclusion is also supported by the experiments in which inhibition of the submitochondrial-particle ATPase activity by mixed anhydrides of mesitylenecarboxylic acid and nucleotides with various types of nucleotide residue is studied.

It is known that the non-catalytic site of factor F_1 exhibits a high specificity in binding nucleotides [for review see Kozlov (1975) and Kozlov & Skulachev (1977)]. Thus ADP or ϵ -ADP binding in the non-catalytic site of mitochondrial ATPase is characterized by $K_{diss.} \approx 0.1\text{--}1\mu\text{M}$, whereas other nucleotides (ATP, AMP, CDP and so on) bind to the enzyme with a $K_{diss.} \geq 1\text{mM}$ (Pedersen *et al.*, 1974; Pedersen, 1975).

The dependence of the effectiveness of inhibition of submitochondrial-particle ATPase on the nature of the nucleotide residue in mixed anhydrides is shown in Fig. 3. The results show that only ϵ -ADP-MC is as effective an inhibitor as ADP-MC. The inhibiting effect of AMP-MC is somewhat weaker. Preincubation of submitochondrial particles with ATP-MC, GDP-MC and CDP-MC did not lead to a noticeable change in the ATPase activity.

Thus, with regard to mixed anhydrides of nucleo-

tides and mesitylenecarboxylic acid (Fig. 3), the enzyme exhibits a specificity of the same nature as for adsorption of nucleotides to the non-catalytic site.

Unlike ATP-MC, ADP-MC does not form a stable nucleotide derivative of factor F_1 . All attempts to obtain factor F_1 with a covalently bound nucleotide residue after treatment of the enzyme with ϵ -ADP-MC have proved unsuccessful. This result may be explained by the low stability of the bond between the nucleotide residue and the corresponding nucleophilic group in the non-catalytic site of the enzyme. Thus some of the model nucleotide peptides synthesized in our laboratory showed high reactivity and can be hydrolysed in comparatively mild conditions (Shabarova, 1970). If an unstable nucleotide derivative forms during the reaction of factor F_1 with ADP-MC, then the separation of submitochondrial particles from the solution containing ADP-MC should lead to the restoration of ATPase activity. In fact, the submitochondrial particles treated with ADP-MC for 15 min and possessing 20% of the initial ATPase activity were re-activated after removal of the inhibitor. Fig. 4 shows that the ATPase activity of the modified particles increases with time, after the ADP-MC concentration has been decreased by a 1000-fold dilution of the reaction mixture. In accordance with the data of Fig. 4, the half-time of the re-activation of ATPase is 3–4 min. In the control experiments it was shown, firstly, that incubation of submitochondrial particles with 1000-fold diluted ($2\mu\text{M}$) ADP-MC does not lead to a change in their ATPase activity, and, secondly, that incubation of non-modified diluted submitochondrial

Fig. 4. Re-activation of submitochondrial particles treated with ADP-MC, after removal of the inhibitor

A small sample of the particles preincubated with 3mM-ADP-MC for 20 min was placed in a medium containing all the components necessary for the measurement of ATPase activity, except for the substrate MgATP. This was added to the system after 1–13 min, and the ATPase reaction rate was measured for 1 min.

particles leads only to an insignificant growth in ATPase activity (up to 20%).

Thus the data obtained may easily be explained by proposing that the reaction between ADP-MC and factor F_1 results in the formation of a comparatively unstable nucleotide derivative of the enzyme which is hydrolysed in a few minutes. If this is the case, submitochondrial particles and factor F_1 should catalyse the hydrolysis of ADP-MC.

In fact, as the analysis of the reaction mixture showed, incubation of submitochondrial particles (or factor F_1) with ADP-MC is followed by the slow hydrolysis of ADP-MC resulting in the formation of AMP. The rate of this process for submitochondrial particles is 0.2–2.0 nmol/min per mg of protein, and for isolated factor F_1 (in the presence of $MgSO_4$) 0.5–5.0 nmol/min per mg of protein. The average time taken for one single action of the ATPase during hydrolysis of ADP-MC coincides in order of magnitude with the time required for the ATPase to be re-activated after separation of submitochondrial particles and solution containing ADP-MC (Fig. 4). This result may be regarded as indicating that both the hydrolysis of ADP-MC and the re-activation of ATPase is the result of the formation and subsequent decomposition of the unstable nucleotide derivative of ATPase. However, it should be pointed out that some contamination with other enzymes in the preparations of submitochondrial particles and factor F_1 may also be responsible for the hydrolysis of ADP-MC. So the preparation of factor F_1 obtained by us had low, but measurable, adenylate kinase activity (the rate of the adenylate kinase reaction is approx. one ten-thousandth of the ATPase reaction). Moreover, it appeared that 3 mM-ADP-MC inhibited this adenylate kinase activity of factor F_1 , as effectively as it did for submitochondrial particles. Thus we cannot yet determine which of the enzymes carries out the hydrolysis of ADP-MC.

The results obtained in this work indicate that mixed anhydrides of nucleotides and mesitylenecarboxylic acid are powerful inhibitors of mitochondrial ATPase and can be used as tools for studying the mechanism of the ATPase reaction. These inhibitors may be divided into two groups: the first includes ADP-MC and ϵ -ADP-MC, which carry out reversible inhibition of ATPase of submitochondrial particles and have no effect on the ATPase activity of isolated factor F_1 ; the second group includes mixed anhydrides of nucleoside triphosphates and mesitylenecarboxylic acid. These compounds inhibit the ATPase and GTPase activities of factor F_1 in water solution and have no effect on the ATPase activity of submitochondrial particles.

In conclusion, we point out two possible ways of using mixed anhydrides of nucleotides and mesity-

lenecarboxylic acid in studying the structure of ATPase and the ATPase reaction mechanism: (1) the incorporation of fluorescent, spectral or paramagnetic labels into the nucleotide-binding sites of the enzymes; (2) the estimation of the accessibility of the nucleotide-binding sites of factor F_1 to inhibitors in various functional states of ATPase.

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