THE INTERACTION OF MITOCHONDRIAL ATPase WITH AN ALKYLATING ATP-ANALOG

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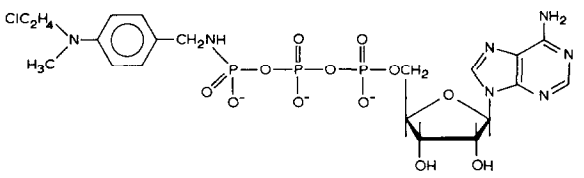
1. Introduction

Coupling factor F_1 (soluble mitochondrial ATPase) plays a key role in the mechanism of oxidative phosphorylation (see reviews [1,2]). Factor F_1 has mol. wt approx. 370 000 and contains five types of subunits (see reviews [1–4]). There is no agreement in the literature about the subunit stoichiometry and molecular weight of the subunits. According to Knowles and Penefsky [5], whose point of view seems to be the most consistent, the molecular weights of the subunits of beef heart mitochondrial factor F_1 are 54 000 (α), 51 000 (β), 33 000 (γ), 16 000 (δ), and 11 000 (ϵ), and the subunit composition of a molecule of the enzyme corresponds to the formula $\alpha_3\beta_3\gamma\delta\epsilon$. Knowles and Penefsky [5] have concluded that the ϵ-subunit is a dimer of the protein inhibitor of mitochondrial ATPase [6]. The results previously obtained in our group indicate that the active site of mitochondrial ATPase is located on the α- and/or β-subunits [7,8].

At first glance it would seem logical that further research on the role of the α- and β-subunits of factor F_1 in the mechanism of the ATPase reaction should be devoted to the study of the structure and catalytic properties of isolated subunits. However, as the research of Rosing et al. [9] shows, even the 'mild' dissociation of factor F_1 into subunits causes the subunits to lose their ability to bind nucleotides. Thus, research connected with the fractionation of factor F_1 into subunits is not very promising in studying the role of individual subunits in the mechanism of ATPase reaction. In this work we have used another approach. Factor F_1 was treated with ATP γ-4-(*N*-2-chloroethyl-*N*-methylamino)benzylamidate, a specific inhibitor that modifies the active site of the enzyme.



The subsequent sodium dodecyl sulfate induced dissocation of factor F_1 into subunits and the separation of the subunits by electrophoresis in polyacrylamide gel showed that this ATP derivative binds to the β-subunit(s) of factor F_1.

2. Methods

The soluble mitochondrial ATPase was obtained according to Horstman and Racker [10]. The ATPase reaction rate was measured by the pH-metric method [11,12]. For measurement of the ATPase reaction rate, the following incubation medium was used: 3 mM Tris–HCl, pH 8.3, 2 mM ATP, 2 mM $MgSO_4$ and factor F_1 from 10–40 μg in overall vol. 8 ml. The reaction was started by adding factor F_1 to the reaction mixture.

The protein was determined according to Lowry et al. [13]. Electrophoresis of factor F_1 in 10% polyacrylamide gel in the presence of dodecyl sulfate was carried out by the method of Weber and Osborn [14]. The gels were stained with amido black and scanned at 630 nm.

ATP γ-4-(*N*-2-chloroethyl-*N*-methylamino)benzyl-

amidate [15] was prepared as described in [16]. The product used for determining the stoichiometry of the reaction of factor F_1 with this derivative was ^{14}C-labelled (1.1 mCi/mmol). The ^{32}P-derivative was used in experiments to determine the location of the active site of mitochondrial ATPase. The radioactivity of the preparations possessing a ^{32}P-label was determined by measuring the Cerenkov radiation in water.

Alkylation of the enzyme by the ATP analog was studied in a buffer containing 10 mM Tris–HCl, pH 7.8 and 0.25 M sucrose. At certain time intervals aliquots were taken and added to the incubation medium to determine the ATPase activity.

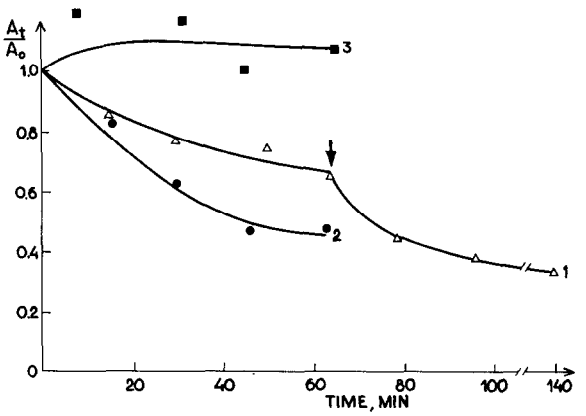


Fig.1. Inhibition of soluble mitochondrial ATPase by the alkylating ATP analog. A_t the activity of mitochondrial ATPase after t min of incubation with the inhibitor at 25°C. At the outset, the reaction mixture contained 1 mg/ml of factor F_1, 10 mM Tris–HCl, pH 7.8, and the ATP analog. The concentration of the reagent was 1 mM (1), 2.3 mM (2) and 0.90 mM (3). In the last case the mixture was supplemented with 10 mM ATP. The arrow shows the second addition of freshly prepared reagent (1 mM).

2. Results and discussion

At present several modifying reagents are known which specifically inhibit soluble mitochondrial ATPase, among them 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol [17–19], *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide [2,20] and mixed anhydrides of nucleosidetriphosphates and mesitylencarboxylic acid [21]. All the above-mentioned compounds suppress the ATPase activity of isolated factor F_1, and the inhibition is accompanied by the modification of one of the amino acid residues in the molecule of the enzyme. *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide and mixed anhydrides of nucleosidetriphosphates and mesitylenecarboxylic acid probably interact with the amino acid located in the active site of the mitochondrial ATPase [2,20,21]. On the other hand, according to Ferguson et al. [19], 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol modifies the tyrosine residue located outside the active site of mitochondrial ATPase.

In this work we have studied the inhibition of soluble mitochondrial ATPase induced by ATP γ-4-(*N*-2-chloroethyl-*N*-methyl-amino)benzylamidate, the alkylating analog of the ATPase reaction substrate. Figures 1 and 2 show the dependence of factor F_1 activity on the time of preincubation with this compound. It can be seen that this ATP analog is an effective inhibitor of the enzyme. The inhibition develops with time, and addition of a fresh portion of the inhibitor results in an additional decrease in the ATPase reaction rate. The inhibiting effect is increased if the preincubation of the enzyme with the ATP analog is carried out in the presence of $MgSO_4$ (figs 1 and 2). This result is not unexpected since the Mg complexes of nucleotides are more firmly bound to the active site of mitochondrial ATPase than free nucleotides [22].

The suggestion that the inhibiting effect of the ATP

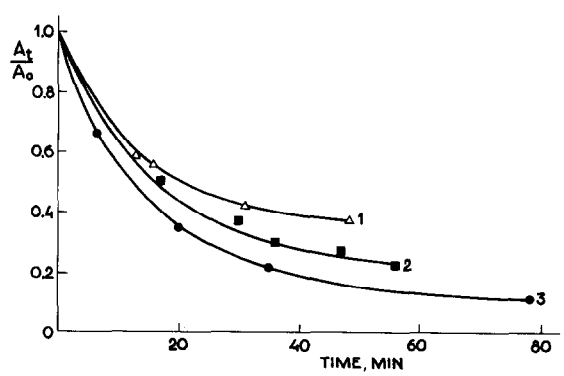


Fig.2. Inhibition of soluble mitochondrial ATPase by the alkylating analog of ATP in the presence of $MgSO_4$. The reaction mixture contained 1 mg/ml of factor F_1, 10 mM Tris–HCl, pH 7.8, the [^{32}P]ATP analog and $MgSO_4$. (1) 0.5 mM reagent, 0.5 mM $MgSO_4$, 25°C; (2) 1 mM reagent, 1 mM $MgSO_4$, 37°C; (3) 3.3 mM reagent, 3.3 mM $MgSO_4$ (37°C).

analog is connected with a modification of the active site of ATPase is confirmed by the results of the experiments in which the reaction between factor F_1 and the inhibitor was carried out in the presence of ATP. The ATP, acting as a competitive inhibitor of the ATPase reaction in the absence of Mg^{2+} [22], protects factor F_1 from alkylation, as is exhibited by curve 3 (fig.1). The results of experiments with 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamine give an additional indication that the alkylating derivative of ATP reacts with the active site of factor F_1. 4-(*N*-2-chloroethyl-*N*-methylamino)benzylamine in a concentration of 2 mM does not inhibit factor F_1 (65 min at 37°C). In its reactivity, this compound differs very little from the ATP analog, the rate constants for the reaction of chloride liberation under comparable conditions being $1 \cdot 10^{-4}$ s^{-1} and $4 \cdot 10^{-4}$ s^{-1}, respectively [15,23]. However, the alkylating amine does not contain any substrate-like moieties, and it is probably for this reason that it does not bind to the active site of ATPase.

At the next stage of investigation we attempted to determine the stoichiometry of the reaction of factor F_1 with the ATP alkylating analog. For this purpose, factor F_1 was treated with 0.5 mM [^{14}C]ATP analog in the presence of 2 mM $MgSO_4$, 10 mM Tris–HCl, at 25°C and pH 7.8. β-Mercaptoethanol up to a concentration of 1% was added to this mixture after 45 min. The protein containing a radioactive label was separated from the free label on a Sephadex G-25 column. Analysis of the protein fraction showed that the radioactive label/factor F_1 molar ratio is 1.4 ± 0.2. If $MgSO_4$ is excluded from the reaction mixture, and the reaction of factor F_1 with the ^{14}C-labelled inhibitor is carried out in the presence of 10 mM ATP, the radioactive label/factor F_1 molar ratio drops from 1.4 to 0.7. Note that 10 mM ATP protects factor F_1 completely from inactivation by the ATP analog (see fig.1). Thus, it seems that the reaction of factor F_1 with the ATP derivative is accompanied by the incorporation of two molecules of the inhibitor into each molecule of the enzyme. One of the molecules of the ATP analog blocks the active site of the enzyme, and the other apparently reacts with amino acid residue(s) outside the active center. The side reaction does not, apparently, cause any changes in the ATPase activity of factor F_1, nor is it prevented by ATP added to alkylation medium.

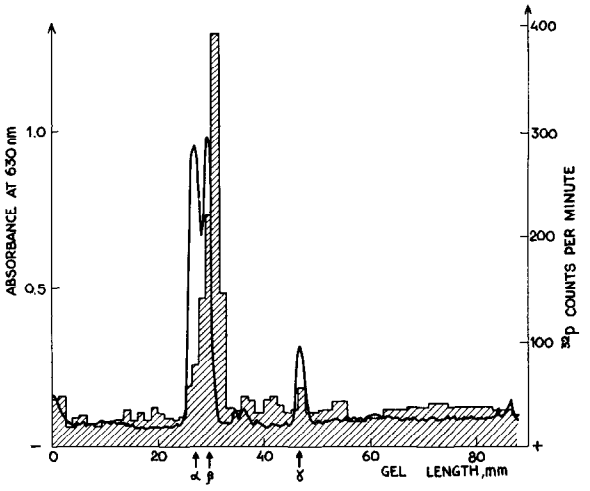


Fig.3. Electrophoresis of factor F_1 modified by ^{32}P-labelled reagent in polyacrylamide gel in the presence of SDS. Factor F_1 treated with 1 mM of the [^{32}P]ATP alkylating analog in 10 mM Tris–HCl, pH 7.8, at 37°C for 45 min was supplemented with mercaptoethanol to a concn. of 1%. The mixture was dialysed against 10 mM Tris–HCl and then against distilled water. The enzyme separated from the free label was treated with 1% sodium dodecylsulfate for 2 h at 37°C. 80 μg of protein were applied on each tube. Electrophoresis was carried out in ten tubes simultaneously. Five samples were stained with amido black, and the remaining 5 gels were sliced into 1 mm thick slices; the slices were placed in separate vials and treated with 0.5 ml 0.5 N HCl for 12 h at 35°C. The acid was neutralised with 2 N NaOH and the volume was brought up to 10 ml with distilled water. The radioactivity of each vial was counted for 4 min. In parallel experiments with different samples of gel, the position of the radioactivity peak varies within 0.5 mm. The solid line represents the scanning of gel at 630 nm. The shaded rectangles show the radioactivity of the gel slices.

In the last series of studies it was shown that the alkylating analog of ATP reacts preferentially with the β-subunit(s) of the enzyme (fig.3). This result indicates that the β-subunits take part in forming the active site of mitochondrial ATPase.

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