The inhibition of mitochondrial F_1-ATPase by 1,5-difluoro-2,4-dinitrobenzene

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1,5-Difluoro-2,4-dinitrobenzene completely inhibits F_1-ATPase when it is used at micromolar concentrations and the inhibitor/enzyme molar ratio is equal to 3. The inhibition can be reversed by dithiothreitol treatment. 7-Chloro-4-nitrobenzofurazan treatment of F_1-ATPase does not prevent the reaction of the enzyme with 1,5-difluoro-2,4-dinitrobenzene. The 1,5-difluoro-2,4-dinitrobenzene-induced inhibition is thought to be a result of the modification of a tyrosine residue with pK 9.1.

Mitochondrial F_1-ATPase *Tyrosine modification*

1. INTRODUCTION

Mitochondrial F_1-ATPase has a molecular mass of approx. 380 kDa and a complex subunit structure (review [1]). But surprisingly enough, of all the numerous amino acid residues of this enzyme there are only a few that react with low concentrations of the reagents modifying the protein functional groups. It is precisely these amino acid residues that take part in the ATPase activity of F_1-ATPase. Thus, *N*-cyclohexyl-*N'*-β-(4-methylmorpholine)ethylcarbodiimide modifies one carboxyl group in F_1-ATPase, and this causes the complete inhibition of its ATPase activity [2]. *N,N'*-Dicyclohexylcarbodiimide behaves in a similar manner, although the other carboxyl group in F_1-ATPase probably serves as the target of the attack in this case [3]. 7-Chloro-4-nitrobenzofurazan reacts with one tyrosine residue in F_1-ATPase. This reaction is also accompanied by the inactivation of F_1-ATPase [4]. Another tyrosine residue controlling the ATPase activity was modified with the ATP analogue in the work of Esch and Allison [5]. Inhibition of F_1-ATPase also occurs as a result of the modification of arginine residues with phenylglyoxal [6,7], and lysine residues with pyridoxal phosphate [8,9] and with 2,4-dinitrofluorobenzene [10,11].

Here, we have studied the reaction of F_1-ATPase with 1,5-difluoro-2,4-dinitrobenzene. This reagent, which is briefly mentioned by Andrews and Allison [11] as an inhibitor of mitochondrial ATPase, suppresses F_1-ATPase activity at micromolar concentrations. Complete inhibition of the F_1-ATPase is achieved when the molar ratio 1,5-difluoro-2,4-dinitrobenzene/F_1-ATPase is equal to 3.

2. MATERIALS AND METHODS

1,5-Difluoro-2,4-dinitrobenzene and 7-chloro-4-nitrobenzofurazan from Fluka, dithiothreitol, Tris, Mops, and SDS from Sigma and ATP from Reanal were used.

The F_1-ATPase was prepared from beef heart mitochondria according to Knowles and Penefsky [12]. Aliquots of the enzyme were desalinated, using Sephadex G-50 (fine) columns in 10 mM Mops-NaOH buffer, pH 7.5, containing 1 mM EDTA and 0.25 M sucrose [13]. Desalinated

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F_1-ATPase was stored in liquid nitrogen. Prior to the experiment, the enzyme preparations were thawed out on a water bath at 37°C.

The ATPase activity was measured in 1 ml of a medium containing 25 mM Tris-HCl, 2 mM $MgSO_4$, 2 mM ATP, pH 8.4. After 3 min, the reaction was stopped by the addition of 0.5 ml of 3.0% SDS and 10 mM EDTA. Inorganic phosphate concentrations were measured according to [14]. Standard preparations of the enzyme have an ATPase activity of 70–80 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

The protein concentration was measured according to Lowry et al. [15].

All the experiments to modify F_1-ATPase were carried out at 25°C in the dark.

3. RESULTS AND DISCUSSION

Fig.1 shows the time course of the inhibition of F_1-ATPase activity by 1,5-difluoro-2,4-dinitrobenzene. It follows from the results obtained that the micromolar concentrations of 1,5-difluoro-2,4-dinitrobenzene readily slow down the ATPase activity, and complete inhibition is achieved when the inhibitor/ATPase ratio is equal to 3.

Low concentrations of the inhibitor, the short duration of the reaction and also the fact that the inhibition is completed at a small molar excess of 1,5-difluoro-2,4-dinitrobenzene all reveal that 1,5-difluoro-2,4-dinitrobenzene is one of the most, if not the most, powerful of the known inhibitors of F_1-ATPase.

Fig.2 shows the kinetics of the inhibition of F_1-ATPase at a great molar excess of 1,5-difluoro-2,4-dinitrobenzene. It follows from the results obtained that the inhibition occurs as a pseudomonomolecular reaction, i.e. (i) 1,5-difluoro-2,4-dinitrobenzene reacts with F_1-ATPase without the step of specific non-covalent binding to the enzyme and (ii) the inhibition is a result of the reaction of 1,5-difluoro-2,4-dinitrobenzene with one amino acid residue of F_1-ATPase.

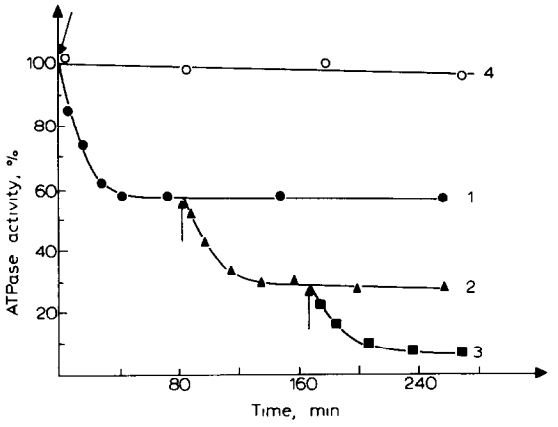

Fig.1. The inhibition of F_1-ATPase by the consecutive equimolar addition of 1,5-difluoro-2,4-dinitrobenzene. F_1-ATPase (1.9 mg/ml, 5.5 μM) was dissolved in a solution containing 80 mM triethanolamine, pH 8.5, 1 mM EDTA and 0.25 M sucrose. The arrows indicate when the methanol solution of 1,5-difluoro-2,4-dinitrobenzene was added to this mixture. In every case, inhibitor additions equimolar to F_1-ATPase were made. The molar ratio inhibitor/F_1-ATPase was equal to 1 (curve 1), 2 (curve 2), and 3 (curve 3). Aliquots of the reaction mixture were taken after the time intervals indicated to measure the ATPase activity. In the control experiments (section of the curves which are parallel to the abscissa), only 1 (curve 1) or 2 (curve 2) additions of 1,5-difluoro-2,4-dinitrobenzene were made. Curve 4: F_1-ATPase was incubated in the same mixture, but without the inhibitor.

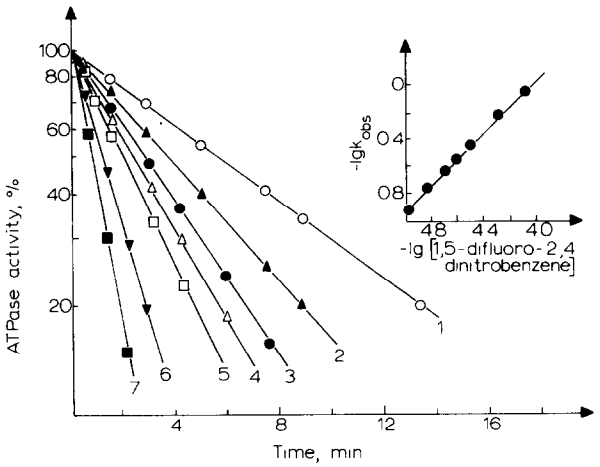

Fig.2. The kinetics of the inhibition of F_1-ATPase by different concentrations of 1,5-difluoro-2,4-dinitrobenzene. For conditions, see the legend to fig.1. The concentration of F_1-ATPase was 0.4 mg/ml; concentration of 1,5-difluoro-2,4-dinitrobenzene – 10 μM (curve 1), 15 μM (curve 2), 20 μM (curve 3), 25 μM (curve 4), 30 μM (curve 5), 50 μM (curve 6) and 80 μM (curve 7). Inset: determination of the order for the reaction of F_1-ATPase with 1,5-difluoro-2,4-dinitrobenzene.

The chemical properties of 1,5-difluoro-2,4-dinitrobenzene and the data on the chemical nature of the groups controlling F_1-ATPase activity [4,5,8–10] indicate that the inhibition is caused by the reaction of this inhibitor with a tyrosine or lysine residue. Cysteine, the other candidate for the reaction with 1,5-difluoro-2,4-dinitrobenzene, was not found in the composition of the β-subunits, which bear the ATPase active site [16]. So, cysteine modification with 1,5-difluoro-2,4-dinitrobenzene cannot be the reason for enzyme inhibition. Histidine modification also seems to be unlikely due to the pH dependence of the reaction (see fig.4) and also because the reagents specific to histidine do not inhibit the ATPase reaction [8]. The addition of 100 mM dithiothreitol to F_1-ATPase preincubated with 80 μM 1,5-difluoro-2,4-dinitrobenzene for 4 min and having 55% of the initial activity, leads to the almost complete restoration of its ATPase activity. This result testifies to the fact that 1,5-difluoro-2,4-dinitrobenzene modifies a tyrosine residue in F_1-ATPase since dinitrobenzene derivatives of tyrosine react with dithiothreitol with the restoration of free tyrosine [17] and, on the other hand, the 1,5-difluoro-2,4-dinitrobenzene reaction with a lysine residue could not be reversed by dithiothreitol.

Another well known inhibitor of F_1-ATPase that reacts with tyrosine is 7-chloro-4-nitrobenzofurazan. Dithiothreitol treatment of F_1-ATPase modified with this inhibitor also leads to the restoration of ATPase activity [4]. We therefore tried to determine whether 1,5-difluoro-2,4-dinitrobenzene and 7-chloro-4-nitrobenzofurazan react with the same tyrosine residue in F_1-ATPase.

F_1-ATPase was consecutively treated with 7-chloro-4-nitrobenzofurazan and 1,5-difluoro-2,4-dinitrobenzene. The enzyme modified in this way was then treated for 5 min with 5 mM dithiothreitol. The time of incubation with dithiothreitol and its concentration were sufficient to reactivate F_1-ATPase treated with 7-chloro-4-nitrobenzofurazan (on the other hand, such dithiothreitol treatment was too mild to cause reversion of the 1,5-difluoro-2,4-dinitrobenzene-induced inhibition). The control preparation of F_1-ATPase was subjected to the same treatment, excluding 7-chloro-4-nitrobenzofurazan. The results of these experiments are given in fig.3. Similar slopes of the curves in fig.3 testify to the fact that a control preparation of F_1-ATPase and F_1-ATPase treated with 7-chloro-4-nitrobenzofurazan reacts with 1,5-difluoro-2,4-dinitrobenzene at the same rate. Thus, there is no competition between these inhibitors for F_1-ATPase, i.e. 1,5-difluoro-2,4-dinitrobenzene modifies another amino acid residue than does 7-chloro-4-nitrobenzofurazan.

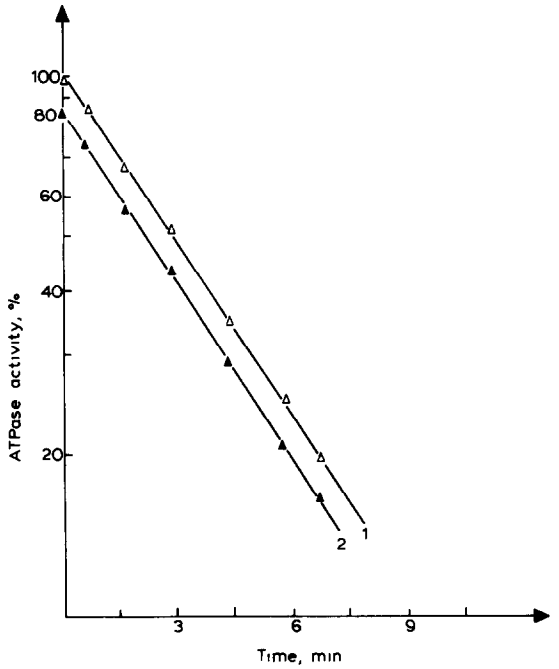

Fig.3. 7-Chloro-4-nitrobenzofurazan treatment of F_1-ATPase does not prevent the reaction of this enzyme with 1,5-difluoro-2,4-dinitrobenzene. F_1-ATPase (5 mg/ml) was incubated for 40 min in a reaction mixture containing 50 mM Mops-NaOH, pH 7.5, 1 mM EDTA, 0.25 M sucrose and 100 μM 7-chloro-4-nitrobenzofurazan. The modified F_1-ATPase containing less than 10% of the initial activity was separated from the reaction mixture by Penefsky's method [13] (see section 2). The control preparation of F_1-ATPase was treated in the same way but without 7-chloro-4-nitrobenzofurazan. To both F_1-ATPase preparations (final protein concentration 1 mg/ml) triethanolamine was added up to a concentration of 80 mM, pH 8.5, and then the protein solutions were incubated with 15 μM 1,5-difluoro-2,4-dinitrobenzene. After the time intervals indicated, aliquots of the reaction mixture were taken and incubated for 5 min with 5 mM dithiothreitol. Then the ATPase activity of these enzyme aliquots was measured. Curve 1, control preparation of F_1-ATPase; curve 2, F_1-ATPase pretreated with 7-chloro-4-nitrobenzofurazan.

At saturating concentrations, ATP and ADP only partially protect F_1-ATPase from the 1,5-difluoro-2,4-dinitrobenzene-induced inhibition. For example, 0.5 mM ATP decreases the rate of inhibition of F_1-ATPase by 1,5-difluoro-2,4-dinitrobenzene (15 μM) 2.7-fold. Thus, the question remains open as to whether the group reacting with this inhibitor is located at or outside the active site of the F_1-ATPase.

Fig.4 shows the pH dependence of the rate constant of F_1-ATPase inhibition by 1,5-difluoro-2,4-dinitrobenzene in the absence (curve 1) and presence (curve 2) of ATP. An analysis of these curves using a computer program revealed that in both cases the pK of the group reacting with 1,5-difluoro-2,4-dinitrobenzene is equal to 9.1 ± 0.1. It follows from these data that the protective effect of ATP is not caused by the ATP-dependent change in the pK of the group reacting with 1,5-difluoro-2,4-dinitrobenzene.

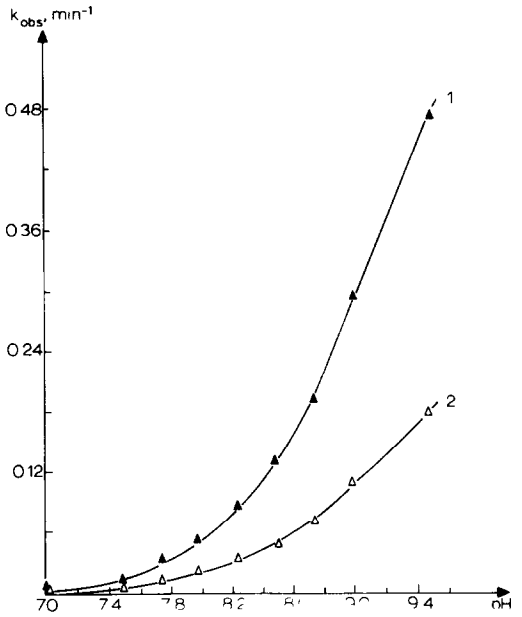

Fig.4. The pH dependence of F_1-ATPase inhibition by 1,5-difluoro-2,4-dinitrobenzene. k_{obs} values of the reaction were determined as shown in fig.2. Conditions as in fig.2 except that the inhibitor concentration was 15 μM and for the buffer mixtures of triethanolamine and Mops were used. In the case of curve 2, incubations of F_1-ATPase with 1,5-difluoro-2,4-dinitrobenzene were carried out in the presence of 0.5 mM ATP.

Our results indicate that 1,5-difluoro-2,4-dinitrobenzene is a very powerful inhibitor of F_1-ATPase. The inhibition is probably caused by the modification of a tyrosine residue with pK 9.1, and this tyrosine residue is not the same one that reacts with 7-chloro-4-nitrobenzofurazan.

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