Tightly bound nucleotides affect phosphate binding to mitochondrial F_1-ATPase

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The interaction of inorganic phosphate with native and nucleotide-depleted F_1-ATPase was studied. F_1-ATPase depleted of tightly bound nucleotides loses the ability to bind inorganic phosphate. The addition of ATP, ADP, GTP and GDP but not AMP, restores the phosphate binding. The nucleotides affecting the phosphate binding to F_1-ATPase are located at the catalytic (exchangeable) site of the enzyme. The phosphate is thought to bind to the same catalytic site where the nucleotide is already bound. It is thought that ADP is the first substrate to bind to F_1-ATPase in the ATP synthesis reaction.

Mitochondrial F_1-ATPase *Phosphate binding* *Nucleotide binding*

1. INTRODUCTION

ATP synthesis in the oxidative phosphorylation system occurs in the catalytic sites of mitochondrial F_1-ATPase [1]. This enzyme consists of 6 large subunits, 3 α- and 3 β-, and 3 small subunits (review [2]). The 6 nucleotide-binding sites of F_1-ATPase are located on the large subunits [3,4]. Three of these sites are catalytic ones and thought to be located on the β-subunits (review [5]). It has been shown that the other 3 sites do not exchange the nucleotides bound to them in the course of ATP hydrolysis and, consequently, they are believed to be non-catalytic sites [4]. These non-catalytic sites are probably located on the α-subunits [4,5]. F_1-ATPase preparations are known to contain nucleotides tightly bound to the enzyme (review [6]). The location and the functions of these nucleotides are unclear.

Mitochondrial F_1-ATPase is able to bind specifically one molecule of inorganic phosphate with K_d equal to 80 μM [7]. Using the photoactivating analogue of phosphate, 4-azido-2-nitrophenylphosphate, Vignais and co-workers [8] showed that the binding site is located on the β-subunit, i.e. probably at the catalytic site of the enzyme.

We recently showed that the binding of ADP to one of the catalytic sites of F_1-ATPase leads to the inhibition of the initial ATP hydrolysis rate [9]. The addition of inorganic phosphate reverses this inhibition [9]. The mechanism of this phosphate-induced reactivation of the enzyme was explained by the formation of the ternary F_1-ATPase–ADP–phosphate complex, which dissociates rapidly as a result of ATP binding to the other catalytic site. It follows from the results obtained [9] that in the preparation of native F_1-ATPase one of the catalytic sites is always occupied by ADP or, in other words, one of the tightly bound nucleotides is located in the catalytic site of F_1-ATPase.

We show here that the removal of the nucleotides tightly bound to F_1-ATPase leads to the enzyme losing its ability to bind inorganic phosphate. We also conclude that the inorganic phosphate can only interact with the catalytic site of F_1-ATPase where the ADP is tightly bound.

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2. EXPERIMENTAL

ATP, ADP, AMP, GTP and GDP were from Reanal; Tris, bovine serum albumin (Vth fraction) from Sigma; [^{3}H]ADP and $^{32}P_i$ from Amersham. The sucrose was recrystallized from ethanol since non-recrystallized preparations inactivate F_1-ATPase depleted of nucleotides.

The mitochondrial F_1-ATPase was isolated and stored according to Knowles and Penefsky [10]. Nucleotide-depleted F_1-ATPase was obtained as described by Garrett and Penefsky [11]. To measure the inorganic phosphate binding, the nucleotide depleted enzyme was diluted 6–9-fold to a final concentration of 1 mg/ml in a medium containing 0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 1.1 mM $MgSO_4$, 0.1 mM EDTA (buffer A). The $MgSO_4$ was added in such an amount that its concentration per mM exceeded that of EDTA. After preincubation for 2 min, the reaction was started by the addition of $Na_2H^{32}PO_4$ ($4–6 \times 10^5$ cpm/nmol). At the intervals indicated, the non-bound ligand was removed by the centrifuge-column procedure [7].

In the experiments with native F_1-ATPase, aliquots of the enzyme were centrifuged for 10 min at 8000 rpm; the protein pellet was dissolved in buffer A and the remaining ammonium sulphate was removed by the centrifuge-column technique [7]. The M_r of the F_1-ATPase was taken to be equal to 360000 [12].

The protein was determined according to Lowry et al. [13], using high-quality crystalline bovine serum albumin as the standard.

3. RESULTS

Fig.1 shows the kinetics of the phosphate binding to native and nucleotide-depleted F_1-ATPase. The native F_1-ATPase binds 0.5 mol phosphate per mol enzyme (fig.1, curve 1), while nucleotide depleted F_1-ATPase binds less than 0.1 mol phosphate per mol enzyme (fig.1, curve 2). Preincubation of nucleotide-depleted F_1-ATPase with an equimolar quantity of ADP restores phosphate binding almost to the value obtained for native F_1-ATPase (fig.1, curve 3). The difference in the phosphate binding for two preparations of the enzyme cannot be explained by the inactivation of nucleotide-depleted F_1-ATPase in the course of the

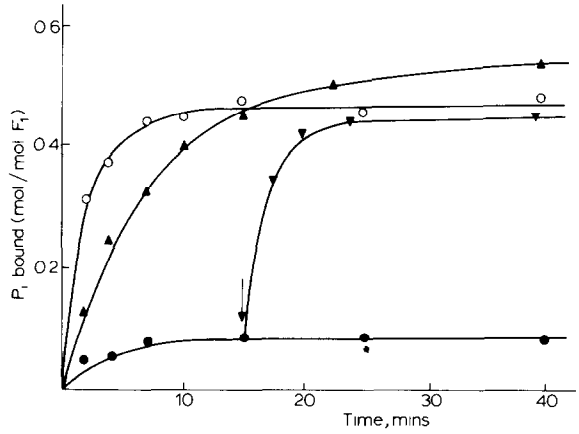

Fig.1. The kinetics of inorganic phosphate binding to native and nucleotide-depleted F_1-ATPase. F_1-ATPase (1 mg/ml) was incubated in buffer A in the absence (1,2) or presence (curve 3) of 2.8 μM ADP for 2 min. Then the phosphate (final concentration 250 μM) was added. (Curve 1) Native F_1-ATPase, (curves 2–4) F_1-ATPase depleted of tightly bound nucleotides. Arrow indicates the moment of addition of ADP (final concentration 2.8 μM).

experiment. The addition of ADP 15 min after the beginning of preincubation of the enzyme with phosphate restores phosphate binding to the same level as ADP addition at the beginning of the incubation (cf. curves 3 and 4, fig.1). The result obtained means that only one of the 3 tightly bound nucleotides is necessary for the binding of phosphate to F_1-ATPase.

ATP, but not AMP, can be substituted for ADP in restoring inorganic phosphate binding (see table 1). Guanine nucleoside di- and triphosphates are also effective in restoring phosphate binding, but in these cases the stoichiometry of phosphate binding is somewhat lower than with adenine nucleotides (cf. 2,3 and 4,5 in table 1). This is possibly caused by the lower affinity of phosphate to the F_1-ATPase complex with GDP than with ADP. In the other paper, we showed that under conditions similar to those in table 1 the nucleotide binding did, in the main, occur to the catalytic (exchangeable) site of the enzyme [9]. Thus, the ATP and GTP added were rapidly hydrolyzed by more than 80%, and the ADP or GDP resulting from this hydrolysis are probably responsible for the effects observed (table 1). Thus, the results obtained in the paper indicate that the preliminary binding

Table 1

Nucleotide-induced restoration of the phosphate binding to nucleotide-depleted F_1-ATPase

Nucleotides added to F_1-ATPase	Quantity of bound phosphate (mol/mol F_1)
–	0.07
ADP	0.53
ATP	0.52
GDP	0.29
GTP	0.27
AMP	0.07

The F_1-ATPase (1 mg/ml) was preincubated for 2 min in buffer A, containing 2.8 μM nucleotides. Then inorganic phosphate ^{32}P was added to a final concentration 0.5 mM and the mixture was incubated for 20 min. For other details, see section 2

of one molecule of nucleoside diphosphate to the catalytic site of F_1-ATPase is needed to bind the inorganic phosphate.

This conclusion was confirmed by the data of fig.2. This figure shows that the quantity of bound

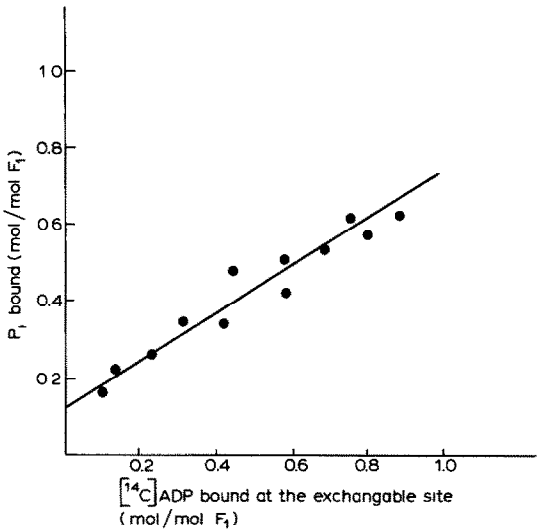

Fig.2. The dependence of the quantity of phosphate bound to F_1-ATPase on the quantity of ADP bound to the catalytic site of the enzyme. F_1-ATPase (1 mg/ml) was preincubated in buffer A containing 0.4–4.0 μM ADP for 2 min. Then phosphate (final concentration 500 μM) was added. After 20 min, aliquots of the enzyme were taken to determine the phosphate binding and the quantity and localization of ADP in the nucleotide-binding sites as described in section 2.

inorganic phosphate is strictly proportional to the quantity of ADP bound to the exchangeable site of F_1-ATPase. The F_1-ATPase can bind approx. 0.8 mol phosphate per mol enzyme, if 1 mol ADP is bound to the exchangeable site.

As can be seen from figs 1,2 and table 1 nucleotide-depleted F_1-ATPase is able to bind a small quantity of the phosphate (less than 0.1 mol per mol enzyme) even in the absence of added ADP. However, this binding is evidently caused by a small fraction of the enzyme which has preserved nucleotides in the course of the treatment by the gel-filtration method of Garrett and Penefsky [11]. It was shown in special experiments that the phosphate binding with both preparations of F_1-ATPase, containing ADP (stoichiometry of binding: 1 mol phosphate per mol enzyme) and nucleotide-depleted (0.07 mol phosphate per mol enzyme), is characterised by a dissociation constant equal to 130 μM (not shown).

4. DISCUSSION

From the data obtained, it can be seen quite clearly that the presence of nucleoside diphosphate in the catalytic site of F_1-ATPase is essential for phosphate binding to the enzyme. The preparations of native F_1-ATPase isolated by the standard method [10] contain 3 tightly bound nucleotides [11]. Recently, the hypothesis was put forward that the non-exchangeable (non-catalytic) sites are identical to the tight binding sites of F_1-ATPase [4,14]. In our group, on the contrary, data have been obtained showing that one of the tightly bound nucleotides is located at the catalytic site of the enzyme [9]. The very fact that the phosphate binding correlates with the presence of ADP in the exchangeable (catalytic) site of F_1-ATPase and that the native F_1-ATPase binds phosphate as well as nucleotide-depleted F_1-ATPase after ADP binding to one of the catalytic sites indicated that, in the case of the native enzyme, one of the tightly bound nucleotides is located in the catalytic site.

The question of whether phosphate binds to the same catalytic site as ADP requires further discussion. We have shown that the binding of one molecule of ADP to one of the F_1-ATPase catalytic sites leads to the complete inhibition of the initial ATP hydrolysis rate [9]. This inhibition was previously observed by Vinogradov and co-

workers [15]. The addition of stoichiometric quantities of ATP (by the method of Grubmeyer et al. [16]) also leads to inhibition of the enzyme, but in this case it develops in time, and the initial rate decreases in parallel to the dissociation of $^{32}P_i$ from [γ-^{32}P]ATP, bound to the enzyme [9]. The phosphate addition to F_1-ATPase inhibited by the incubation with equimolar quantities of ATP leads to the restoration of ATPase activity. Moreover, this reactivated enzyme could be inactivated again as a result of the phosphate release and the kinetics of this inactivation was the same as in the case of the incubation of F_1-ATPase with stoichiometric quantities of ATP (not shown). All these results are evidence of the localization of ADP and phosphate bound at the same catalytic site. Moreover, Feldman and Sigman [17] have shown that ATP may be synthesized by chloroplast F_1-ATPase from tightly bound ADP and added inorganic phosphate. The numerous data testify to the fact that this ADP is located at the catalytic site of the enzyme [18–20].

Yet another problem requiring discussion in the light of our data is the order of the substrate binding to F_1-ATPase in the ATP synthesis reaction. The results on this subject previously obtained are contradictory [21–23]. The data of this paper point to the fact that ADP is the first substrate to bind to the F_1-ATPase catalytic site and inorganic phosphate can only bind to the F_1-ATPase–ADP complex.

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