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Characterization of the nucleotide tight-binding sites of the isolated mitochondrial F_1-ATPase

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The properties of the nucleotides tightly bound with mitochondrial F_1-ATPase were examined. One of three bound nucleotide molecules is localized at the site with $K_d \sim 10^{-7}$ M and released with $k_{off} \sim 0.1$ s^{-1}. The second nucleotide molecule is bound with the enzyme with $K_d \sim 10^{-8}$ M and k_{off} for its dissociation is 3×10^{-4} s^{-1}. The third is never released even in the presence of 1 mM ATP or ADP. The last two nucleotides are believed to be bound at the noncatalytic sites of F_1-ATPase. Pyrophosphate promotes liberation of two releasable nucleotide molecules, decreasing the affinity of the enzyme to AD(T)P. From the results obtained it follows that the only suitable criterion for localization of the nucleotide at the F_1-ATPase catalytic site is the high rate ($k_{off} \geqq 0.1$ s^{-1}) of its spontaneous release.

F_1-ATPase; Tightly bound nucleotide; Nucleotide-binding site

1. INTRODUCTION

Isolated mitochondrial F_1-ATPase contains six nucleotide-binding sites [1,2] localized on the major subunits α and β (review [3]). Three of these sites are able to exchange bound nucleotides with those in the medium [1]. Purified preparations of mitochondrial F_1-ATPase (so-called 'native' F_1-ATPase) usually contain about three tightly bound nucleotide molecules which are not removed from the enzyme after repeated ammonium sulfate precipitation, charcoal treatment or gel filtration [1,4,5].

Recently we have shown that one of the tightly bound nucleotide molecules under particular conditions is rapidly (within 0.5 min) released into the medium [6,7] and that in the presence of an ATP-regenerating system, it serves as the substrate for steady-state uni-site catalysis by F_1-ATPase [7]. This nucleotide is bound at the exchangeable (catalytic?) site of the enzyme. This conclusion was confirmed by Kironde and Cross [8], who also have shown that one of the tightly bound nucleotides is localized at the exchangeable site of F_1-ATPase.

Here, we have examined the properties of all the nucleotides bound with F_1-ATPase. It is shown that two of three tightly bound nucleotide molecules of F_1-ATPase can be relatively rapidly exchanged with the medium ADP in EDTA-containing as well as in Mg^{2+}-containing medium. In the latter case the process is somewhat slower. The third nucleotide bound with the F_1-ATPase cannot be replaced even by millimolar concentrations of nucleotides (ATP or ADP). We also have measured the dissociation constants for two exchangeable tightly bound nucleotides.

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Abbreviation: Mops, morpholinepropanesulfonic acid

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2. MATERIALS AND METHODS

Sources of the chemicals were as published [6,7]. Native F_1-ATPase was obtained using the method of Knowles and Penefsky [9] and nucleotide-depleted F_1-ATPase prepared according to Garrett and Penefsky [4].

The tightly bound nucleotides of F_1-ATPase were labelled as follows. An ammonium sulfate suspension of native F_1-ATPase was centrifuged and the enzyme dissolved in buffer A (50 mM sucrose, 20 mM Mops-Tris, pH 8.0, 1 mM EDTA) at a concentration of 12 μM. The enzyme was desalted using the centrifuge-column method [10] on Sephadex G-50 preswollen in buffer A. To 30 μl eluate 230 μl carrier-free [α-^{32}P]ATP (5×10^5 cpm) in buffer A additionally containing 1.1 mg/ml BSA were added. The mixture was incubated for 30 min and then 120-μl samples were passed through Sephadex columns [10] equilibrated with buffer A, containing 1.1 mg/ml BSA. To study dissociation of the nucleotides, the labelled F_1-ATPase was diluted to ~50 nM with buffer A or buffer B (50 mM sucrose, 20 mM Mops-Tris, pH 8.0, 0.2 mM EDTA, 2.5 mM $MgCl_2$) both containing 1.1 mg/ml BSA and the additions specified in the figure legends. 200-μl portions of the mixtures were withdrawn and passed through Sephadex [10] equilibrated with the corresponding buffer.

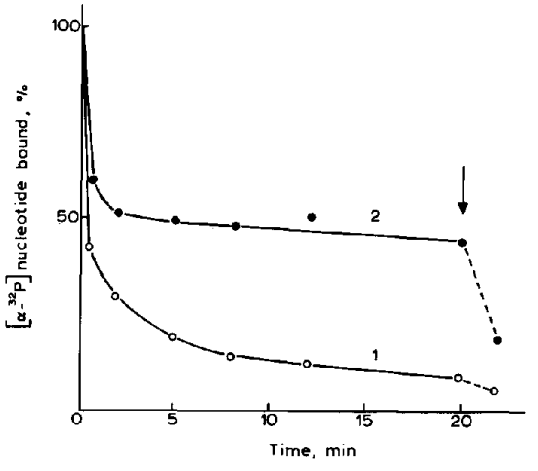

Fig.1. Release of (α-^{32}P)-labelled nucleotides bound with native F_1-ATPase. The enzyme was prelabelled with [α-^{32}P]ATP as described in section 2 and then diluted to ~50 nM with buffer A containing 1.1 mg/ml BSA and 0.5 μM ADP (curve 1) or with the same mixture without ADP (curve 2). The arrow indicates the moment of addition of ATP and Mg^{2+} to final concentrations of 1.0 and 2.0 mM, respectively.

In the case of nucleotide-depleted F_1-ATPase, to the enzyme solution in buffer containing 50% glycerol, 100 mM Tris-H_2SO_4, pH 8.0, and 4 mM EDTA, an equal volume of saturated ammonium sulfate solution was added. After standing in an ice-water bath for 20 min F_1-ATPase was sedimented by centrifugation at 30000 $\times$ g for 15 min at 4°C. The pellet was dissolved in buffer A containing 1 mM [α-^{32}P]ATP (~10^5 cpm/nmol) and 20 min later an equal volume of saturated $(NH_4)_2SO_4$ solution was added. The suspension was stored at 4°C. To study the release of bound nucleotides the enzyme was sedimented and desalted using the centrifuge-column method [10]. Desalted F_1-ATPase usually contained 1.5–2.0 mol α-^{32}P-labelled nucleotide per mol enzyme. The low stoichiometry of nucleotide binding is explained by the partial irreversible dissociation of F_1-ATPase.

3. RESULTS

The kinetics of release of (α-^{32}P)-labelled nucleotides bound with native enzyme is shown in fig.1. It can be seen (curve 1) that all bound label dissociates from the enzyme in EDTA-containing medium within 10 min when 0.5 μM ADP was added as a trap. About half of the bound ^{32}P-labelled nucleotide is released very rapidly – within 20 s, and the remainder – with $\tau_{1/2}$, 3–4 min. In the absence of an ADP trap only half of the bound radioactivity is liberated (fig.1, curve 2).

In Mg^{2+}-containing medium as in the absence of divalent cations all the bound radioactivity is released from the enzyme in the presence of the ADP trap (not shown). However, in the presence of Mg^{2+} this process is slower: half of the label is released within 1 min, the other half doing so with $\tau_{1/2}$ ~10 min. The significant release of bound nucleotides in the presence of Mg^{2+} at about 50 nM F_1-ATPase occurred only in the presence of an ADP trap.

The same pattern of nucleotide dissociation is observed in the case of nucleotide-depleted F_1-ATPase preloaded with (α-^{32}P)-labelled

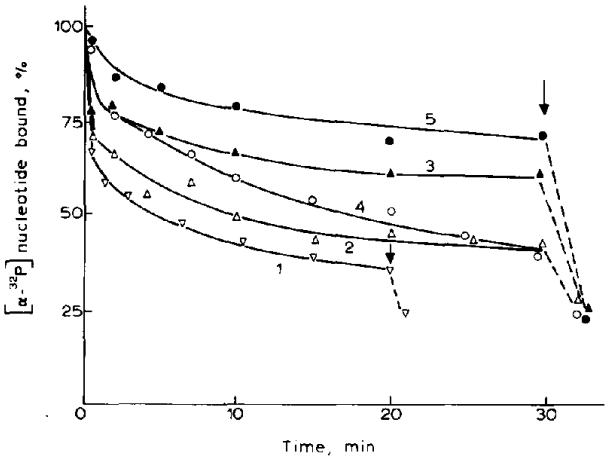

Fig.2. Release of (α-^{32}P)-labelled nucleotides bound with reconstituted nucleotide-depleted F_1-ATPase. The enzyme prepared as described in section 2 was diluted to ~50 nM with buffer A (curve 1) or buffer B (curves 2–5), both containing 1.1 mg/ml BSA. In the case of curves 1, 2 and 4, the medium contained additionally 0.5 μM ADP. In the experiments represented by curves 4 and 5 the enzyme before dilution was preincubated in the presence of 1 mM free Mg^{2+}. The arrows indicate the moment of addition of ATP and Mg^{2+} to final concentrations of 1 and 2 mM, respectively.

nucleotide (fig.2). However, in this case both the rapidly and slowly releasable fractions of the bound radioactivity are about 30–35% (curve 1). Nearly one-third of the bound ^{32}P-labelled nucleotides is not released from the enzyme even in the presence of 1 mM MgATP (2 min). In the presence of Mg^{2+} and an ADP trap, about 70% of the label is released from the enzyme: half with $\tau_{1/2}$ 20 s and half with $\tau_{1/2} \geqslant 20$ min (curve 2). In the absence of the ADP trap, only 30% of the bound radioactivity is liberated (curve 3). After preincubation of F_1-ATPase with Mg^{2+} for 2 min the nucleotides disappeared from the enzyme at somewhat slower rates in the presence of the ADP trap (cf. curves 2,4) but only minute release occurred in the absence of ADP (curve 5). At 1.0 μM F_1-ATPase the bound radioactivity is never released from the enzyme in the absence of the ADP trap (not shown).

It is well known that the native F_1-ATPase molecule contains three molecules of tightly bound nucleotides [1,4,5,8]. Therefore, the results presented in figs 1 and 2 can be easily explained as follows. Two of three bound nucleotides of the enzyme are readily exchanged with those in the medium. During the course of incubation of native F_1-ATPase with carrier-free [α-^{32}P]ATP these enzyme-binding sites containing nucleotides become labelled. Under conditions suitable for the release of bound nucleotides (low enzyme concentration or trapping by cold ADP) these ^{32}P-labelled nucleotides dissociate from the enzyme. The third tightly bound nucleotide is not capable of being released at measurable rates even in the presence of high nucleotide concentrations.

Fig.3 shows the effect of pyrophosphate on the time course of bound nucleotide release. It can be seen that in the case of the prelabelled native F_1-ATPase all bound radioactivity (curve 1) and for reconstituted nucleotide-depleted enzyme about 70% of the bound label (curve 2) are released into solution in the presence of 1 mM pyrophosphate. The half-time of the process is about 2 min. In terms of the amount of nucleotides, 1 mM pyrophosphate promotes the release of two out of three bound nucleotides (fig.3, curves 1,2). This finding is in contrast with the results of Kironde and Cross [8] that

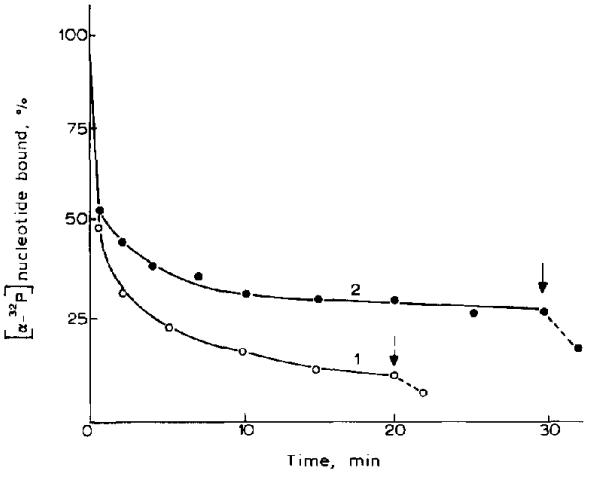

Fig.3. Effect of 1 mM pyrophosphate on release of (α-^{32}P)-labelled nucleotides bound with native (curve 1) or reconstituted nucleotide-depleted (curve 2) F_1-ATPase. The enzyme preparations (see section 2) were diluted to ~50 nM with buffer B, containing 1.1 mg/ml BSA and 0.5 μM ADP. The arrows indicate the moment of addition of ATP and Mg^{2+} to final concentrations 1 and 2 mM, respectively.

pyrophosphate treatment causes the release of only one molecule of the tightly bound nucleotide. This discrepancy may be explained by the high enzyme concentrations used (about 5–10 μM) in [8].

The affinities of the exchangeable tight-binding sites of the enzyme to adenine nucleotides (most probably ADP) can be easily estimated. In the presence of Mg^{2+} rapidly releasable nucleotide in the absence of an ADP trap completely dissociates from the enzyme at 50 nM F_1-ATPase (figs 1,2) but is not released at 0.5 μM F_1-ATPase (not shown). Thus, estimation of the K_d value gives approx. 0.1–0.2 μM. Direct measurement of K_d for this site cannot be made using the centrifuge-column method [10] because on the one hand the rate of nucleotide release is high ($\tau_{1/2} \leqslant 10$ s) and, on the other, the time of free ligand separation is relatively long (about 20 s [10]).

The affinity of the slowly releasable site to ADP can be measured directly using nucleotide-depleted F_1-ATPase. Fig.4 shows a double-reciprocal plot of 0.5 nM [α-^{32}P]ADP binding vs F_1-ATPase concentration ranging from 5 to 30 nM: it can be seen that the K_d value is equal to 10 nM. It should be noted that nonexchangeable sites of F_1-ATPase are not loaded with ADP for at least 10 min under the conditions of fig.4. The nucleotide bound under the specified conditions (fig.4) is released from the enzyme with k_{off} about 3×10^{-4} s^{-1} [6].

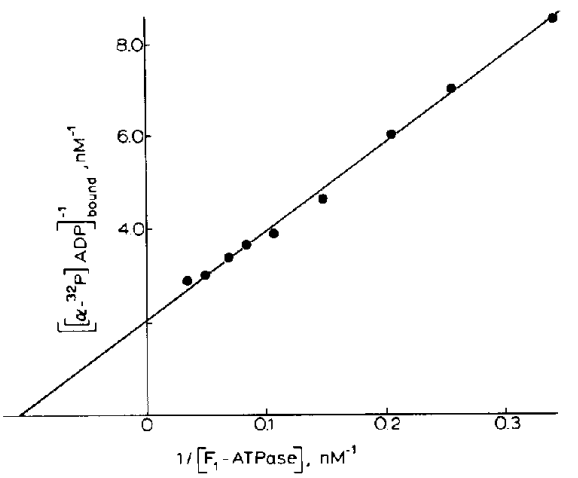

Fig.4. Double-reciprocal plot of 0.5 nM [α-^{32}P]ADP (5×10^7 cpm/nmol) binding as a function of nucleotide-depleted F_1-ATPase concentration. The reaction was carried out in buffer B for 1–5 min and unbound ligand was separated using the centrifuge-column method.

4. DISCUSSION

Here, we have characterised the properties of all three tightly bound nucleotides of isolated F_1-ATPase from beef heart mitochondria. One is localized at the binding site that is able to release the nucleotide with $k_{off} \sim 0.1$ s^{-1}. The K_d value for this site is about 0.1–0.2 μM (see also [6]). We believe that it is this nucleotide which serves as the substrate for uni-site catalysis by native F_1-ATPase in the presence of an ATP-regenerating system [7].

The second nucleotide of the native F_1-ATPase molecule is bound at the high-affinity site ($K_d \sim 10^{-8}$ M, fig.4) and is released with $k_{off} \sim 3 \times 10^{-4}$ s^{-1} (see also [6]). It appears that this is the site which was believed to be catalytic by Grubmeyer et al. [11]. However, two facts indicate that this ADP-binding site is not catalytic. Firstly, the rate of ADP liberation from the catalytic site even during uni-site catalysis is faster by two orders of magnitude [7,12,13]. Secondly, ADP liberation from the active site in the course of single-site hydrolysis was not affected by NaN_3 whereas the release of ADP from the tight-binding, non-catalytic site is prevented by azide [6]. The third nucleotide molecule is bound with the enzyme non-exchangeably and is not released into solution under the usual conditions.

Thus, three nucleotide molecules are bound at the different binding sites of F_1-ATPase. This suggests the existence of asymmetry of the nucleotide tight-binding sites of F_1-ATPase.

Cross and Nalin [1] have proposed that nucleotides bound with F_1-ATPase exchangeably are localized at the catalytic sites. It follows from the results presented that one exchangeable nucleotide is bound at the noncatalytic site of F_1-ATPase. Therefore, the suggestion as to the equivalence of the catalytic and exchangeable sites of the enzyme [1] is not adequate. Thus, the only reliable criterion for localization of the nucleotide at the enzyme catalytic site is the rate of its spontaneous release. Because the rate of ADP liberation even during the course of uni-site catalysis is equal to 0.1 s^{-1} [7,12,13], only those nucleotides that are released spontaneously with $\tau_{1/2} \leqslant 10$ s can be assumed to be bound at the F_1-ATPase catalytic site(s).

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