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On the rate of F_1-ATPase turnover during ATP hydrolysis by the single catalytic site

Evidence that hydrolysis with a slow rate of product release does not occur at the alternating active site

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Under conditions of molar excess of enzyme, isolated F_1-ATPase from beef heart mitochondria catalyses ATP hydrolysis biphasically. The rate constants for product release are $\sim 10^{-1}$ and $10^{-4}-10^{-3}$ s^{-1}, respectively. The slow phase of ATP hydrolysis is insensitive to EDTA. [γ-^{32}P]ATP splitting in the slow phase cannot be chased from the enzyme during several catalytic turnovers. It follows from these results that the slow single-site hydrolysis of ATP ($k_{cat} \sim 10^{-4}$ s^{-1}), initially observed by Grubmeyer et al. [(1982) J. Biol. Chem. 257, 12092–12100], is not carried out by the normal catalytic site. In contrast, the phase of rapid ATP hydrolysis ($k_{cat} \sim 10^{-1}$ s^{-1}) is completely prevented by EDTA and is believed to be the normal function of the normal catalytic site of F_1-ATPase.

F_1-ATPase; Single-site catalysis; Turnover rate

1. INTRODUCTION

It is well known that six major subunits of F_1-ATPase (3 α and 3 β) form six nucleotide-binding sites [1,2]. Three of these sites are believed to be catalytic [3,4]. There is a lot of data that confirm the alternating-site cooperativity mechanism of Repke [5] for the active sites of F_1-ATPase [6]. According to this mechanism the rate of product (ADP and P_i) release from one active site of the enzyme is accelerated several-fold when ATP is bound at the alternating catalytic site(s) [5,6]. The clearest manifestation of alternating-site cooperativity for F_1-ATPase is the ATP dependence of intermediate water-phosphate oxygen exchange [7–9] (review [6]).

Grubmeyer et al. [10] have recently shown that F_1-ATPase catalyses ATP hydrolysis by a single catalytic site with a turnover rate of 10^{-4} s^{-1}. Recently we have studied single-site catalysis by nucleotide-depleted F_1-ATPase [11–13]. We have found the rate of F_1-ATPase turnover by the single catalytic site to be $\sim 10^{-1}$ s^{-1} [11] which is three orders of magnitude faster than that measured in Penefsky's group [10]. We obtained support for the results on single-turnover experiments by steady-state measurements: the enzyme catalyses steady-state ATP hydrolysis by the single catalytic site and k_{cat} and K_m values have been measured, amounting to ~ 0.1 s^{-1} and 10^{-8} M, respectively [12,13]. In parallel we have also shown that the native F_1-ATPase catalyses single-site hydrolysis with parameters close to that for the nucleotide-

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depleted enzyme [11,12].

This discrepancy in k_{cat} values obtained by different groups (cf. [10] and [11–13]) raises the question: Which out of two values of k_{cat} is correct and belongs to the catalytic site participating in the alternating-site cooperative mechanism of F_1-ATPase? We have therefore tested this problem here using two approaches: the requirement of Mg^{2+} for catalysis and chasing of the bound nucleotide by excess MgATP.

2. MATERIALS AND METHODS

ATP, Tris, Mops and bovine serum albumin from Sigma; phospho*enol*pyruvate and pyruvate kinase from Reanal; Sephadex G-50 (fine) from Pharmacia and [γ-^{32}P]ATP from Isotop (USSR) were used.

F_1-ATPase was isolated according to Knowles and Penefsky [14]. Nucleotide-depleted F_1-ATPase was obtained according to Garrett and Penefsky [15]. Protein fractions with $A_{280}/A_{260} > 1.90$ were used. The specific activity of both enzyme preparations was 80–90 μmol P_i/min per mg at 25°C and 2 mM MgATP.

[γ-^{32}P]ATP hydrolysis was measured as follows. The enzyme was incubated with [γ-^{32}P]ATP for the times indicated in buffer A (20 mM Tris-Mops, pH 8.0, 2.2 mM $MgCl_2$, 0.2 mM EDTA). The reaction was stopped by the addition of an equal volume of 1 M $HClO_4$ containing 0.5 mM NaH_2PO_4, and P_i was precipitated using the reagent of Sugino and Miyoshi [16]. After centrifugation for 5 min at 5000 rpm aliquots of the supernatant were taken for Cerenkov counting. When [γ-^{32}P]ATP hydrolysis in the presence of EDTA was studied buffer A was replaced by buffer B (20 mM Tris-Mops, pH 8.0, 0.5 mM EDTA).

Binding of nucleotides was measured by the centrifuge-column method [17]. Sephadex was equilibrated and the reaction was carried out in buffer A or buffer B, both containing additionally 1.1 mg/ml BSA (for details see the figure legends).

Native F_1-ATPase was treated as described by Grubmeyer et al. [10]. Briefly, the ammonium sulphate suspension of F_1-ATPase was centrifuged, the pellet was dissolved in buffer C (0.25 M sucrose, 20 mM Mops-KOH, pH 7.5, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4) and F_1-ATPase was passed through a centrifuge column [17] equilibrated with the same buffer.

Protein was determined according to Lowry et al. [18] using BSA as the standard.

3. RESULTS AND DISCUSSION

In studying single-site ATP hydrolysis by F_1-ATPase, Grubmeyer et al. [10] did not discuss the biphasic nature of the process. At least half of the hydrolysable ATP is cleaved within 1 min and almost all ADP and P_i formed are released into solution (during 1 min incubation) [10] (see also [11]). The remaining ATP that comprises the smaller proportion of hydrolysable nucleotides (30–50%) is indeed split very slowly with a rate of product release of $\sim 10^{-4}$ s^{-1} [10,11]. Thus, the question arises as to which phase of hydrolysis should be attributed to the normal catalytic site participating in cooperative F_1-ATPase functioning? To resolve this problem we have exploited two peculiarities in the F_1-ATPase catalysis. The first is the divalent cation requirement for ATP hydrolysis, the second being the acceleration of product release from one catalytic site when MgATP is bound at the alternating catalytic site.

3.1. *The requirement for Mg^{2+}*

It is well known that F_1-ATPase catalyses ATP

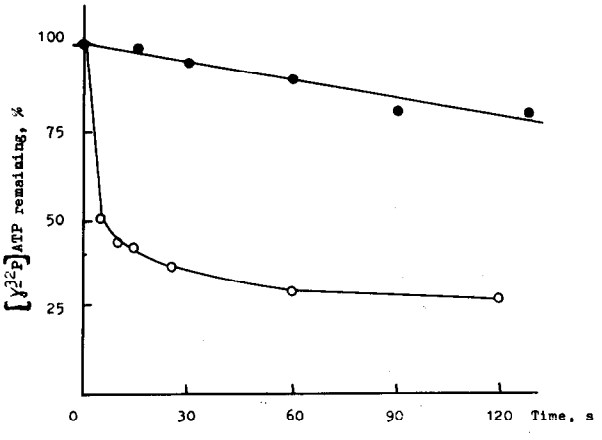

Fig.1. Kinetics of [γ-^{32}P]ATP hydrolysis by a molar excess of nucleotide-depleted F_1-ATPase in the presence of $MgCl_2$ (○) or EDTA (●). The enzyme at 2 μM was incubated with 0.7 μM [γ-^{32}P]ATP (2.5×10^5 cpm/nmol) in buffer A (○) or buffer B (●). The reaction was stopped and P_i was separated as described in section 2.

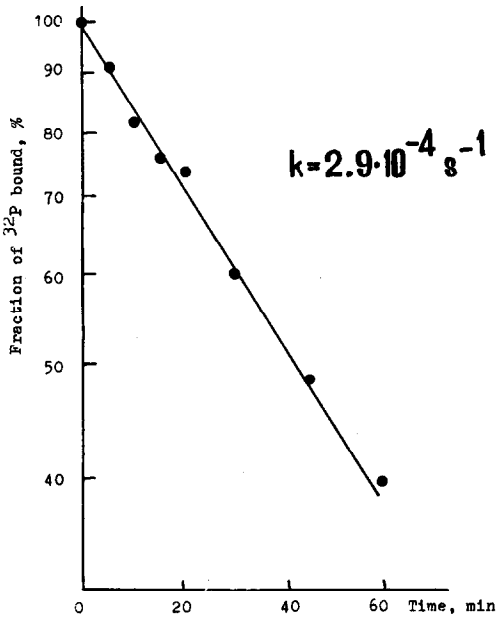

Fig.2. Kinetics of $^{32}P_i$ release during [γ-^{32}P]ATP hydrolysis by nucleotide-depleted F_1-ATPase in the presence of EDTA. 250 μl of 3 μM F_1-ATPase in buffer B were mixed with 250 μl of 1.0 μM [γ-^{32}P]ATP (1.6×10^6 cpm/nmol) in buffer B and the mixture was incubated for 1 min. The mixture was then passed through a 5 ml centrifuge column. This point was taken as zero time. At the indicated times, 50-μl aliquots of the eluate were taken for determination of ^{32}P label bound with F_1-ATPase. 100% binding corresponds to 0.27 mol ^{32}P/mol F_1-ATPase.

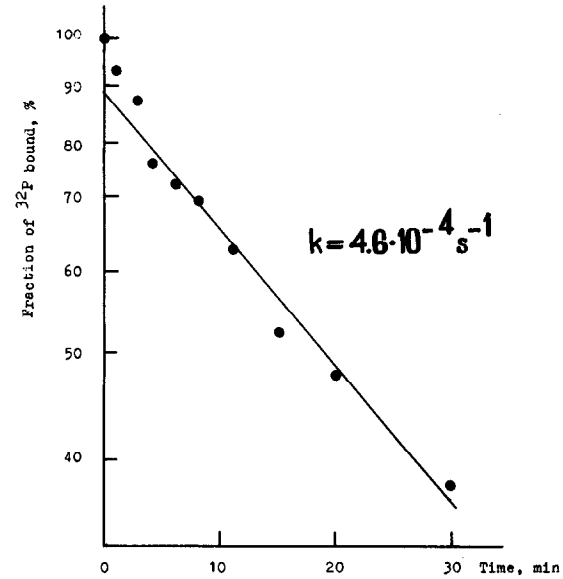

Fig.3. Release of enzyme-bound ^{32}P label from the [γ-^{32}P]ATP·F_1-ATPase complex obtained after cold ATP chase. Native F_1-ATPase was prepared as described by Grubmeyer et al. [10] (see also section 2). 250 μl of 2 μM enzyme in buffer C were mixed with 250 μl of 0.8 μM [γ-^{32}P]ATP (4.8×10^6 cpm/nmol) in the same buffer and the mixture was incubated for 1 min. Then ATP, phospho*enol*pyruvate and pyruvate kinase were simultaneously added to the reaction mixture to final concentrations of 20 μM, 2 mM and 0.2 mg/ml, respectively. After incubation for 20 s unbound radioactivity was separated using the centrifuge-column method on a 5 ml column and at the times indicated 50 μl eluate were taken for determination of bound ^{32}P. Zero time corresponds to 2 min after the first separation of unbound ligands. 100% binding corresponds to 0.06 mol ^{32}P/mol F_1-ATPase.

hydrolysis at a high rate only in the presence of divalent cations. In other words, Mg^{2+} is obligatory for the proper functioning of normally interacting F_1-ATPase active sites. Fig.1 shows the kinetics of ATP hydrolysis by a molar excess of F_1-ATPase in the presence and absence of $MgCl_2$. It can be seen that 70–75% of [γ-^{32}P]ATP added is hydrolysed with $\tau_{1/2}$ ~5 s in the presence of $MgCl_2$. As we have shown recently, the ADP and P_i formed are released from the enzyme within 20–30 s [11]. The remaining [γ-^{32}P]ATP is hydrolysed approx. 100-times slower with an extremely slow rate of product release [10,11].

In the absence of $MgCl_2$ the nucleotide added is hydrolysed very slowly (fig.1). Under these conditions nearly all [γ-^{32}P]ATP added is bound with F_1-ATPase (not shown) and the fate of this nucleotide is the same as reported by Grubmeyer et al. [10] (fig.2). In the absence of Mg^{2+} about 30 and 70% of the enzyme-bound ^{32}P label exists as P_i and ATP, respectively, and consequently the rate constant of P_i release is 10^{-3} s^{-1} (see fig.2). The slow phase of Mg^{2+}-dependent ATP hydrolysis coincides with the kinetics presented in fig.2 (not shown; see [10,11]).

Thus, we can conclude that because the slow kinetics of ATP hydrolysis by a molar excess of F_1-ATPase is insensitive to the presence of EDTA (see figs 1 and 2) this process does not occur at the normal catalytic site of the enzyme. In contrast, the rapid phase of single-site ATP hydrolysis is completely inhibited by EDTA (fig.1) and,

therefore, it is this phase that is carried out by the normal catalytic site of F_1-ATPase. Evidence of the single-site nature of rapid ATP hydrolysis by a molar excess of F_1-ATPase has been reported elsewhere [11–13].

3.2. *Chasing of the bound reactants*

Another demonstration that the slow phase of ATP hydrolysis does not occur at the normal active site of F_1-ATPase is presented in fig.3. Native F_1-ATPase was prepared as described by Grubmeyer et al. [10]. Then 1 μM enzyme was incubated with 0.3 μM [γ-^{32}P]ATP for 1 min and passed through centrifuge columns immediately or after incubation for an additional 20 s in the presence of 20 μM ATP and an ATP-regenerating system. Without such an ATP chase F_1-ATPase contains 0.14 mol ^{32}P label per mol enzyme and after incubation in the presence of ATP F_1-ATPase still contains 0.06 mol label per mol enzyme. Taking into account that during incubation with ATP F_1-ATPase carries out 700–800 catalytic turnovers (the activity of the enzyme at 20 μM ATP is about 40 s^{-1}), it is obvious that the bound ^{32}P label is chased from the enzyme by ATP more slowly than the catalytic turnover of F_1-ATPase is carried out. The bound radioactivity remaining after such an ATP chase is released from the enzyme (fig.3) with the kinetics of the slow single-site catalysis of Grubmeyer et al. [10] and with that for EDTA-insensitive F_1-ATPase function (fig.2). Again, the bound radioactivity exists as P_i (30%) and ATP (70%) and k_{off} for P_i release is 1.5×10^{-3} s^{-1}. Therefore, the enzyme site(s) which exhibit(s) slow single-site catalysis do(es) not normally participate in the sequential interactions between alternating catalytic sites of F_1-ATPase during ATP hydrolysis.

Thus, the present results together with our previous data [11] demonstrate that the ATP hydrolysis with $k \sim 10^{-4}$ s^{-1} is an artefactual activity of F_1-ATPase. Presumably, this is the result of an inactive or sluggish form of the enzyme functioning during catalysis. Consequently, the ratio of bound reactants measured [10] is not the equilibrium constant for the reaction at the catalytic site of F_1-ATPase.

Recently some papers on single-site catalysis by bacterial F_1-ATPases have appeared [19–21]. The authors have measured the rate of single-site turnover of the enzymes to be equal to 10^{-4} s^{-1}. But in all cases of bacterial enzymes the bimolecular rate constants for ATP binding to the enzyme are too low to permit the correct estimation of the partial rate constants.

In the present paper we have shown unequivocally that the F_1-ATPase activity with $k \sim 10^{-4}$ s^{-1} termed 'single-site catalysis' is not carried out by the normal catalytic site of the enzyme but is an artefactual activity. We believe that the real rate of ATP hydrolysis by the single catalytic site is ~0.1 s^{-1} and that the Michaelis constant for ATP is $\sim 10^{-8}$ M [11–13].

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