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

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Under the conditions of ATP regeneration and molar excess of nucleotide-depleted F_1-ATPase the enzyme catalyses steady-state ATP hydrolysis by the single catalytic site. Values of $K_m = 10^{-8}$ M and $V_m = 0.05$ s^{-1} for the single-site catalysis have been determined. ADP release limits single-site ATP hydrolysis under steady-state conditions. The equilibrium constant for ATP hydrolysis at the F_1-ATPase catalytic site is $\leqslant 0.7$.

F_1-ATPase; Single-site catalysis; Active site; Equilibrium constant

1. INTRODUCTION

The mechanism of alternating catalytic sites for ATP hydrolysis by F_1-ATPase with sequential participation of all three enzyme active sites in catalysis was proposed by Boyer et al. [1,2]. Grubmeyer and co-workers [3,4] obtained results which indicate that mitochondrial F_1-ATPase can catalyse ATP hydrolysis by the single-, two- and three-site modes with participation in the F_1-ATPase cycle of only one, only two or all three catalytic sites, respectively. F_1-ATPase functioning in single-site catalysis is characterized by extremely low rates of ADP and P_i release ($\sim 10^{-4}$ s^{-1}) [3]. Independently, Gresser et al. [5] calculated the kinetic parameters for various modes of action of F_1-ATPase from the ATP dependences of enzyme activity and intermediate oxygen-exchange reaction. The calculated rate constant of product release for single-site catalysis is about 10 s^{-1} [5]. We have recently shown that nucleotide-depleted F_1-ATPase in a molar excess catalyses single-site ATP hydrolysis, the ADP and P_i formed being released from the enzyme with $\tau_{1/2} \sim 10$ s [6].

These discrepancies in the value for the single-site F_1-ATPase catalysis rate obtained in various laboratories [3,5,6] prompted us to reinvestigate this process. Here, we have studied the steady-state kinetics of ATP hydrolysis by nucleotide-depleted F_1-ATPase at different ATP/enzyme ratios. It follows from the results obtained that nucleotide-depleted F_1-ATPase catalyses steady-state ATP hydrolysis by the single catalytic site. Values of $K_m = 10^{-8}$ M and $V_m = 0.05$ s^{-1} have been determined. The rate of ADP release from the enzyme active site limits the F_1-ATPase catalytic turnover. In the course of steady-state ATP hydrolysis the equilibrium ATP $\rightleftharpoons$ ADP + P_i is established at the F_1-ATPase catalytic site with $K_{eq.} \leqslant 0.7$.

2. MATERIALS AND METHODS

PEI-cellulose plates from Merck, ATP-monitoring reagent from LKB and pyruvate kinase and phospho*enol*pyruvate from Reanal were used. The sources of other chemicals were the same as those in our previous paper [6].

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Nucleotide-depleted F_1-ATPase was prepared using the procedure of Garrett and Penefsky [7] with a minor modification [8]. Protein fractions with $A_{280}/A_{260} \geqslant 1.95$ were used in experiments.

The rate of steady-state ATP hydrolysis at F_1-ATPase concentrations comparable with that of substrate was measured in a medium containing 50 mM Mops-KOH (pH 8.0), 50 mM KCl, 2.2 mM $MgCl_2$ and 0.2 mM EDTA (buffer A) in the presence of 1.0 mM phospho*enol*pyruvate and 1.0 mg/ml pyruvate kinase. The reaction was started by the addition of aliquots of 5–10 μM F_1-ATPase. 0.2 ml samples were incubated at 25°C for 2–15 min and the reaction terminated by the addition of 20 μl of 15% SDS solution containing 50 mM EDTA. P_i liberated was determined according to Lin and Morales [9]. In all cases P_i formation was linear during the time of incubation. Pyruvate kinase activity at 10 μM ADP amounted to 10.0 μmol/min per mg and the amount of pyruvate kinase used did not limit the reaction of P_i formation catalyzed by F_1-ATPase. It was verified that pyruvate kinase neither catalysed P_i formation nor bound ^{14}C-labelled nucleotides in the absence of F_1-ATPase.

(α-^{32}P)- and ^{14}C-labelled nucleotide binding by F_1-ATPase was determined using the centrifuge-column method [10]. The concentration of free ATP in the reaction mixture was determined using the luciferin-luciferase method. Protein concentration was determined according to Lowry et al. [11]. The molecular mass of the F_1-ATPase was taken as being equal to 360 kDa.

3. RESULTS

Incubation of ATP in the presence of a molar excess of nucleotide-depleted F_1-ATPase and ATP-regenerating system results in P_i formation. From curve 1 of fig.1 it can be seen that when [ATP] < [F_1-ATPase] the rate of P_i formation is proportional to the amount of ATP added. Nearly all ^{14}C-labelled nucleotide added is bound to F_1-ATPase (curve 2, fig.1), the concentration of free ATP in the medium is very low (curve 3, fig.1), and consequently the rate of P_i formation is proportional to the concentration of enzyme-substrate complex. The turnover rate of the F_1-ATPase·ATP complex is 0.05 s^{-1}. The steady-state ADP concentration in the reaction mixture

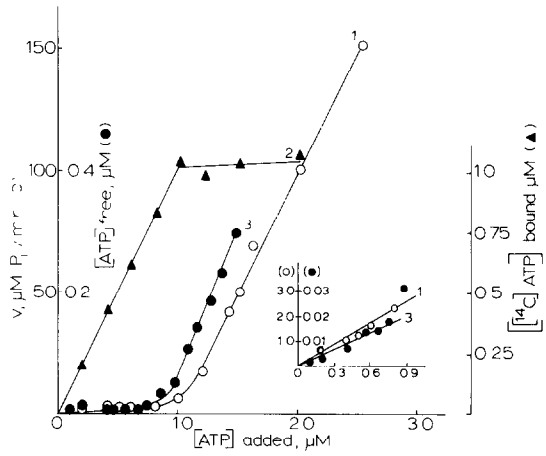


Fig.1. Dependence of (1) rate of P_i formation, (2) bound ^{14}C-labelled nucleotide and (3) free ATP concentrations on the concentration of ATP added in the presence of 1.0 μM nucleotide-depleted F_1-ATPase and ATP-regenerating system. For details see section 2. From the part of curve 1 at [ATP] > 1.0 μM a k_{+1} of 1.8 × 10^6 $M^{-1}\cdot s^{-1}$ can be calculated. (Inset) Curves 1 and 3 when [ATP] added < 0.9 μM.

calculated as in [12] under single-site catalysis conditions is lower than 1.0 nM. When [ATP] > [F_1-ATPase] the rate of the ATPase reaction increases linearly with the rise in substrate concentration (curve 1, fig.1) and the bimolecular rate constant of ATP binding in cooperative ATP hydrolysis is equal to 1.8 × 10^6 $M^{-1}\cdot s^{-1}$.

When [ATP] is fixed at 1.0 μM the rate of the ATPase reaction rises with increasing F_1-ATPase concentration up to 0.5 μM (fig.2, curve 1) as a result of the increase in the amount of enzyme functioning in a cooperative manner. Further increase of the F_1-ATPase concentration up to 1.0 μM leads to lowering of the ATP hydrolysis rate because the relative fraction of F_1-ATPase catalysing cooperative ATP hydrolysis is decreased and the amount of enzyme catalysing single-site ATP hydrolysis is increased. Under these conditions F_1-ATPase contains only 1 mol bound nucleotide per mol enzyme (fig.2, curve 2). At [F_1-ATPase] > [ATP] all the nucleotide is bound to the enzyme and F_1-ATPase catalyses single-site ATP hydrolysis.

At a fixed ATP/F_1-ATPase ratio equal to 0.5, the rate of single-site F_1-ATPase turnover is not dependent on enzyme concentration over the range 0.15–2.0 μM and amounts to 0.05 s^{-1} (not

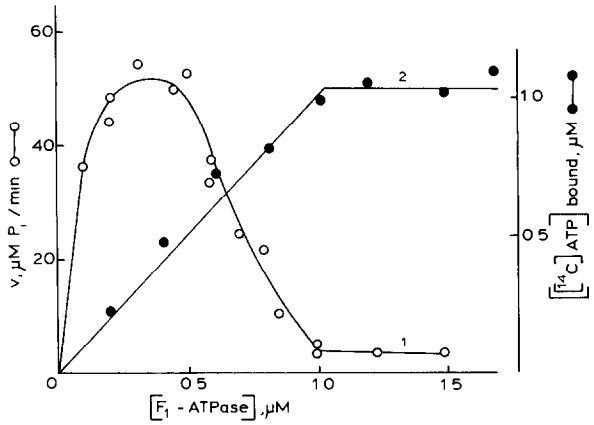


Fig.2. Dependence of (1) rate of P_i formation and (2) concentration of bound ^{14}C-labelled nucleotide on concentration of F_1-ATPase in the presence of an ATP-regenerating system when [ATP] added is fixed at 1.0 μM.

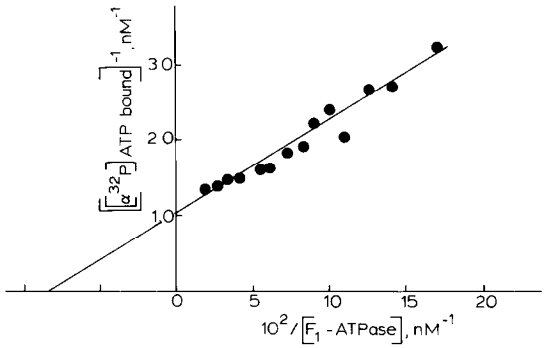


Fig.3. Double-reciprocal plot of [α-^{32}P]ATP binding by nucleotide-depleted F_1-ATPase. 1.0 nM [α-^{32}P]ATP (5×10^7 cpm/nmol) was incubated with nucleotide-depleted F_1-ATPase over the concentration range 6–50 nM for 5 min in buffer A containing 1.0 mg/ml BSA, 1.0 mg/ml pyruvate kinase and 1.0 mM phospho*enol*pyruvate. The concentration of bound nucleotide was determined in 100-μl aliquots by means of the centrifuge-column method [10].

shown). We have measured K_m for ATP under single-site catalysis conditions from [α-^{32}P]ATP binding at the F_1-ATPase catalytic site. Fig.3 shows the dependence on concentration of the nucleotide-depleted F_1-ATPase of the binding of 1.0 nM [α-^{32}P]ATP in the presence of an ATP-regenerating system. The value of K_m is about 10 nM.

Table 1

Partition between ADP and ATP of the ^{14}C-labelled nucleotide bound at the F_1-ATPase catalytic site during steady-state ATP hydrolysis

[^{14}C]ATP added (μM)	Concentration of ^{14}C-labelled nucleotide in reaction mixture (μM)		Ratio ADP/ATP
	ADP	ATP	
0.5	0.20	0.27	0.74
1.0	0.40	0.56	0.71

1.0 μM F_1-ATPase was incubated with [^{14}C]ATP in the mixture for the determination of ATPase activity. The reaction was allowed to proceed for 2 min, 20 μl samples were then quenched by the addition of 10 μl of 1.5 N $HClO_4$ containing 20 mM EDTA, neutralized by 1.0 M K_2CO_3 and aliquots of the supernatant were spotted on PEI-cellulose plates. The plates were developed using 0.5 M KH_2PO_4 (pH 3.4) according to [13]. Zones corresponding to ADP and ATP were cut out and the amount of ^{14}C-labelled nucleotide was determined by liquid-scintillation counting

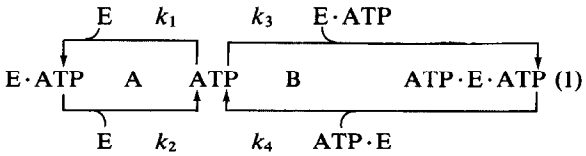
Pyruvate kinase activity does not limit the rate of ATPase reaction under the single-site catalysis conditions and, therefore, the steady-state rate of ATP hydrolysis in the presence of a molar excess of F_1-ATPase is limited by the rate of some step(s) of the F_1-ATPase catalytic cycle: namely by either ATP binding, ATP hydrolysis or product release. The apparent rate constant of the hydrolysis of 2.0 nM [γ-^{32}P]ATP is linearly dependent on the concentration of nucleotide-depleted F_1-ATPase (enzyme excess, not shown). The bimolecular rate constant calculated for ATP binding under single-site catalysis conditions is 3.0×10^6 $M^{-1} \cdot s^{-1}$ and at 0.1 μM F_1-ATPase the rate of [γ-^{32}P]ATP binding and hydrolysis is 0.3 s^{-1}. Since the rate of single-site F_1-ATPase turnover is significantly lower (0.05 s^{-1}, figs 1,2) we can conclude that in the course of steady-state F_1-ATPase functioning the step of ADP release limits overall enzyme turnover.

We have also investigated in which form, ATP or ADP, the nucleotide is transitorily bound at the F_1-ATPase catalytic site in the course of single-site catalysis. Our results are summarized in table 1. It can be seen that ~60% of bound ^{14}C-labelled

nucleotide is ATP and the remaining ~40% is ADP, the ratio of bound ADP/ATP being ~0.7.

4. DISCUSSION

Under our experimental conditions the rate of ADP phosphorylation by pyruvate kinase is very high and the rate of ATP release from the F_1-ATPase catalytic site is extremely low [3]. In the presence of an ATP-regenerating system and a molar excess of F_1-ATPase interaction of the nucleotide-depleted F_1-ATPase with ATP can be represented by the following scheme:



Cycles A and B represent the single-site and cooperative mechanisms of ATP hydrolysis by F_1-ATPase, respectively. k_1 and k_3 are the bimolecular rate constants for ATP binding with E and E·ATP forms of F_1-ATPase which are equal to 3.0×10^6 and 1.8×10^6 $M^{-1} \cdot s^{-1}$, respectively (see above). k_2 is the first-order rate constant for ADP release from the catalytic site (the rate-limiting step of single-site ATP hydrolysis). k_4 is the rate constant for ADP formation in cooperative F_1-ATPase functioning. From the data in fig.1, it follows that at low ATP concentrations (<5 μM) cooperative ATP hydrolysis is a second-order reaction and that the overall ATP hydrolysis is limited by the rate of ATP binding. Returning to scheme 1: $k_4 \gg k_3[ATP]$ and consequently $[ATP \cdot E \cdot ATP] \ll [E \cdot ATP]$, i.e.

$$[E_t] \simeq [E \cdot ATP] + [E_f] \quad (2)$$

where subscripts t and f denote total and free concentrations, respectively. When $[ATP_t] < [E_t]$ it can be shown that the concentration of free ATP is defined by the relation:

$$[ATP_f] = k_2[E \cdot ATP]/k_1[E_f] \quad (3)$$

From the experimental conditions of fig.1 at $[E_t] = 1.0\ \mu M$ and $[ATP_t] = 0.5\ \mu M$, more than 95% of the nucleotide added is bound with F_1-ATPase, i.e. $[ATP_f] < [ATP_t]$. Thus $[E \cdot ATP] \sim 0.5\ \mu M$ can be easily obtained from eqn 2. Using the rate constants obtained here, namely $k_1 = 3.0 \times 10^6$ $M^{-1} \cdot s^{-1}$ and $k_2 = 0.05$ s^{-1}, $[ATP_f]$ should be equal to 1.7×10^{-8} M. From curve 2 of fig.1 it can be seen that at 1.0 μM nucleotide-depleted F_1-ATPase and 0.5 μM ATP added, $[ATP_f]$ is about 15 nM which agrees well with the calculated value. From the rate constant values reported in [3,5] the calculated values of $[ATP_f]$ differ from the measured concentration by at least one order of magnitude.

Thus, we can conclude that nucleotide-depleted F_1-ATPase catalyses steady-state ATP hydrolysis by the single catalytic site. The K_m value for ATP in this mode of enzyme action is about 10^{-8} M and the catalytic turnover number of F_1-ATPase is 0.05 s^{-1}. In the course of single-site ATP hydrolysis the dynamic equilibrium ATP $\rightleftharpoons$ ADP + P_i with $K_{eq.} \leqslant 0.7$ is established at the F_1-ATPase catalytic site and the ADP release step limits the overall enzyme turnover.

The present results support the concept of Boyer and colleagues [14,15] on the energy requirement for the net ATP-synthesis reaction catalysed by F_oF_1-ATPases. Indeed, the equilibrium constant for the reaction ATP $\rightleftharpoons$ ADP + P_i at the enzyme catalytic site is close to unity and pyrophosphate bond formation occurs with a negligible free energy change. Thus, the energy-consuming steps of the net ATP-synthesis reaction must be those involved in changes of the affinities of the enzyme catalytic sites to substrates (products).

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