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Uni-site catalysis by *Escherichia coli* F_1-ATPase with different numbers of bound nucleotides

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We prepared two types of *E. coli* F_1 by slightly different gel filtration procedures of the purified F_1: F_1(II) contained about 2 mol, and F_1(V) about 5 mol of bound adenine nucleotides per mol of the enzyme. Thus F_1(II) had more than 2, possibly 3, vacant catalytic sites, while F_1(V) had less than one vacant catalytic site. The rate of ATP hydrolysis in uni-site catalysis (in the presence of inorganic phosphate) was about 3-fold higher with F_1(II) than with F_1(V), suggesting that ADP and inorganic phosphate bound at the catalytic sites of F_1(V) changed the kinetics of uni-site catalysis significantly.

ATPase, H^+-; F_1; Uni-site catalysis; Bound nucleotide; (*Escherichia coli*)

1. INTRODUCTION

The H^+-ATPase of oxidative phosphorylation is formed from a catalytic sector F_1 (F_1-ATPase) and a H^+ pathway F_0 (for reviews, see [1–4]). Six nucleotide-binding sites have been found in F_1 from bovine mitochondria [5,6] and *E. coli* [7,8], and classified as 3 catalytic (exchangeable) and 3 non-catalytic (non-exchangeable) sites according to their abilities to exchange bound nucleotide rapidly during multi-site (steady-state) catalysis in the presence of excess ATP. Mitochondrial F_1 preparations with different numbers of bound nucleotides have been prepared [9–11]. Kinetic studies on ATP hydrolysis by F_1 indicated the presence of positive cooperativity in multi-site catalysis. ATP is hydrolyzed only slowly in uni-site (single site or non-steady-state) catalysis in which the molar ratio of ATP to F_1 is less than 1, but the rate of ATP hydrolysis becomes 10^4–10^6-fold faster on addition of excess ATP (multi-site catalysis) [12,13]. Thus the rate of ATP hydrolysis and release of ADP and phosphate from the first catalytic site is accelerated by the binding of ATP to the second and third catalytic sites. However, the effects of binding of nucleotides to the catalytic or non-catalytic sites on uni-site catalysis have not been reported.

In this study we prepared two types of *E. coli* F_1, F_1(II) and F_1(V) containing 2 and 5 mol, respectively, of nucleotides bound per mol of F_1, and found that uni-site catalysis was significantly faster with F_1(II) than with F_1(V).

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2. EXPERIMENTAL PROCEDURES

F_1 was purified from *E. coli* strain KY7485 as described [14], and stored at −80°C in 50 mM Tris-Cl (pH 8.0) containing 1.0 mM ATP, 0.5 mM EDTA/Na and 10% glycerol. Samples of F_1 with defined numbers of bound nucleotides (F_1(II) and F_1(V)) were prepared at 22–25°C by passing the stored F_1 (1–4 mg/ml) through a centrifuge column [15] (Sephadex G-50 fine, 0.5 × 5.5 cm) equilibrated with TMP buffer (50 mM Tris-SO_4 (pH 8.0) containing 0.5 mM $MgSO_4$ and 1 mM K_2HPO_4) and TEP buffer (50 mM Tris-SO_4, pH 8.0, containing 0.5 mM EDTA/Na and 1 mM K_2HPO_4). The same buffers without 1.0 mM K_2HPO_4 were also used. The two preparations of F_1 had similar subunit ratios as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Coomassie brilliant blue staining) [14].

Uni-site hydrolysis of ATP was assayed as described [16]: 0.25 μM [γ-^{32}P]ATP was incubated at 25–27°C in 50 mM Tris-SO_4, pH 8.0, containing 1 mM K_2HPO_4 and 0.5 mM $MgSO_4$. For measurement of bound nucleotides, F_1 was incubated at 70°C for 10 min, stood on ice for 5 min, and centrifuged at 12000 × *g* for 10 min. The supernatant was subjected to high-performance liquid chromatography (HPLC) in a C_{18} column (MS Pack 0.46 × 15 cm) in 50 mM KH_2PO_4 containing 0.6 mM tetra-*N*-butylammonium bromide and 11–12% (v/v) methanol, pH 6.4 (flow rate 0.4 ml/min) and amounts of ATP and ADP were determined from the absorbances of the respective peaks at 259 nm. Protein was measured by the methods of Bradford et al. [17] (Bio-Rad protein assay reagent) and Smith et al. [18] (BCA protein assay reagent, Pierce) with bovine serum albumin (Pierce) as a standard. [α-^{32}P]ATP (21 Ci/mmol) and [γ-^{32}P]ATP (9 Ci/mmol) were purchased from DuPont/NEN Research Products. Radioactive bound nucleotides were measured in solvent-free scintillation medium (Ready Cap, Beckman). Other chemicals used were of the highest grade available commercially.

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3. RESULTS AND DISCUSSION

Kironde and Cross [9] reported that bovine F_1 stored as an ammonium sulfate precipitate contained 3 mol of adenine nucleotides per mol enzyme (2 at non-catalytic sites and 1 at a catalytic site) after its gel filtration through a centrifuge column. F_1 containing 5 mol of adenine nucleotides was obtained by incubating this F_1 with excess MgATP. Similarly, *E. coli* F_1 containing about 5 mol of ADP (F_1(V)) could be obtained by passing the F_1 (stored frozen in solution containing 1 mM ATP and 0.5 mM EDTA/Na) through a centrifuge column equilibrated with TMP buffer (table 1). F_1 containing only two adenine nucleotides (about 1 mol each of ADP and ATP) was obtained by passing the stored F_1 through two successive centrifuge columns in TEP buffer (F_1(II)*). The bound ATP in F_1(II)* was hydrolyzed to ADP in a centrifuge column equilibrated with TMP buffer (table 1, F_1(II)). F_1(II) could bind 3 more mol of ADP incubated with 2 mM ATP and 0.5 mM $MgSO_4$.

On incubation of F_1(II) with [α-^{32}P]ATP, F_1(V) with 2 unlabeled and 3 radioactive adenine nucleotides was obtained. When this radioactive F_1 was incubated with 2 mM ATP and 0.5 mM $MgSO_4$, the 2 radioactive nucleotides were rapidly exchanged with unlabeled nucleotides (fig.1), although the total number of bound nucleotides, estimated by HPLC did not change. The radioactive nucleotides were not exchanged during incubation without ATP. These results suggest that two radioactive adenine nucleotides bound to the catalytic sites were rapidly replaced by unlabeled nucleotides during multi-site catalysis of the enzyme. The other nucleotides (two unlabeled and one radioactive) may be at non-catalytic sites. The radioactive adenine nucleotide remaining in F_1 was released from the non-catalytic site when F_1 was passed through two centrifuge columns equilibrated with TEP buffer, indicating that the nucleotide was at the non-catalytic site, as shown for bovine F_1 [10]. As the radioactive F_1(V) formed from F_1(II) had 2 adenine nucleotides at the catalytic sites, F_1(V) had less than one vacant catalytic site, and F_1(II) had at least 2, possibly 3, vacant catalytic sites. F_1(II) and F_1(V) could also be prepared using centrifuge columns equilibrated with phosphate-free buffer (data not shown).

We compared the kinetics of uni-site catalysis with F_1(II) and F_1(V), which have different numbers of va-

Table 1

Preparations of F_1 with defined numbers of bound nucleotides

F_1	Procedure	Bound nucleotides (mol/mol F_1)		
		ADP	ATP	Total
F_1(V)	F_1 ⟶ cc(TMP)	4.9	0.0	4.9
F_1(II)*	F_1 ⟶ cc(TEP) × 2	1.2	0.9	2.1
F_1(II)	F_1(II)* ⟶ cc(TMP)	2.3	0.0	2.3
F_1(V)	F_1(II) ⟶ cc(TMP) ⟶ +Mg·ATP ⟶ cc(TMP)	5.1	0.0	5.1

F_1 that had been stored at −80°C in 50 mM Tris-Cl, pH 8.0, containing ATP, EDTA and glycerol was thawed and applied to a centrifuge column (cc) equilibrated with TMP or TEP buffer. When necessary, samples were applied to two successive centrifuge columns (× 2). Values are averages for multiple assays (at least 8 assays). Deviations were within 10–20%. F_1s prepared by the same procedures but with potassium phosphate-free buffers had essentially the same amounts of bound nucleotides as those prepared in the above conditions

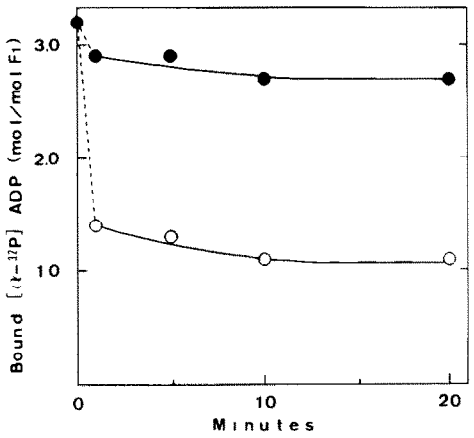

Fig.1. Release of radioactive adenine nucleotides from catalytic and non-catalytic sites of F_1(V). F_1(V), containing two unlabeled and 3 radioactive adenine nucleotides, was incubated in 50 mM Tris-SO_4, pH 8.0, containing 0.5 mM $MgSO_4$ with (○) or without (●) 2 mM ATP. At the times indicated, aliquots of the incubation mixtures were introduced into centrifuge columns equilibrated with TMP buffer, and the radioactivity in F_1 was determined.

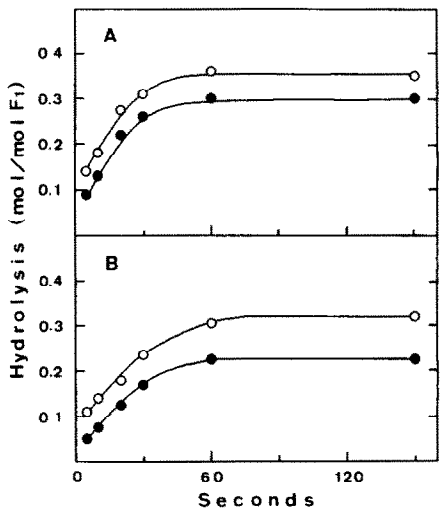

Fig.2. Uni-site hydrolysis of ATP by F_1(II) and F_1(V). Mixtures of 0.25 μM [γ-^{32}P]ATP and 0.5 μM F_1(II) (A) or F_1(V) (B) in 50 mM Tris-SO_4, pH 8.0, containing 1 mM K_2HPO_4 and 0.5 mM $MgSO_4$ were incubated for the indicated times. Reactions were terminated by addition of perchloric acid (●) (acid quench) or 1 min after addition of 2 × 10^4 molar excess of non-radioactive ATP (○) (cold chase).

Table 2

Uni-site hydrolysis of ATP by F_1 preparations with defined numbers of bound nucleotides

Assay condition	F_1	k_1 ($\times 10^{-4}$ $M^{-1} \cdot s^{-1}$)	Initial rate ($\times 10^{-9}$ mol/mg·min)
+ K_2HPO_4	F_1(V)	4.2	1.6
	F_1(II)	8.3	4.7
	F_1(V)[a]	5.3	1.6
− K_2HPO_4	F_1(V)	6.9	5.1
	F_1(II)	7.7	5.3

[a] F_1(V) obtained from F_1(II) (see table 1)

k_1 is the rate constant of binding of ATP to F_1 in uni-site hydrolysis and was calculated from the results of cold chase experiments (fig.2). The initial rate of ATP hydrolysis was determined from the amount of inorganic phosphate released after acid quench. Preparations of F_1 had the numbers of nucleotides shown in table 1

cant catalytic sites. The initial rate (acid quench) of ATP hydrolysis by uni-site catalysis (determined by measuring radioactive inorganic phosphate bound to F_1 and released into the medium) by F_1(II) was about 4.7×10^{-9} mol/mg·min and was 3-fold faster than the initial rate (1.6×10^{-9} mol/mg·min) catalyzed by F_1(V) (fig.2). The release of radioactive inorganic phosphate on addition of excess unlabeled ATP (cold chase) was also measured: the value of k_1 (the rate of binding of ATP) of F_1(II) was 1.6 ~ 2.0 times that of F_1(V) (table 2). The F_1(V) formed from F_1(II) showed essentially the same values of k_1 and initial rate of uni-site hydrolysis as F_1(V) obtained directly from the stored F_1 (table 2). Similarly, F_1(II) formed from F_1(V) showed essentially the same kinetic properties as F_1(II) prepared directly from the stored F_1 (data not shown). These results indicate that the low activity of F_1(V) was not due to irreversible change (e.g. denaturation) of the enzyme.

The F_1(V) showed a lower rate of uni-site catalysis than F_1(II) when the hydrolysis was assayed in the presence of inorganic phosphate, but essentially the same values of k_1 and initial rate of hydrolysis as F_1(II) in the absence of inorganic phosphate (table 2, K_2HPO_4). It should be noted that we could not obtain F_1 without bound nucleotides by passing F_1 through a gel filtration column with 50% glycerol, as previously reported for *E. coli* F_1 [7,8,19].

The present results clearly indicate that change in the kinetics of uni-site catalysis by F_1 in the presence of inorganic phosphate depends on the preparation of F_1, possibly due to differences in the numbers of vacant catalytic sites in the F_1 molecule. As described above, F_1(V) had only a single vacant catalytic site, whereas F_1(II) had possibly 3 vacant catalytic sites. Thus we conclude that the presence of ADP in the catalytic sites of F_1(V) together with inorganic phosphate significantly lowered the rate of uni-site catalysis of ATP at the third catalytic site.

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