A minimum catalytic unit of F_1-ATPase shows non-cooperative ATPase activity inherent in a single catalytic site with a K_m 70 μM

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Abstract **F_1-ATPase has three interacting catalytic sites and shows complicated kinetics. Here, we report reconstitution of a complex, most likely composed of one α subunit and one β subunit, with a single catalytic site from thermophilic *Bacillus* PS3 F_1-ATPase on the solid surface. The complex has an ATPase activity which obeys a simple non-cooperative kinetics with a K_m(ATP) of 70 μM and a V_{max} of 0.1 unit/mg. Different from F_1-ATPase, the complex is not inactivated by 7-chrolo-4-nitrobenzofrazan. Thus, the inherent activity attributable to a single catalytic site unaffected by other catalytic sites of F_1-ATPase is characterized.**

Key words: F_1-ATPase; F_1-ATPase core unit; F_1-ATPase α/β heterodimer; F_1-ATPase cooperativity; 7-Chrolo-4-nitrobenzofrazan

1. Introduction

F_oF_1-ATP synthase catalyzes ATP synthesis coupled with the proton flow across membrane [1–3]. The catalytic portion of the enzyme, F_1-ATPase, is consisted of five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ [4]. Both α and β subunits have AT(D)P binding sites [5,6] but none of them alone has ATPase activity [7]. Subunit complexes of F_1-ATPase from a thermophilic *Bacillus* strain PS3 (TF_1) with various subunit compositions have been reconstituted [8] and the ATPase-active complex with minimum subunit composition so far purified is the $\alpha_3\beta_3$ complex [9,10]. The reason of requirement for *both* α and β subunits for the ATPase activity is now well understood from crystal structure of bovine heart mitochondrial F_1-ATPase in which catalytic sites of F_1-ATPase are constructed mainly by the residues of β subunits but a part of the α subunit also contributes [11].

F_1-ATPase exhibits kinetic complexity; it shows positive cooperativity in catalysis at low concentration of ATP (uni-site catalysis and chase promotion), negative cooperativity with respect to ATP binding, and, under steady state catalysis condition, apparently negative cooperativity [12–16]. These complexity has been interpreted as a result of sequential alternation of characteristics of each of three catalytic sites on F_1-ATPase [3]. It has been proposed that this alternation is possibly mediated by the rotation of central 'axis' part consisting of single copy subunits such as the γ subunit, and the structure of bovine mitochondrial F_1-ATPase at 2.8 Å resolution is thought to be compatible with physical rotation of the γ subunit in the center of $\alpha_3\beta_3$ structure [11]. If so, the $\alpha_3\beta_3$ complex of TF_1 is expected to show simple Michaelis-Menten type kinetics because it lacks single copy subunits.

However, kinetic analysis of the $\alpha_3\beta_3$ complex is not easy because the $\alpha_3\beta_3$ complex of TF_1 is in dynamic equilibrium with the $\alpha\beta$ heterodimers during ATP hydrolysis as shown by gel filtration and X-ray scattering [17,18]. From analysis of photoaffinity labeling by benzoyl-benzoyl ADP [19] and from the fact that ATPase activity was detected at a protein band corresponding to the $\alpha_1\beta_1$ heterodimer in non-denaturing polyacrylamide gel electrophoresis [20], Kagawa and his colleagues indicated that the $\alpha\beta$ heterodimer is ATPase-active. An ATPase-active $\alpha\beta$ heterodimer was also isolated from chromatophores of *Rhodospirillum rubrum* [21]. The V_{max} and K_m values of the *R. rubrum* $\alpha\beta$ heterodimer are 0.11 unit per mg of protein and 0.1 mM, respectively. Therefore, it is obvious now that ATPase-active minimum unit of F_1-ATPase is $\alpha\beta$ heterodimer. However, the *R. rubrum* $\alpha\beta$ heterodimer tends to dissociate into α and β subunit monomers and it is stable only in the presence of ATP at more than 0.1 mM and Mg^{2+}. Dilution of the protein concentration of the *R. rubrum* $\alpha\beta$ heterodimer also induces the dissociation. By this reason, ATPase activities were measured only at ATP concentration range higher than 0.1 mM, and the question whether ATPase activity of the $\alpha\beta$ heterodimer obeys Michaelis-Menten type or cooperative kinetics has not been answered. Here, we report reconstitution of an ATPase-active subunit complex which contains only a single β subunit, most likely $\alpha\beta$ heterodimer, using TF_1-β subunit immobilized on a solid surface. This complex reasonably obeys simple Michaelis-Menten type kinetics and the Km for ATP inherent in a single catalytic site, unaffected by other catalytic sites, is 70 μM.

2. Materials and methods

The wild-type α and β subunits of TF_1 were expressed in the *E. coli* DK8 strain which lacks whole F_oF_1 genes [22]. A mutant β subunit (βCAS) which contains an insertion of three amino acids, Cys-Ala-Ser, between the amino-terminal Met and the next Thr was expressed in the *E. coli* NM522 strain. These subunits were individually isolated as described in the previous report [23].

The complex containing a single β subunit was reconstituted on a solid surface with modifications of the procedures described previously (Fig. 1) [9]. Prior to use, 8 mg of the βCAS was dissolved in 1 ml of 20 mM Tris-SO_4 buffer (pH 8.0) containing 1 mM dithiothreitol and incubated for 30 min at room temperature to cleave residual disulfide bonds between the subunits. Dithiothreitol was removed by gel filtration through a Sephadex G-50 column equilibrated with 20 mM Tris-SO_4, 50 mM Na_2SO_4 (pH 7.5) (buffer A) and 0.4 g of thiopropyl-Sepharose 6B beads (TS-6B, Pharmacia) preswollen in 4 ml of the buffer A was added to the βCAS solution. The mixture was incubated for 30 min at room temperature with gentle stirring and then beads were washed

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Abbreviations: Nbf-Cl, 7-chrolo-4-nitrobenzofrazan; TF_1, F_1-ATPase from thermophilic *Bacillus* PS3; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; TS-6B, thiopropyl-Sepharose 6B.

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thoroughly with 200 ml of the buffer A. Most of the βCAS were immobilized on the beads. The α subunit (10 mg) in 1 ml of 20 mM Tris-SO_4 (pH 8.0) was mixed with the βCAS loaded beads and incubated for 2 h at 30°C with gentle stirring. The beads were washed with 200 ml of the buffer A to remove free α subunit. Reconstituted subunit complexes containing a single β subunit were used without detaching from the beads to avoid generation of $\alpha_3\beta_3$ complex.

ATPase activity was determined by measuring liberated P_i [24]. Reaction was started by addition of 20 μl of the concentrated (5×) assay mixture containing 250 mM Tris-Cl, 250 mM Na_2SO_4, 25 mM ATP-Na and 25 mM $MgCl_2$ to 80 μl of the beads suspension which contained 20–40 μg (protein) of immobilized complexes. The reaction was stopped by adding 100 μl of 2% perchrolic acid at an appropriate time and, after addition of 900 μl of the acetate buffer for P_i measurement [24], the beads were removed from the suspension by centrifugation. From the supernatant, 900 μl was transferred to another test tube and used for determination of the amounts of released P_i. When the ATPase activity was examined as a function of ATP concentration, the concentrated (5×) assay mixture containing 250 mM Tris-Cl, 250 mM Na_2SO_4, 25 mM $MgCl_2$, 100 μg/ml pyruvate kinase, 25 mM sodium phosphoenolpyruvate, 50 mM KCl, and appropriate concentration of ATP-Na was used. All assays were performed at 30°C. One unit of activity is defined as that hydrolyzes 1 μmol of ATP per min.

Inactivation by 7-chrolo-4-nitrobenzofrazan (Nbf-Cl) was started by mixing 2 ml of 400 μM Nbf-Cl solution in the buffer A with 2 ml of the suspension of beads with immobilized complexes (0.1 g beads/ml). As control experiments, TF_1 and $\alpha_3\beta_3$ complex were suspended separately in 2 ml of untreated TS-6B beads suspensions and used for inactivation assays. The mixtures were incubated at 30°C. At the time indicated, 400 μl of beads suspension was withdrawn and the beads were precipitated by centrifugation. The supernatant (300 μl) was removed and 500 μl of the buffer A was added to the precipitated beads. Then, 30 μl of the beads suspension was withdrawn and ATPase activity was measured as described above. For inactivation assays of TF_1 and $\alpha_3\beta_3$ complex, 5 μl of the inactivation suspension was withdrawn, diluted to 80 μl with water, and ATPase activity was measured.

Protein concentrations were determined by the method of Bradford [25] using bovine serum albumin as a standard. For the complexes immobilized on TS-6B, protein concentration was measured after the complexes were released from the beads by washing with 5 mM dithiothreitol. The concentration of acrylamide in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was 13% [26].

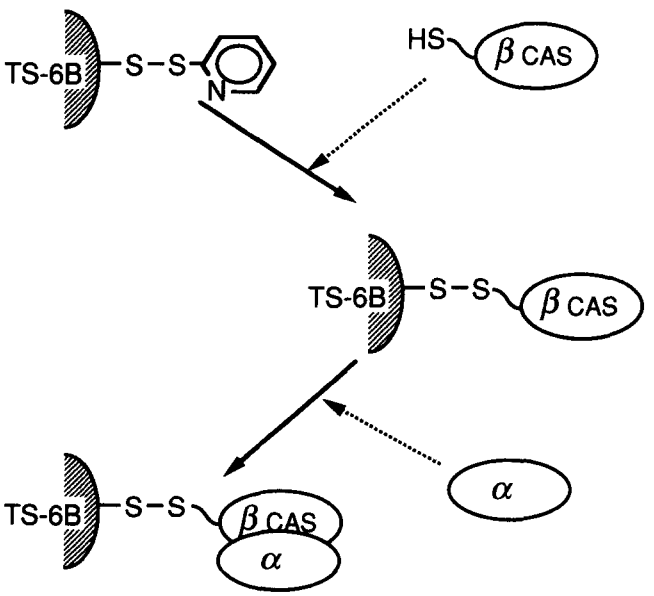

Fig. 1. Schematic illustration of the procedure to reconstitute a minimum catalytic unit on the surface of TS-6B beads.

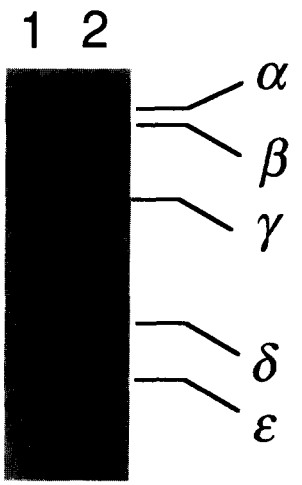

Fig. 2. SDS-PAGE analysis of the protein fraction released from TS-6B beads with the buffer containing dithiothreitol. Lane 1 = released proteins; lane 2 = TF_1. The positions of each subunit are indicated.

3. Results and discussion

3.1. Reconstitution of the complexes on the beads

For reconstitution of the complexes on the TS-6B beads, βCAS was immobilized on the surface of TS-6B beads by disulfide bond (Fig. 1) [27]. Introduction of Cys-Ala-Ser sequence at the amino-terminus of the β subunit did not have significant effect on the activity of the subunit complex containing this mutant [27]. The wild-type β subunit does not contain Cys and mutationally induced Cys in the βCAS is a sole sulfhydryl group in the protein. The location of Cys in the βCAS is next to amino-terminal Met and from crystal structure of mitochondrial F_1-ATPase it turns out to be in the amino-terminal flexible tail at the 'top' of the molecule, an ideal location for other subunits to get contact to form assembly [11]. The α subunit has one Cys residue, but the sulfhydryl group of this Cys is inaccessible by sulfhydryl reagents unless the protein is denatured [4]. Actually, α subunit was not bound or absorbed to TS-6B beads unless βCAS was immobilized previously (data not shown). Reconstitution of the complexes was confirmed by SDS-PAGE analysis of the proteins released from beads by washing with 5 mM dithiothreitol (Fig. 2). The released protein solution contained only α and β subunits and the staining intensity of the β subunit in SDS-PAGE was about double of that of the α subunit indicating that, given that the reconstituted complex was αβ heterodimer, about 40–60% of immobilized βCAS was successfully utilized for reconstitution. In the crystal structure of mitochondrial F_1-ATPase, there are two kinds of α/β contact regions; one has a catalytic AT(D)P site and another has a non-catalytic AT(D)P site [11]. The complex reconstituted on TS-6B beads described here, as well as αβ heterodimer of *R. rubrum*, should contain α/β contact region where a catalytic AT(D)P site resides because the complex has catalytic activity as shown below. A mention should also be added on a possibility that αβα type complexes are formed on TS-6B beads. This possibility is unlikely since $\alpha_3\beta_3$ structure has been thought to be three pairs of αβ units rather than αβα plus βαβ units [11,17,20,28]. Furthermore, when the immobilized complex on the beads was released into solution, it readily formed the $\alpha_3\beta_3$

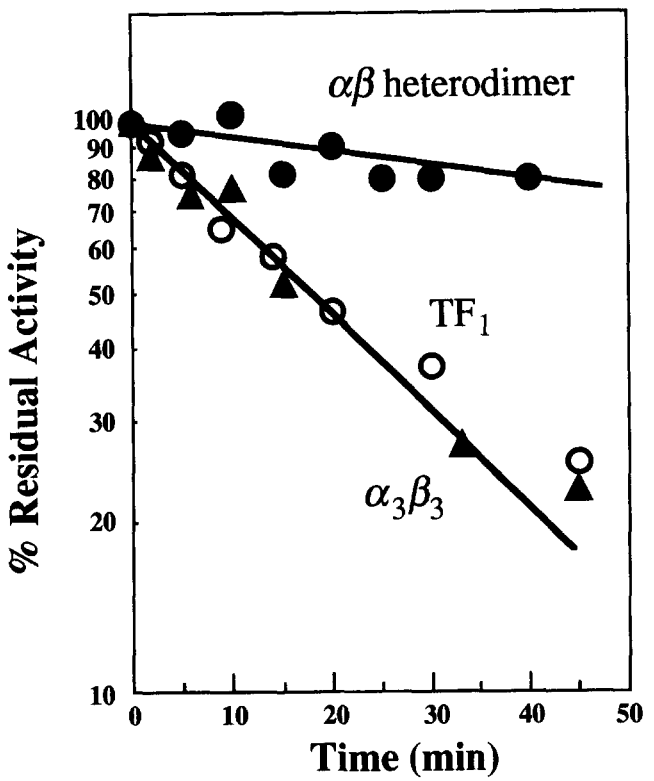

Fig. 3. Inactivation of the minimum catalytic unit (indicated as $\alpha\beta$ heterodimer) (●), the $\alpha_3\beta_3$ complex (▲), and TF_1 (○) by 200 μM Nbf-Cl. Experimental details are described in section 2.

complex which was confirmed by gel filtration chromatography (data not shown). This was impossible if the released complex had $\alpha\beta\alpha$ composition. Therefore, we hereafter call the reconstituted complex on the beads as $\alpha\beta$ heterodimer with a reservation that existence of $\alpha\beta\alpha$ type complexes is not absolutely denied.

3.2. *ATPase activity of the ab heterodimer*

We measured ATPase activity of the immobilized $\alpha\beta$ heterodimers without releasing them from beads since, as mentioned above, free $\alpha\beta$ heterodimers in solution can readily reassemble into the $\alpha_3\beta_3$ complexes. As shown in Table 1, the $\alpha\beta$ heterodimer reconstituted on the TS-6B beads retained about 15% of ATPase activity of that of the $\alpha_3\beta_3$ complex. This specific activity might be an underestimation because, as described above, 40–60% of the immobilized βCAS did not form $\alpha\beta$ heterodimer and values of protein concentrations used for calculating specific activity was the sum of the $\alpha\beta$ heterodimers and βCAS which failed to catch α subunit. Taking this fact into consideration, $\alpha\beta$ heterodimer may have ~25% of ATPase activity of that of the $\alpha_3\beta_3$ complex and ~5% of TF_1. Free βCAS, as well as immobilized βCAS, have no detectable ATPase activity. Thus, it is clear that both α and β subunits are necessary for ATPase activity and this $\alpha\beta$ heterodimer is the minimum unit for the ATPase activity. The specific ATPase activity of the $\alpha\beta$ heterodimer of TF_1 is about double of that of *R. rubrum* $\alpha\beta$ heterodimer (0.11 unit/mg) [21].

3.3. *Large Ca^{2+}-dependent activity and resistance to azide inhibition*

Similar to TF_1 and the $\alpha_3\beta_3$ complex, ATPase activity of the $\alpha\beta$ heterodimer is dependent on divalent cation. However, different from TF_1 and the $\alpha_3\beta_3$ complex, Ca^{2+}-dependent ATPase activity of the $\alpha\beta$ heterodimer is extraordinarily large, 850% of the Mg^{2+}-dependent activity, whereas those of TF_1 and the $\alpha_3\beta_3$ complex are 80% and 250% of the Mg^{2+}-dependent activities, respectively. The $\alpha\beta$ heterodimer from *R. rubrum* is activated by Ca^{2+} but the extent of activation is about two-fold of the Mg^{2+}-dependent activity [21]. As expected from previous results that the $\alpha_3\beta_3$ complex and the $\alpha_3\beta_3\delta$ complex are insensitive to azide inhibition [8,9], the $\alpha\beta$ heterodimer is not inhibited by sodium azide at all.

3.4. *Resistance to Nbf-Cl inactivation*

As was first demonstrated for the mitochondrial F_1-ATPase [29] and later for all of F_1-ATPases from various sources [3], TF_1 is inactivated by Nbf-Cl when Tyr-307 is modified in a single β subunit [30]. The $\alpha_3\beta_3$ complex is also inactivated by modification of a single β subunit and thus one-third of the sites' reactivity of Nbf-Cl is not attributable to the presence of single copy subunit of F_1-ATPase such as γ, δ, and ε subunits [31]. The isolated free β subunit of TF_1 does not react with Nbf-Cl [31]. Then, we examined sensitivity of the $\alpha\beta$ heterodimer to Nbf-Cl inactivation. As shown in Fig. 3, the $\alpha\beta$ heterodimer is totally resistant to Nbf-Cl inactivation. When the concentration of Nbf-Cl was increased up to 500 μM, the result was the same (data not shown). For Tyr-307 to be reactive with Nbf-Cl, the β subunit should be incorporated into $\alpha_3\beta_3$ structure. Resistance of our preparation to Nbf-Cl inactivation also confirmed that our preparation of $\alpha\beta$ heterodimer was not contaminated by accidentally generated $\alpha_3\beta_3$ complex.

3.5. *Non-cooperative kinetics of the $\alpha\beta$* heterodimer

ATPase activity of immobilized $\alpha\beta$ heterodimer was carefully examined as a function of ATP concentrations from 2 μM to 3 mM. As mentioned, the isolated α and β subunits can bind AT(D)P and hence two AT(D)P molecules can bind to the $\alpha\beta$ heterodimer; one to the α/β contact region (catalytic site) and another to the α subunit (non-catalytic site). If kinetic cooperativity is generated from interaction between a catalytic AT(D)P binding site and an adjacent non-catalytic AT(D)P site, the $\alpha\beta$ heterodimer should show cooperative kinetics. However, in sharp contrast with the $\alpha_3\beta_3\gamma$, the $\alpha_3\beta_3\delta$ complexes, and TF_1 [8,32], the $\alpha\beta$ heterodimer exhibited a simple Michaelis-Menten type kinetics (Fig. 4). Eadie-Hofstee plot of the data showed a straight line indicating that there is no cata-

Table 1
ATPase activity of the $\alpha\beta$ heterodimer

Sample	ATPase activity (unit/mg)
TF_1	3.7 ± 0.66
$\alpha_3\beta_3$	0.75 ± 0.070
$\alpha\beta$ heterodimer	0.10 ± 0.012
β (CAS), free	≤0.002
β (CAS), immobilized	≤0.0006

ATPase activities were measured as described in section 2 except that 0.2 M, instead of 50 mM, of Na_2SO_4 was included in the assay mixture in the case of the $\alpha_3\beta_3$ complex. The minimum catalytic unit is expressed as $\alpha\beta$ heterodimer.

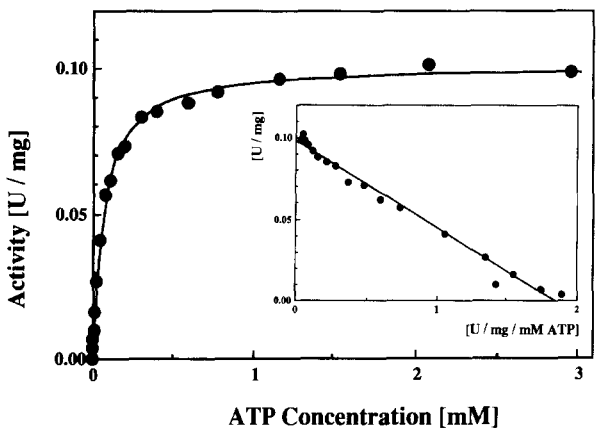

Fig. 4. ATPase activity of the minimum catalytic unit immobilized on TS-6B beads as a function of ATP concentrations. Inset: Eadie-Hofstee plots of the same data. Experimental details are described in section 2.

lytic cooperativity (Fig. 4, inset). This implies that catalytic cooperativity arises from interaction between two catalytic sites or, in other words, at least two catalytic sites are necessary for cooperativity. Probably, occupation of one catalytic site by the first substrate introduces an asymmetry to the enzyme in such a way that the second catalytic site gains the ability to stimulate catalytic efficiency upon occupied by the second substrate [33]. One-third of the sites' reactivity of the $\alpha_3\beta_3$ complex to Nbf-Cl is also explained by this induced asymmetry [31]. A K_m value obtained from Fig. 4 is 70 μM. Obviously, this value neither corresponds to the highest binding site where the single-site (uni-site) hydrolysis occurs when substoichiometric ATP is present [34] nor the lowest K_m values obtained from steady state kinetics of mitochondrial F_1-ATPase (1.7 μM) [13] and TF_1 (<10 μM) [16]. The K_m of 70 μM observed here should be the inherent one in a single isolated catalytic site without any influence from other catalytic sites and the catalytic site with a very high affinity to ATP may be generated either when the α and β subunits are organized in a hexamer structure or when a single copy subunit is attached to the $\alpha_3\beta_3$ structure.

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