ATP-binding affinity of the ε subunit of thermophilic F₁-ATPase under label-free conditions

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A B S T R A C T

The ε subunits of several bacterial F₁-ATPases bind ATP. ATP binding to the ε subunit has been shown to be involved in the regulation of F₁-ATPase from thermophilic *Bacillus* sp. PS3 (TF₁). We previously reported that the dissociation constant for ATP of wild-type ε subunit of TF₁ at 25 °C is 4.3 μM by measuring changes in the fluorescence of the dye attached to the ε subunit (Kato, S. et al. (2007) *J. Biol. Chem.* **282**, 37618). However, we have recently noticed that this varies with the dye used. In this report, to determine the affinity for ATP under label-free conditions, we have measured the competitive displacement of 2′(3′)-O-*N*′-methylaniloyl-aminoadenosine-5′-triphosphate (Mant-ATP), a fluorescent analog of ATP, by ATP. The dissociation constant for ATP of wild-type ε subunit of TF₁ at 25 °C was determined to be 0.29 μM, which is one order of magnitude higher affinity than previously reported values.

1. Introduction

ATP synthase (EC 7.1.2.2) catalyzes ATP synthesis from ADP and inorganic phosphate by using electrochemical H⁺ motive force across the various biological energy-transducing membranes such as bacterial cell membrane, mitochondrial inner membrane or thylakoid membrane of chloroplast through a unique subunit-rotating mechanism (reviewed in Refs. [1,2]). The ε subunit of the bacterial ATP synthase has a molecular mass of 14 kDa and regulates activity of ATP synthase, and its water-soluble subcomplex, F₁-ATPase [3]. The ε subunit consists of two distinct domains, N-terminal β-sandwich domain and C-terminal α-helical domain [4–6]. The former has a structural role as connecting the γ subunit of F₁ and the c-subunit ring of Fₒ to ensure the coupling of ATP hydrolysis/synthesis and H⁺ flow. The latter is not essential for the coupling, but has a regulatory role as an intrinsic ATPase inhibitor. When the C-terminal domain takes extended conformation, in which C-terminal α helices are inserted into the central cavity of F₁ along with the γ subunit, ATPase activity is inhibited [7,8]. When the C-terminal domain takes folded conformation, in which C-terminal α helices expelled out from the central cavity and folded into hairpin like structure beside the N-terminal domain, ATPase activity is recovered [9–11]. It has been shown that the ε subunits of F₁-ATPases from *Bacillus* sp. PS3 [12–14], *Bacillus subtilis* [15], *Caldalkalibacillus thermarum* [16], and

Escherichia coli [6] have the ability to bind ATP. The ATP binding stabilizes folded conformation [6]. In the case of F₁-ATPase from *Bacillus* sp. PS3 (TF₁), the ATP binding to the ε subunit directly concerns with the regulation and the coupling of ATPase and H⁺- pumping activities [14,17].

The isolated ε subunit also changes its conformation drastically depending on the ATP binding [6]. Owing to this characteristic and very high specificity for ATP, the ε subunit is utilized in various types of ATP-biosensor proteins [18–23]. ATP binding to the ε subunit of TF₁ has been shown first qualitatively by gel-filtration analysis [12], then quantitatively by fluorescence change induced by ATP binding to the fluorescently labeled ε subunit [13,14]. We have reported that the dissociation constant of wild type TF₁ ε subunit for ATP at 25 °C was 4.3 μM according to the measurement with *N*-ethyl-*N*′-{5-[*N*″-(2-maleimidoethyl) pyperazinocarbonyl] pentyl} indocarbocyanine (IC3)-labeled ε subunit [14]. However, we have recently noticed that the apparent affinity seemed to change by the dye attached to the ε subunit. From these observations, we attempted to measure ATP binding to TF₁ ε with non-labeled protein and non-labeled ATP. Although it was reported on *Escherichia coli* F₁ ε subunit that ATP binding affinity under label-free conditions by means of changes in the NMR chemical shift [6], there are no quantitative measurements on ATP binding to the ε subunit of TF₁ without any labeling.

To determine ATP-binding affinity of TF$_1$ ε subunit under label-free conditions, we first measure the binding of the fluorescent analog of ATP, 2′(3′)-O-N'-methylaniloyl-aminoadenosine-5′-triphosphate (Mant-ATP) [24] and determine its binding affinity. Then, by analyzing

$$\text{Fluorescence} = C_1$$
$$+ \frac{C_2\{K_{d-ATP}[\varepsilon] + K_{d-ATP}[\text{Man} - \text{ATP}] + K_{d-\text{Mant}}K_{d-ATP} + K_{d-\text{Mant}}[\text{ATP}] - \sqrt{(K_{d-ATP}[\varepsilon] + K_{d-ATP}[\text{Mant} - \text{ATP}] + K_{d-\text{Mant}}K_{d-ATP} + K_{d-\text{Mant}}[\text{ATP}])^2 - 4K_{d-ATP}^2[\varepsilon][\text{Mant} - \text{ATP}]}\}}{2K_{d-ATP}}$$

(Equation 1)

competitive binding of ATP and Mant-ATP, we have determined ATP-binding affinity of wild type TF$_1$ ε subunit under label-free condition.

2. Materials and methods

2.1. Measurement of ATP binding to Cy3-labeled ε subunit

ATP binding to TF$_1$ ε subunit (Q107C) labeled by Cy3-maleimide (GE healthcare) at the sole cysteine was measured as described previously [13,25]. Briefly, the cuvette containing 2 mL of Cy3-ε (2 nM) in the buffer consisted of 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl$_2$, and 0.1 mg/mL BSA was placed in an FP-6500 fluorescence spectrometer (JASCO) at 25 °C with continuous stirring. Then ATP was added consecutively from 1 nM to 50 μM. The fluorescence was recorded at 2 Hz with excitation and emission wavelengths at 522 nm and 559 nm, respectively.

2.2. Measurement of Mant-ATP binding to ε subunits

Titration of Mant-ATP binding with ε subunit was carried out as follows. The cuvette containing 2 mL of Mant-ATP (0.1 μM) in the buffer consisted of 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, and 10 mM MgCl$_2$ was placed in an FP-6500 fluorescence spectrometer (JASCO) at 25 °C with continuous stirring. Then, the ε subunit was added consecutively from 1 nM to 10 μM. The fluorescence was recorded at 2 Hz with excitation and emission wavelengths at 360 nm and 440 nm, respectively.

2.3. Measurement of displacement of Mant-ATP from ε subunit by ATP

Displacement of Mant-ATP from the ε subunit by ATP was measured as follows. The cuvette containing 2 mL of Mant-ATP (0.1 μM) in the buffer consisted of 50 mM HEPES-KOH (pH 7.5), 100 mM KCl, and 10 mM MgCl$_2$ was placed in an FP-6500 fluorescence spectrometer (JASCO) at 25 °C with continuous stirring. Then the ε subunit was added at 0.5–1.1 μM. After the fluorescence reached steady value, ATP solution was added consecutively from 1 nM to 20 μM. The fluorescence was recorded at 2 Hz with excitation and emission wavelengths at 360 nm and 440 nm, respectively.

2.4. Data analysis

The fluorescence value was taken at the time point where the fluorescence became steady after transient changes. ATP binding to the Cy3-labeled ε subunit was analyzed as described previously [13,25]. In the measurements for Mant-ATP binding, relative changes in the fluorescence to its initial value before addition of the ε subunit was plotted against the ε subunit concentration. The plot was fitted with a hyperbolic curve assuming 1:1 binding.

Displacement of Mant-ATP from the ε subunit by ATP was analyzed

assuming simple competitive binding. The fluorescence after addition of the ε subunit was set as 100%. The relative fluorescence was plotted against added ATP concentrations. The plot was analyzed by curve fitting with the following equation:

where, C_1 and C_2 are the constants, K_{d-ATP} is the dissociation constant of the ε subunit for ATP, K_{d-Mant} is the dissociation constant of the ε subunit for Mant-ATP, and the values in the square brackets represent total concentrations of each reactant [26]. OriginPro 9.0J (Origin Lab) was used for data analysis.

2.5. Other methods

ε subunit was prepared as described previously [25,27]. Mutant ε subunit containing Q107C in addition to R103A/R115A was used here and solely noted as R103A/R115A, hereafter. Protein concentrations were determined by the method of Bradford [28] using BSA as the standard and corrected by multiplying a factor 0.54 as described [12]. Mant-ATP was purchased from Sigma-Aldrich.

3. Results and discussion

3.1. ATP binding to Cy3-labeled ε subunit

From the titration experiment of Cy3-labeled ε subunit (Q107C) with ATP (Fig. 1), K_d for ATP (K_{d-Cy3}) was determined to be 0.50 μM at 25 °C. This value is significantly different from the value previously we reported (4.3 μM) by using IC3-labeled ε subunit [14]. These dyes share almost the same structure except that Cy3-maleimide has two sulfonate groups to increase solubility in water, although the linker structure is also slightly different [29,30]. These small differences may affect the ATP-binding property of the ε subunit. For example, hydrophobic
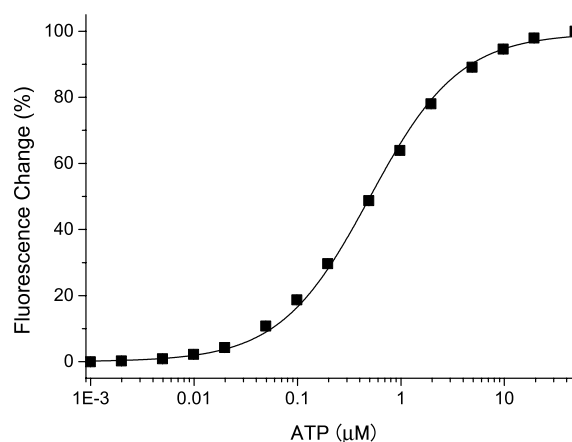

Fig. 1. ATP binding to Cy3-labeled ε subunit. Relative fluorescence changes induced by the addition of ATP are plotted against ATP concentration. Fluorescence change at 50 μM ATP was set as 100%. Solid line represents a theoretical curve obtained from curve fitting with experimental data by simple binding scheme (Fluorescence Change = %ΔFL$_{max}$ × [ATP]/(K_{d-Cy3} + [ATP])). The K_{d-Cy3} and %ΔFL$_{max}$ obtained (± standard error) are 0.50 ± 0.02 μM and 99.4 ± 0.7%.
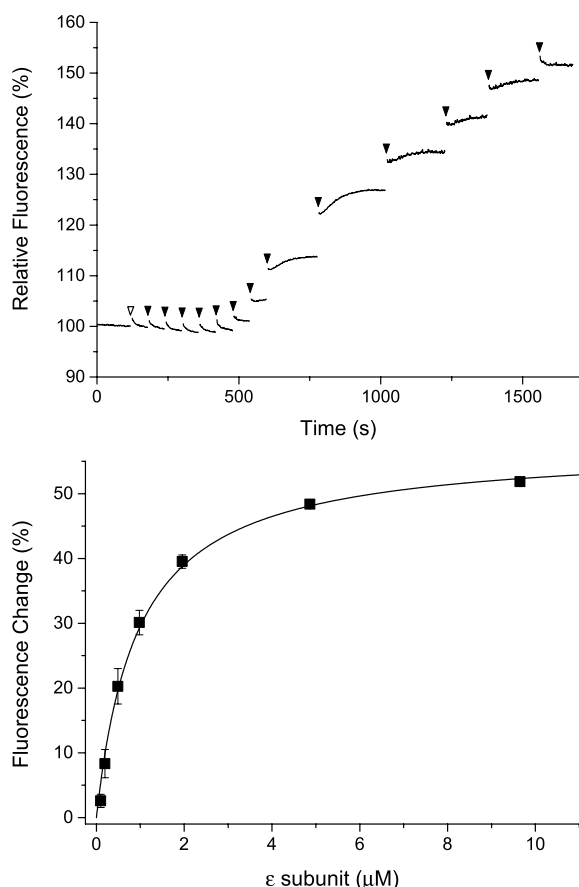
Fig. 2. Mant-ATP binding to label-free ε subunit. (A) An example of time-course of changes in the fluorescence of 0.1 μM Mant-ATP induced by the ε subunit is shown. Open arrowhead represents buffer addition as a control. Closed arrowheads represent addition of the ε subunit. ε subunit was added consecutively to achieve final concentration at 1 nM to 10 μM. Initial fluorescence in the absence of ε subunit was set as 100%. (B) Fluorescence changes are plotted against ε subunit concentration. Solid line represents a theoretical curve obtained from curve fitting with experimental data by simple binding scheme (Fluorescence Change = %ΔFL_{max} × [ε]/(K_{d-Mant} + [ε])). The K_{d-Mant} and %ΔFL_{max} obtained (± standard error) are 0.94 ± 0.11 μM and 57 ± 1%.
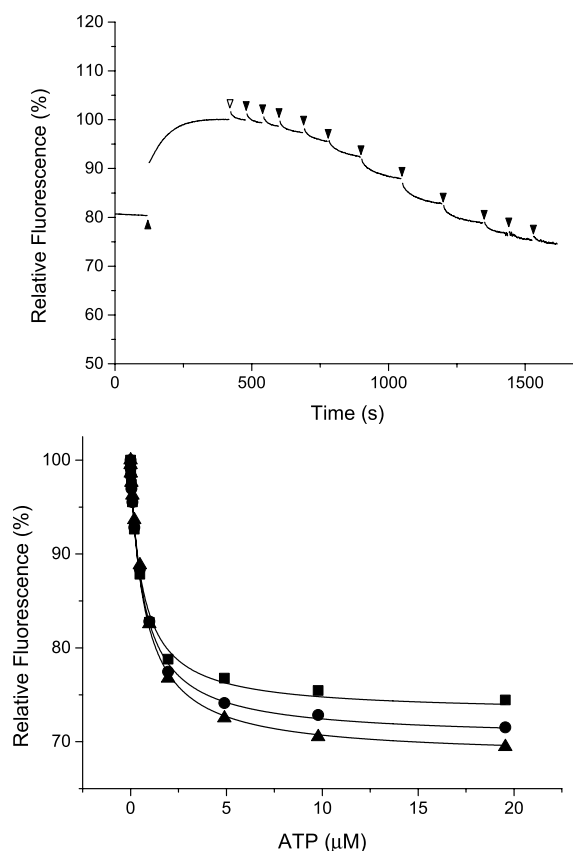

Fig. 3. Displacement of Mant-ATP from ε subunit by ATP. (A) An example of time-course of changes in the fluorescence of 0.1 μM Mant-ATP induced by the ε subunit followed by ATP is shown. An upward closed arrowhead represents addition of 0.52 μM ε subunit. An downward open arrowhead represents buffer addition as a control. Downward closed arrowheads represent addition of ATP. ATP was added consecutively to achieve final concentration at 10 nM to 20 μM. Fluorescence in the presence of ε subunit was set as 100%. (B) Relative fluorescence is plotted against ATP concentration. Three experiments with different ε subunit concentrations (solid square, solid circle, and solid triangle represent 0.52, 0.93, and 1.08 μM, respectively) are shown. Solid lines represent theoretical curves obtained from global curve fitting with experimental data by the equation (1). The K_{d-ATP} obtained (± standard error) is 0.29 ± 0.01 μM.

interactions between IC3- and hydrophobic region in the N-terminal domain of the ε subunit, which binds to the γ subunit, may affect conformational flexibility and impede ATP-binding to the ε subunit. From these observations, we have attempted to determine the ATP binding to the ε subunit under non-labeled conditions.

3.2. Mant-ATP binding to wild-type ε subunit

Mant-ATP, a fluorescent analog of ATP modified at 2′ or 3′ of ribose with N-methylanthranilate, was first synthesized by Hiratsuka [24]. Addition of the ε subunit to Mant-ATP resulted in the increase in its fluorescence (Fig. 2A) as previously reported on other ATP-binding proteins [24,26]. The increase in the fluorescence saturated above 5 μM of the ε subunit, indicating that the changes in the fluorescence are due to the specific binding of Mant-ATP to the ε subunit, not by the non-specific one. Although the stoichiometry of the ATP binding cannot be determined by this measurement, we assumed 1:1 binding based on the crystal structure [6]. Titration of fluorescence by the ε subunit followed simple binding scheme (Fig. 2B), and the K_d of the ε subunit for Mant-ATP binding (K_{d-Mant}) was determined to be 0.94 μM.

3.3. Mant-ATP displacement by ATP from ε subunit

The affinity measured in above section (K_{d-Mant}) is for Mant-ATP, not for ATP. To measure the ATP-binding affinity with label-free ε subunit and ATP, the displacement of Mant-ATP by ATP was measured. Fluorescence of the Mant-ATP bound ε subunit was decreased by the addition of ATP (Fig. 3A). The decrease in fluorescence was saturated with increasing ATP concentration. The degree of displacement depends on concentrations of both ATP and Mant-ATP and affinities for both ATP and Mant-ATP (equation (1)). Global fitting with simple competitive binding scheme (equation (1)) to three individual sets of measurements yielded K_d value for ATP (K_{d-ATP}) as 0.29 μM (Fig. 3B). This value is lower than previously reported value obtained with IC3-labeled ε subunit (4.3 μM) and in the same range as that obtained from Cy3-labeled ε subunit (K_{d-Cy3}:0.50 μM). From these observations, it can be said that Cy3-is better than IC3- to monitor the ATP binding to the ε subunit at least in this particular case. The lower affinity in the previous report may be mostly due to the attachment of IC3-, which may impede ATP binding to the ε subunit as discussed in the section 3.1.

The K_{d-Mant} is in the same range with the K_{d-ATP}, indicating that Mant-group attached to the ribose moiety of ATP does not impede binding to the ε subunit very much. This agrees with the crystal structure of the ATP-bound ε subunit, in which the ribose moiety of the
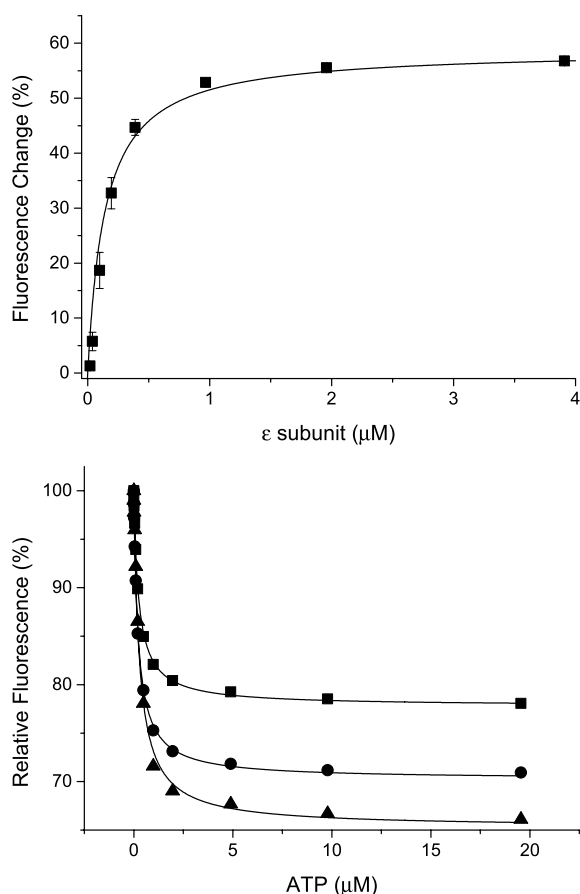
Fig. 4. Mant-ATP binding to and displacement from R103A/R115A mutant ε subunit. (A) Fluorescence changes upon Mant-ATP binding to the R103A/R115A mutant ε subunit were measured as in Fig. 2A and plotted as Fig. 2B. The solid line represents a theoretical curve obtained from curve fitting with experimental data by simple binding scheme (Fluorescence Change = %ΔFL$_{max}$ × [ε]/($K_{d\text{-Mant}}$ + [ε])). The $K_{d\text{-Mant}}$ and %ΔFL$_{max}$ obtained (± standard error) are 0.14 ± 0.03 μM and 59 ± 1%, respectively. **(B)** Displacement of Mant-ATP from the R103A/R115A mutant ε subunit was observed as Fig. 3A and relative fluorescence is plotted as Fig. 3B. Three experiments with different ε subunit concentrations (solid square, solid circle, and solid triangle represent 0.17, 0.12, and 0.21 μM, respectively) are shown. Solid lines represent theoretical curves obtained from global curve fitting with experimental data by the equation (1). The $K_{d\text{-ATP}}$ obtained (± standard error) is 0.10 ± 0.00 μM.

ATP bound to the ε subunit opens to the solvent [6].

3.4. Measurement with mutant ε subunit

Measurement with high-affinity ATP-binding mutant ε subunit (R103A/R115A) was carried out (Fig. 4). The $K_{d\text{-Mant}}$ and $K_{d\text{-ATP}}$ of the R103A/R115A mutant were determined to be 0.14 μM and 0.10 μM, respectively. These values are again in the same range, indicating that the above discussion on the wild type is also the case for this mutant. The $K_{d\text{-ATP}}$ is similar to the $K_{d\text{-Cy3}}$ (52–160 nM) [25], implying that Cy3-labeling does not affect ATP-binding very much also in the case of the mutant. Comparison with the wild-type ε subunit indicates that the mutation increases ATP binding affinity about 3-fold.

It should be noted that, it is practically difficult to apply the method described here to the low affinity mutant or ε subunit with low ATP-binding affinity, such as *Bacillus subtilis* F$_1$ ε subunit [15] since very high concentration of ε subunit is required. Actually, we have attempted to measure Mant-ATP binding to the R92A mutant TF$_1$-ε subunit, which has been shown to have low affinity for ATP [14], no

fluorescence change of Mant-ATP was observed upon addition of up to 16 μM of the ε subunit (data not shown). Although we should be careful that the fluorescent dye may affect the ATP-binding property of the ε subunit as shown here, the measurement with fluorescence is still a useful and practical method to determine binding affinity.

One should carefully consider ATP-binding affinity of the ε subunit when designing ATP-sensor proteins and calibrate thus constructed protein as they may also be varied by the introduction of reporter domains such as green fluorescent protein.

CRediT authorship contribution statement

Miria Fujiwara: Investigation, Formal analysis, Writing - original draft. **Yasuyuki Kato-Yamada:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition.

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