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

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ABSTRACT

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 [12–14]. 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-′-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 ε-subunit ring of F0, 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-maleimidooethyl) perpyrazinocarboxyl] 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.

**Abbreviations:** $K_d$-ATP, dissociation constant of ε subunit for ATP; $K_d$-Cy3, dissociation constant of Cy3-labeled ε subunit for ATP; $K_{d,Mant}$-ATP, dissociation constant of ε subunit for Mant-ATP; Mant-ATP, 2′(3′)-O-′-methylaniloyl-aminoadenosine-5′-triphosphate; TF1, F1-ATPase from *Bacillus* sp. PS3

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To determine ATP-binding affinity of TF1 ε subunit under label-free conditions, we first measure the binding of the fluorescent analog of ATP, 2′(3′)-O′-methylaniloyl-aminoadenosine-5′-triphosphate (Mant-ATP) [24] and determine its binding affinity. Then, by analyzing competitive binding of ATP and Mant-ATP, we have determined ATP-binding affinity of wild type TF1 ε subunit under label-free condition.

2. Materials and methods

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

ATP binding to TF1 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:

$$\text{Fluorescence} = C_1 + \frac{C_2 [\text{Mant-ATP}] + K_{d\text{Mant}} [\text{Mant-ATP}] + K_{d\text{ATP}} [\text{ATP}] + K_{d\text{Mant}K_d\text{ATP}} + K_{d\text{Mant}K_{d\text{ATP}}}[\text{ATP}]^2 - 4K_{d\text{ATP}} [\text{Mant-ATP}]}{2K_{d\text{ATP}}} \times [\text{ATP}]$$

(Equation 1)

where, $C_1$ and $C_2$ are the constants, $K_{d\text{ATP}}$ is the dissociation constant of the ε subunit for ATP, $K_{d\text{Mant}}$ 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 ($K_d\text{Cy3}$) was determined to be 0.50 μM at 25 °C. This value is significantly different from the value previously 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

![Graph](https://via.placeholder.com/150)  
**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 = %ΔF$_{\text{max}}$ × [ATP]/($K_{d\text{Cy3}}$ + [ATP])). The $K_{d\text{Cy3}}$ and %ΔF$_{\text{max}}$ obtained (± standard error) are 0.50 ± 0.02 μM and 99.4 ± 0.7%.
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 fluorescence in the absence of ε subunit was set as 100%.

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 = %ΔFmax × [ε]/(Kd-Mant+ [ε])). The Kd-Mant and %ΔFmax obtained (± standard error) are 0.94 ± 0.11 μM and 57 ± 1%.

3.3. Mant-ATP displacement by ATP from ε subunit

The affinity measured in above section (Kd-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 Kd value for ATP (Kd-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 (Kd-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 Kd-Mant is in the same range with the Kd-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
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 \(\mu M\) and 0.10 \(\mu M\), 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 \(K_{d,\text{ATP}}\) obtained (± standard error) is 0.10 ± 0.00 \(\mu M\). ATP bound to the ε subunit opens to the solvent [6].

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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 F1 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 TFε 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 \(\mu M\) 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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