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High affinity nucleotide-binding mutant of the ε subunit of thermophilic F_1-ATPase

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ABSTRACT

Specific ATP binding to the ε subunit of thermophilic F_1-ATPase has been utilized for the biosensors of ATP *in vivo*. I report here that the ε subunit containing R103A/R115A mutations can bind ATP with a dissociation constant at 52 nM, which is two orders of magnitude higher affinity than the wild type. The mutant retained specificity for ATP; ADP and GTP bound to the mutant with dissociation constants 16 and 53 μM, respectively. Thus, the mutant would be a good platform for various types of nucleotide biosensor with appropriate modifications.

1. Introduction

ATP synthase (EC 3.6.3.14) catalyzes ATP synthesis from ADP and inorganic phosphate by using 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 bacterial and chloroplast ATP synthase has a molecular mass of 14 kDa and has a regulatory role in ATP synthase [3]. The ε subunit has two distinct domains [4–6]. The N-terminal two thirds forms N-terminal domain with β-sandwich structure, which has a structural role connecting γ subunit of F_1 and the c-subunit ring of Fo. The rest forms α-helical C-terminal domain, which has regulatory role and does not essential for the coupling ATP synthesis and H^+ flow [7–10]. Highly specific ATP binding to the ε subunit of F_1-ATPase from *Bacillus* sp. PS3 (TF_1) was confirmed by the biochemical [11–13] and structural analyses [6] (Fig. 1). The affinity for ATP of wild type TF_1 ε subunit at 25 °C is 4.3 μM [12]. It turned out that ε subunits of F_1-ATPases from *Bacillus subtilis* and *Escherichia coli* also bind ATP although with lower affinity (about 2 mM [6], and 22 mM [14], respectively). Although most of the residues necessary for ATP binding in TF_1 ε subunit are preserved in the ε subunit of *B. subtilis* F_1-ATPase (BF_1), there is a significant difference between ATP binding affinities of them. This large difference is possibly due to the differences in the Mg^{2+} binding or the slight differences between their structures remote from the ATP binding site [15]. It was also shown that the ε subunit changes its conformation upon binding of ATP [6,13,16]. In the presence of bound ATP, the C-terminal domain of the ε subunit takes folded conformation in which two α helices in the C-terminal domain form hairpin-like structure. The bound ATP has contacts with both the first and the second α helices in the C-terminal domain and several other residues of the N-terminal domain. The binding of ATP may weaken charge repulsion between basic residues consisting ATP binding site [6]. In the absence of bound ATP, two α helices in the C-terminal domain become apart from each other, and the ε subunit takes extended conformation. Upon this conformational change, the distance between N- and C- termini of the ε subunit changes about 50 Å [6]. The ATP binding to the ε subunit is related to its regulatory role in F_1 or FoF_1 [12,17].

By applying this drastic conformational change and very specific ATP binding to the ε subunit, Imamura et al. constructed a genetically encoded ATP biosensor, ATeam (Adenosine 5′-Triphosphate indicator based on Epsilon subunit for Analytical Measurements), by sandwiching ε subunit with two green fluorescent protein (GFP) variants [18]. In the presence of bound ATP, the ε subunit takes folded conformation and two GFP variants become in close

Abbreviations: ATeam, ATP indicator based on epsilon subunit for analytical measurements; BF_1, F_1-ATPase from *Bacillus subtilis*; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; TF_1, F_1-ATPase from *Bacillus* sp. PS3.

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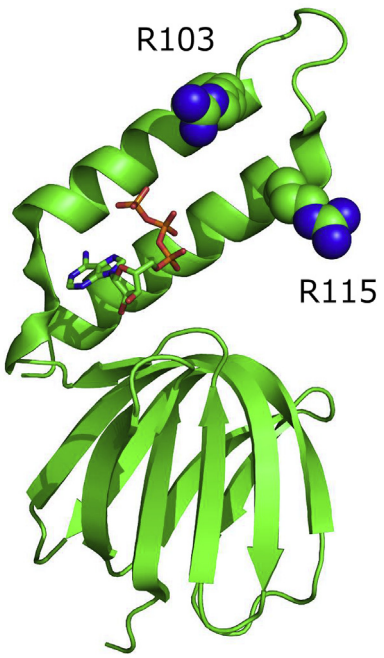

Fig. 1. Positions of the mutations.Crystal structure of the ε subunit (2E5Y, chain A) is shown in cartoon representation. ATP is shown as sticks. The side chains of the residues mutated to Ala (R103 and R115) are shown as spheres. This figure was generated with PyMOL (DeLano Scientific).

proximity leading Fluorescence Resonance Energy Transfer (FRET) between them. On the other hand, in the absence of ATP, the C-terminal domain of the ε subunit takes elongated conformation, two GFP variants are well separated, and the FRET efficiency becomes low. Thus, ATeam allowed to monitor cellular ATP concentration in real time along with various cellular activities [19–21]. There are also other types of ATP-biosensor proteins utilizing ε subunit as the sensing core [22,23].

To date, the affinity for ATP of these biosensors ranges from μM to mM, by introducing appropriate mutant forms of ε subunit of F_1-ATPases from *Bacillus* sp. PS3 or *B. subtilis* with various affinity for ATP. However, to monitor even lower concentrations of ATP to examine, for example, the role of ATP as the signal transducer in the extracellular spaces, the higher affinity variant of the ε subunit is demanded. In addition, it would be much easier to create lower affinity mutant than to create a higher affinity mutant. Thus, high affinity mutant can be a useful platform to create different types of biosensors (*e.g.* GTP biosensor).

I report here, a mutant of TF_1 ε subunit has two orders of magnitude higher affinity for ATP, which may be useful for various applications.

2. Materials and methods

2.1. Protein preparation

The DNA fragment containing TF_1 ε subunit gene with R103A/Q107C/R115A mutations was prepared by overlap-extension PCR method [24,25] applied to the expression plasmid for R115A [12] mutant TF_1 ε subunit with appropriate mutation primers and T7 promoter and terminator primers. Then, the DNA fragment was cut with *Nde*I and *Hin*dIII and ligated into the respective sites of pET-21c expression vector (Novagen) to obtain pET-21c-TF1ε (R103A/Q107C/R115A). The protein expression and purification were performed as described previously [12,26]. The purified protein was labeled with Cy3-maleimide (GE Healthcare) at a sole Cys at 107 as described previously [12,13]. From the absorption spectrum, the labeling ratio was estimated to >0.7 (data not shown). The labeled protein was snap frozen by liquid nitrogen and stored at −80 °C.

2.2. Fluorescence measurement

Titration with nucleotides was performed as described previously. Briefly, the cuvette containing 2 mL of Cy3-ε subunit at 2 or 5 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 bovine serum albumin was placed in an FP-6500 fluorescence spectrometer (JASCO) at 25 °C with continuous stirring. In the case of the measurements with ADP, 200 mM glucose and 6 UmL^{-1} hexokinase (Sigma−Aldrich) were included in the assay solution. Fluorescence was recorded at 2 Hz sampling with excitation and emission wavelengths set at 522 and 559 nm, respectively. The concentrated nucleotide solution was added sequentially at every 30 s to monitor the changes in the fluorescence upon nucleotide addition. Time-course measurements for ATP binding were performed with the same setup with 100 Hz sampling.

2.3. Data analysis

For the titration experiment, fluorescence at 25 s after each nucleotide addition was corrected for baseline measurement with buffer addition and plotted against nucleotide concentration. Every point in the plot represents the average of at least three measurements. The dissociation constants (K_d) were determined by the nonlinear regression analysis to the titration data with a simple binding scheme by OriginPro 9.0J (Origin Lab). The rate constant for the time-course data was obtained by nonlinear regression fitting with an exponential function. The apparent first-order rate constants (k_{app}) were plotted against the ATP concentration and the on rate (k_{on}) and the off rate (k_{off}) were determined from linear regression and their relationship; $k_{app} = k_{on}[ATP] + k_{off}$.

2.4. Other methods

Protein concentrations were determined by the method of Bradford [27] using BSA as the standard and corrected by multiplying a factor 0.54 as described [11,12].

3. Results and discussion

3.1. Titration measurements

Titration with ATP resulted in dramatic change from that with wild type (Fig. 2A). The K_d for ATP of the mutant was 52 nM, which is two orders of magnitude lower value than that of the wild type (4.3 μM) [12]. The K_d values of the mutant for other nucleotides were 16 μM and 53 μM, for ADP and GTP, respectively (Fig. 2B and C). Although there were no reported values of K_d for ADP or GTP at 25 °C, those reported for 37 °C indicated that ADP binds two orders of magnitude lower affinity than that for ATP, and GTP binds even one order lower than ADP [13]. Thus, in the mutant, the relative specificity for ADP compared to ATP was altered to slightly lower levels while that for GTP was essentially preserved.

The maximum relative fluorescence change with the mutant was 22% with ATP (Fig. 2A). This value is considerably lower than that with the wild type (79%) [12], indicating that structural change in the mutant upon ATP binding may differ considerably from the wild type, or specific Cy3-side chain interactions causing changes in the fluorescence are altered in the mutant.

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