Effect of ε subunit on the rotation of thermophilic Bacillus F₁-ATPase

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ARTICLE INFO
Article history:
Received 7 February 2009
Revised 24 February 2009
Accepted 26 February 2009
Available online 4 March 2009
Edited by Peter Brzezinski

Keywords:
F₁, F₁-ATPase
ATP synthase
Motor
Single-molecule
Epsilon subunit

1. Introduction

F₁ is a water soluble portion of ATP synthase (F_{0}F_{1}) that catalyzes synthesis of ATP [1,2]. The simplest F₁ from bacteria, such as Escherichia coli (EF₁) and thermophilic Bacillus PS3 (TF₁), has a subunit composition of αβ₃γδεc. F₁ is an ATP-driven rotary motor in which γ rotates in the αβ₃-cylinder [3,4]. Starting from γ angle at 0° (ATP-waiting dwell), ATP-binding induces the 80°-substep rotation of γ, the ATP previously bound is hydrolyzed (catalytic dwell), and Pi-release induces 40°-substep rotation [5–8]. Duration of ATP-waiting dwell is inversely proportional to [ATP] but that of catalytic dwell is independent from [ATP], always few milliseconds.

ATP synthase can catalyze a back reaction, ATP hydrolysis. When a cell is starved and ATP production has ceased, wasteful ATP hydrolysis by ATP synthase should be avoided by the regulatory mechanisms that respond to the change of cellular energy level, typically cellular [ATP] and [ADP] (and [AMP] as well). Actual physiological significance is yet unclear but several mechanisms that inhibit ATPase activity of F₁ and ATP synthase have been known. A common mechanism observed for F₁ from most organisms is “MgADP inhibition” in which occasional persistent occup-
ATPase activity, as well as rotation, of \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \) are the same at [ATP] > 0.1 mM where \( \varepsilon \) in \( \alpha_{3} \beta_{3} \gamma \) is stabilized in the non-inhibitory form by ATP-binding. Here, we extend the study to sub-micromolar ATP range where ATP no longer can bind to \( \varepsilon \).

2. Materials and methods

2.1. Proteins

Plasmids for expression of \( \alpha_{3} \beta_{3} \gamma \), \( \alpha_{3} \beta_{3} \gamma_{c} \) (containing a stop codon after \( \varepsilon \)-D87) and \( \alpha_{3} \beta_{3} \gamma \) complexes derived from thermophilic Bacillus PS9 carrying mutations of \( \alpha_{3}-C193S \), His\(_{32} \) at N-terminus of \( \beta \), e-I214C and e-D73C (for \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \)), or \( \gamma_{-}S107C \) and \( \gamma_{-}L210C \) (for \( \alpha_{3} \beta_{3} \gamma \)), were introduced into E. coli strain JM103Dumbc-D. The expressed complexes were purified as described [27].

2.2. ATPase activity

ATPase activity was measured at 25°C by an ATP regenerating coupling assay monitored by NADH oxidation at 340 nm [7]. The reaction was initiated by adding enzyme into the assay solution (1.2 ml) containing indicated amount of Mg–ATP (1:1 mixture of MgCl\(_2\) and ATP). ATP hydrolysis by \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \) started with an initial rapid phase and then reached the steady-state activity. The activities at final 150 s (2 mM ATP), 200 s (600 mM, 200 \( \mu \)M ATP) or 600 s ([ATP] < 600 \( \mu \)M) were taken as the steady-state activities. In the case of \( \alpha_{3} \beta_{3} \gamma \), ATP hydrolysis at low [ATP] started with a very long initial lag and therefore the activity below 60 \( \mu \)M ATP was measured as follows. The \( \alpha_{3} \beta_{3} \gamma_{c} \) was preincubated at 25°C in Mg–ATP at a concentration 120-fold of the final one, 2.5 mM phosphoenolpyruvate, 0.2 mg ml\(^{-1}\) pyruvate kinase, 5 mM MgCl\(_2\), 100 mM potassium phosphate, pH 7.5, 100 mM KCl, 2 mM EDTA for 5 min (final [ATP] 60 \( \mu \)M), 10 min (20 \( \mu \)M), 15 min (6 \( \mu \)M), 20 min (2 \( \mu \)M), 25 min (600 nM), or 35 min (200 nM). The mixture (10 \( \mu \)l) was added to 1.2 ml of the assay mixture. Steady-state activity was defined by NADH oxidation rate between 0 s and 200 s (for \( \alpha_{3} \beta_{3} \gamma \)). At 600 \( \mu \)M ATP, the activity of \( \alpha_{3} \beta_{3} \gamma_{c} \) was ~40% of \( \alpha_{3} \beta_{3} \gamma_{c} \), and at 200 nM ATP, it became barely detectable level. Consistently, when excess amount of isolated \( \varepsilon \) was added to the \( \alpha_{3} \beta_{3} \gamma \) catalyzing the steady-state ATP hydrolysis at 2 \( \mu \)M, 600 nM and 200 nM ATP, activity at 2 \( \mu \)M ATP was unchanged but activities at 600 nM and 200 nM ATP were slowed down to ~70% and ~20%, respectively (Fig. 1B, inset). Thus, the effect of C-terminal helical domain of \( \varepsilon \) on the steady-state ATPase activity is evident only at sub-micromolar [ATP]. Because initial and steady-state ATPase activities of \( \alpha_{3} \beta_{3} \gamma_{c} \) were similar to those of \( \alpha_{3} \beta_{3} \gamma \) at all [ATP], the N-terminal domain of \( \varepsilon \) has minimum effect on enzyme kinetics of \( \alpha_{3} \beta_{3} \gamma \).

2.3. Observation of rotation

Two cysteines (e-I214C, e-D73C) in \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \) were biotinylated by incubation with 5-fold molar excess of 6-[N-[2-(N-maleimidoethyl]-N-piperazinylamido]hexyl]-1,6-diaminonitrile (Dojindo, Kumamoto, Japan) for 2 h at room temperature, and unbound biotins were removed by a PD10 column (GE Healthcare, UK). Setup for observing system and observation of rotation are described in Supplement.

3. Results

3.1. Steady-state ATPase activity

Steady-state ATPase activities of \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \) in bulk solution were measured. Also examined was \( \alpha_{3} \beta_{3} \gamma_{c} \) in which \( \varepsilon_{c} \) lacks C-terminal helices and ability to bind ATP. ATPase activities of \( \alpha_{3} \beta_{3} \gamma \) and \( \alpha_{3} \beta_{3} \gamma_{c} \) started with initial burst at almost the same velocities at all [ATP] (data not shown). The activity was rapidly slowed down as MgADP inhibition progressed. ATPase activity of \( \alpha_{3} \beta_{3} \gamma \) started with a slow lag phase [17]. In the purified \( \alpha_{3} \beta_{3} \gamma_{c} \), \( \varepsilon \) adopts an inhibitory form, and the recovery from initial lag reflects the transition of \( \varepsilon \) from the extended to hairpin form [26]. This lag phase becomes longer as [ATP] decreases [17]. To avoid a long initial lag phase of \( \alpha_{3} \beta_{3} \gamma \) at low [ATP], we preincubated \( \alpha_{3} \beta_{3} \gamma_{c} \) with high [ATP] in the presence of an ATP-regenerating system and diluted it into the reaction mixture to give the final [ATP]. With these cautions, data of steady-state ATPase activities of \( \alpha_{3} \beta_{3} \gamma \), \( \alpha_{3} \beta_{3} \gamma_{c} \) and \( \alpha_{3} \beta_{3} \gamma \) were collected. Remarkably, although the initial phases were very different, steady-state ATPase activities of \( \alpha_{3} \beta_{3} \gamma \), \( \alpha_{3} \beta_{3} \gamma_{c} \) and \( \alpha_{3} \beta_{3} \gamma \) were very similar at [ATP] > 2 \( \mu \)M (Fig. 1A). Since the steady-state activity is mostly determined by the dynamic equilibrium between active and MgADP-inhibited state, \( \varepsilon \) does not seem to have significant effect on this equilibrium at this [ATP] range. Below 2 \( \mu \)M ATP, the steady-state activity of \( \alpha_{3} \beta_{3} \gamma_{c} \) and \( \alpha_{3} \beta_{3} \gamma \) decreased in proportion to the decrease in [ATP], but the steady-state activity of \( \alpha_{3} \beta_{3} \gamma \) decreased more sharply (Fig. 1A, inset). At 600 nM ATP, the steady-state activity of \( \alpha_{3} \beta_{3} \gamma_{c} \) was ~40% of \( \alpha_{3} \beta_{3} \gamma_{c} \), and at 200 nM ATP, it became barely detectable level. Consistently, when excess amount of isolated \( \varepsilon \) was added to the \( \alpha_{3} \beta_{3} \gamma \) catalyzing the steady-state ATP hydrolysis at 2 \( \mu \)M, 600 nM and 200 nM ATP, activity at 2 \( \mu \)M ATP was unchanged but activities at 600 nM and 200 nM ATP were slowed down to ~70% and ~20%, respectively (Fig. 1B, inset). Thus, the effect of C-terminal helical domain of \( \varepsilon \) on the steady-state ATPase activity is evident only at sub-micromolar [ATP]. Because initial and steady-state ATPase activities of \( \alpha_{3} \beta_{3} \gamma_{c} \) were similar to those of \( \alpha_{3} \beta_{3} \gamma \) at all [ATP], the N-terminal domain of \( \varepsilon \) has minimum effect on enzyme kinetics of \( \alpha_{3} \beta_{3} \gamma \).

Fig. 1. (A) Steady-state ATP hydrolysis rates by \( \alpha_{3} \beta_{3} \gamma \), \( \alpha_{3} \beta_{3} \gamma_{c} \) and \( \alpha_{3} \beta_{3} \gamma \). Measurement and definition of the steady-state hydrolytic activity are described in Section 2. Inset: steady-state ATP hydrolysis rates below 1 \( \mu \)M ATP. (B) Trace of ATP hydrolysis activities of \( \alpha_{3} \beta_{3} \gamma \) at 200 nM, 600 nM, and 2 \( \mu \)M ATP. Excess amount of \( \varepsilon \) was added as indicated. Inset: comparison of ATP hydrolysis rates with and without addition of \( \varepsilon \).
3.2. Observation of rotating molecules for several seconds

We observed the rotation of α3β3γε and α3β3γεAC at various [ATP] in a short time-scale (2–8 s) under dark-field illumination on a fast-framing camera (8000 frames s⁻¹). In either case, rotation was probed by a 40-nm bead attached to the β-sandwich region of ε through biotinylated residues (ε-I47C, ε-D73C), rather than γ, to avoid a possible mistaken observation of rotation of the ε-less complex. The viscous friction derived from the 40-nm bead is small enough to observe the rotation without load at this framing speed [5]. Below 2 μM ATP only very few rotating molecules of α3β3γε were found and we improved this by preincubation of biotinylated

![Graphs showing rotation rate vs. [ATP] for α3β3γε and α3β3γεAC.](image)

Fig. 2. Rotation of 40 nm-beads attached to ε (or εAC) of α3β3γε and α3β3γεAC observed for several seconds with a fast-framing camera. (A) [ATP] dependence of rotation speed. (B–E) Time courses of stepping rotation at indicated [ATP]. The time courses shown in (E) were not typical time segments but those happened to contain short ATP-waiting dwells. Horizontal gray lines indicate 0°-position and 80°-positions in a 120°-unit rotation. *Insets*, centroid position of beads. Experimental details are described in Section 2.
The rotation rates of $\alpha_3\beta_3\gamma e$ and $\alpha_3\beta_3\gamma e^{AC}$ averaged over $\sim 1$ s were very similar each other at all [ATP] ranging 200 nM to 2 mM (Fig. 2A) and can be fitted well with a simple Michaelis–Menten kinetics with $V_{\text{max}} = 200$ revolution s$^{-1}$ and $K_m = 17$ µM for $\alpha_3\beta_3\gamma e$ and $V_{\text{max}} = 220$ revolution s$^{-1}$ and $K_m = 20$ µM for $\alpha_3\beta_3\gamma e^{AC}$. These values are also very close to those of $\alpha_3\beta_3\gamma c$ [5, 8]. Both $\alpha_3\beta_3\gamma e$ and $\alpha_3\beta_3\gamma e^{AC}$ exhibited stepping rotation that was similar to $\alpha_3\beta_3\gamma c$. At 2 mM ATP, only the catalytic dwell was observed since ATP-waiting dwell was too short (Fig. 2B). At 20 µM ($\sim K_m$) ATP, both ATP-waiting dwell at 0° and catalytic dwell at 80° with nearly equal durations repeated in every 120°-unit rotation (Fig. 2C). At 2 µM ATP, ATP-waiting dwell became longer than catalytic dwell (Fig. 2D). At 200 nM ATP, catalytic dwell was barely visible (Fig. 2E). Analysis of the duration histogram of the catalytic dwells at different [ATP] (Supplementary, Fig. S1A–C) showed that two sequential catalytic reactions occurred during the catalytic dwell, of which averaged rate constants were $k_1 = 8.6 \times 10^2$ s$^{-1}$ and $k_2 = 7.6 \times 10^3$ s$^{-1}$ for $\alpha_3\beta_3\gamma e$ and $k_1 = 9.0 \times 10^2$ s$^{-1}$ and $k_2 = 7.3 \times 10^3$ s$^{-1}$ for $\alpha_3\beta_3\gamma e^{AC}$. These values are close to those of $\alpha_3\beta_3\gamma c$ ($k_1 = 7.5 \times 10^2$ s$^{-1}$ and $k_2 = 8.6 \times 10^3$ s$^{-1}$) [8]. Therefore, the presence of $e$ (and $e^{AC}$ as well) in $F_1$ does not have significant effect on catalytic events occurring in catalytic dwell, namely ATP-hydrolysis and Pi-release [7, 8]. Apparent ATP-binding rates were obtained from the analysis of duration histograms of ATP-waiting dwells at 2 µM and 200 nM ATP (Supplementary, Fig. S1D and E). These histograms could be fitted by a single-reaction scheme and the ATP-binding rate constants were calculated to be $3.8 \times 10^{7}$ M$^{-1}$ s$^{-1}$ for $\alpha_3\beta_3\gamma e$ and $3.6 \times 10^7$ M$^{-1}$ s$^{-1}$ for $\alpha_3\beta_3\gamma e^{AC}$. These values are in the same range of $\alpha_3\beta_3\gamma c$ ($2-3 \times 10^7$ M$^{-1}$ s$^{-1}$) [5, 8]. To summarize, as far as we observed actively rotating molecules of $\alpha_3\beta_3\gamma e$ and $\alpha_3\beta_3\gamma e^{AC}$ by a fast-framing camera for short period, their rotation kinetics are very similar to those of $\alpha_3\beta_3\gamma c$ at all [ATP] ranging 200 nM–2 mM and effect of the presence of $e$ in the complex is very little if any.

3.3. Observation of rotation for several tens of minutes

Next, we observed rotation for long period. A 40-nm bead and fast-framing CCD camera were not suitable for long (up to 2 h) observation because of limitation of computational memory...
capacity. Instead, we attached polystyrene bead of 200-nm diameter to ε of αβγε and observed rotation at 30 frames s⁻¹ (200 nM ATP) or at 150 frames s⁻¹ (2 mM ATP). The catalytic dwell at 80° after ATP-waiting dwell was no longer observable because of slow temporal resolution of the camera and of the slow stepping motion by viscous load imposed on rotating 200-nm beads. We chose rotating αβγε that happened to bind duplex beads, which showed clearer rotation than a single bead. In the case of αβγεNC, rotating polystyrene beads were shortly detached from the complex probably together with εNC, from the complex shortly, possibly because viscous load might break weakened association between εNC and γ. Therefore, we were obliged to use αβγε with polystyrene beads attached on γ, given that bulk-phase activity of αβγεNC are the same to that of αβγε. At 2 mM ATP, αβγε repeated two phases, i.e., continuous regular rotations and long pauses, and their life-times (a reciprocal of rate constant, τ) were 33 s and 28 s, respectively (data not shown). Under the same conditions, αβγε also showed very similar rotation characteristics with life-times of 22 s for regular rotation and 32 s for long pause [11]. Since the long pause of αβγε was previously assigned to be due to MgADP inhibition, the above observation indicates that ε does not have effect on the MgADP inhibition at high ATP.

At 200 nM ATP, regular rotation was also interrupted by long pauses (Fig. 3A). ATP-waiting dwells were seen at every 120 s for both αβγε and αβγε, and the positions of the long pauses were always at ~80° after the ATP-waiting dwells (Fig. 3A, inset). We collected the data from all pauses at 80° longer than 10 s. The duration histogram of the 80°-pause was fitted with a single-reaction scheme assuming transition of inactive state (long pause) to active state (regular rotation) (Fig. 3B). The rate constants were estimated to be 1.5 × 10⁻³ s⁻¹ for αβγε and 9.5 × 10⁻³ s⁻¹ for αβγε. Therefore, life-time of the pause of αβγε, 670 s, was 6-fold longer than that of αβγε, 110 s. Also, the periods of contiguous regular rotation between one long pause (>10 s) and the next long pause (>10 s) were collected and analyzed. In both αβγε and αβγε, the histogram could be fitted with a single-reaction scheme assuming transition from active state (regular rotation) to inactive state (long pause) (Fig. 3C). The rate constants of the transitions were estimated to be 3.0 × 10⁻³ s⁻¹ for αβγε and 3.3 × 10⁻³ s⁻¹ for αβγε. Thus, life-time of the active state of αβγε (330 s), in which regular rotation continues, is similar to that of its (300 s). These results suggest that ε does not increase the frequency of incidence of at 80°-pause but, once the regular rotation is lapsed into the pause, it prolongs the pause duration 6-fold.

4. Discussion

We found here that as far as we analyzed actively rotating αβγε molecules for several seconds, there was no obvious difference in rotation kinetics between αβγε and αβγε (and αβγε) at any [ATP] tested. Rate constants of ATP binding (2–4 × 10⁻¹⁰ M⁻¹ s⁻¹) and those of two events in the catalytic dwell (k₁ = 7.9 × 10⁻⁵ s⁻¹, k₂ = 7.9 × 10⁻⁵ s⁻¹) are all similar among these complexes. This result is not surprising because C-terminal helices of ε in rotating αβγε molecule should be folded in the non-inhibitory hairpin conformation and ε does not have any contact with αβγε-cylinder part that is driving catalysis and rotation.

When we observed rotating molecules as long as several tens of minutes, we noticed that long pauses at 80°-position interrupted the continuous regular rotation. Our observations are summarized and explained by a model shown in Fig. 4. At 2 mM ATP, period of the regular rotation (τ = 20–30 s) and duration of long pause (τ = ~10 s) are common to αβγε and αβγε (Fig. 4A). However, the effect of ε becomes evident at sub-micromolar [ATP] where long pause of αβγε (τ = 670 s) is 6-fold longer than that of αβγε (τ = 110 s) (Fig. 4B). During the prolonged pauses, C-terminal helices of ε in αβγε are supposed to be inhibitory extended form and can interact with catalytic αβγε-cylinder. Interestingly, frequency of the incidence of the pauses of αβγε, expressed as the life-time of actively rotating species of molecules (τ = 330 s), was actually very similar to those of αβγε, 300 s. Therefore, the effect of ε is not to interrupt the regular rotation but to prolong the pause duration. Taking into account that Kₐₐ value of ATP-binding to the isolated ε in the hairpin form is in the range of micromolar [ATP] at 25 °C [26], a simple explanation for the above observations is as follows. At [ATP] higher than micromolar, ATP binds and stabilizes ε in αβγε in non-inhibitory hairpin form and the transition to inhibitory extended form is prevented. At sub-micromolar [ATP], ATP can no longer bind and stabilize the non-inhibitory hairpin form of ε and reversible transition from non-inhibitory to inhibitory form becomes allowed. A possibility is not excluded, however, that the change of nucleotide occupancy at catalytic β subunits at low [ATP] also contributes to the reversible transition of ε.

The position of the long pause of αβγε is at the 80°-position, in agreement with the case of cyanobacterial F₁ [18], suggesting that the conformational transition of ε is allowed only when the complex is at 80°-position. Since it has been known for αβγε that MgADP inhibition is responsible for the long pause at 80° [11], it is likely that the transition of ε is allowed only when αβγε is in the MgADP-inhibited state at low [ATP] where ε is free from ATP-dependent stabilization of the hairpin form (Fig. 4B). The model does not assume the enzyme species that is escaped from MgADP inhibition but is inhibited by extended ε. Indeed, if this species exists, active αβγε molecule with hairpin ε can decay via two pathways and its life-time should be shorter than that of αβγε that decays via a single pathway. Relation between ε and MgADP inhibition was also suggested [25] and confirmed experimentally for TF ε [29]. It seems worth testing whether the mutant αβγε resistant to MgADP inhibition is also resistant to inhibition by ε.

The manner of inhibition of F₁-ATPase by ε varies among species. At least there are two types: in one type, the inhibition is observed only at low [ATP], while in the other type, it is observed at

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<tr>
<td>αβγε</td>
<td>F₁(ε) F₁(ε)</td>
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<td>τ=22 s</td>
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<tr>
<td>αβγε</td>
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<th>B</th>
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<tr>
<td>αβγε</td>
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<td>τ=300 s</td>
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<td>αβγε</td>
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<td>τ=330 s</td>
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<td>αβγε</td>
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all [ATP]. The inhibition is reversible at low [ATP] in the former but actually irreversible in the latter at all [ATP]. TF1 belongs to the former, and cyanobacterial F1 and CF1 belong to the latter [13,18]. Rotation of cyanobacterial F1 stops completely and irreversibly by ε [18]. Most likely, EF1 also belongs to the latter. ATPase of ε-less EF1 was largely (80–90%) inhibited at all [ATP] by addition of excess ε [16] and apparent reversibility of inhibition is due to the dissociation of ε from EF1. Time-averaged rate of rotation and steady-state ATPase activity of ε-containing EF1 was reduced about 50% by addition of excess ε [15] and this incomplete, reversible inhibition is also probably due to the same reason. The difference between two types of ε inhibition can be originated from the ability of ε of TF1 to bind ATP; at high [ATP], ATP binds and stabilizes the non-inhibitory hairpin conformation of ε of TF1. The interaction of ε of EF1 with ATP was detected but the affinity was very weak (Kd ~ 20 mM) [25]. It should be added that the affinity of ε of TF1 is strong at 25 °C but would be weak (Kd ~ 0.6 mM) at 65 °C, a physiological temperature of Bacillus PS3 [26]. It is interesting to see the distribution of ε capable of ATP-binding and its physiological function in various organisms.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.02.038.

References