Real-time Monitoring of Conformational Dynamics of the ε Subunit in F₁-ATPase

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2 The abbreviations used are: F₁, F₁-ATPase; F₀F₁, F₀F₁-ATP synthase; FRET, fluorescence resonance energy transfer; AMPPNP, 5′-adenyl-imidodiphosphate.

It has been proposed that C-terminal two α-helices of the ε subunit of F₁-ATPase can undergo conformational transition between retracted folded-hairpin form and extended form. Here, using F₁ from thermophilic Bacillus PS3, we monitored this transition in real time by fluorescence resonance energy transfer (FRET) between a donor dye and an acceptor dye attached to N terminus of the γ subunit and C terminus of the ε subunit, respectively. High FRET (extended form) of F₁ turned to low FRET (retracted form) by ATP, which then reverted as ATP was hydrolyzed to ADP. 5′-Adenyl-β,γ-imidodiphosphate, ADP + AlF₄⁻, ADP + NaN₃, and GTP also caused the retracted form, indicating that ATP binding to the catalytic β subunits induces the transition. The ATP-induced transition from high FRET to low FRET occurred in a similar time scale to the ATP-induced activation of ATPase from inhibition by the ε subunit, although detailed kinetics were not the same. The transition became faster as temperature increased, but the extrapolated rate at 65 °C (physiological temperature of Bacillus PS3) was still too slow to assign the transition as an obligate step in the catalytic turnover. Furthermore, binding affinity of ATP to the isolated ε subunit was weakened as temperature increased, and the dissociation constant extrapolated to 65 °C reached to 0.67 mM, a consistent value to assume that the ε subunit acts as a sensor of ATP concentration in the cell.

A rotary motor F₁-ATPase (F₁)³ is a water-soluble portion of F₀F₁-ATP synthase, which catalyzes ATP synthesis/hydrolysis coupled with transmembrane proton translocation (1, 2). F₁ has a subunit structure of α₃β₃γδε; α and β subunits have a non-catalytic and catalytic nucleotide binding sites, respectively; γ subunit rotates in the αβ ring; δ subunit connects the ring to the stator part of F₀, and ε subunit rotates together with γ subunit as a body. The ε subunit (~14 kDa) has a regulatory function and consists of N-terminal β-sandwich and C-terminal two α-helices (3, 4).

Previous structural studies of F₁ indicated two conformations of the ε subunit with different arrangement of the two α-helices, that is, retracted folded-hairpin form and partly extended form (Fig. 1, A and B) (5–8). Cross-linking studies suggested the third conformation with fully extended α-helices³ (Fig. 1C) (9). Biochemical data have indicated that the ε subunit adopts the extended form in the absence of nucleotide or in the presence of ADP, in which ATPase activity is inhibited, and that ATP counteracts ADP by favoring the retracted form, which is a non-inhibitory conformation (9). Thus, it appears that the regulatory function of the ε subunit is dependent on the drastic conformational transition that is affected by nucleotide and other factors. However, previous studies have not provided kinetic information on how these dynamic conformational transitions occur in the enzyme at work.

Fluorescence resonance energy transfer (FRET) is a powerful technique that enables us to probe conformational dynamics of biological molecular machines (10). In this study, this technique was applied to the ε subunit in F₁ from thermophilic Bacillus PS3. Real-time FRET monitoring revealed slow, reversible conformational change of the ε subunit, indicating that the ε subunit exerts a slow switch-like regulation. In addition, direct binding of ATP to the isolated ε subunit was probed by fluorescence change of the dye attached to the ε subunit. Submillimolar affinity of the ε subunit to ATP at the physiological temperature of Bacillus PS3 (65 °C) suggests a novel function of the ε subunit as an ATP concentration sensor in vivo.

**EXPERIMENTAL PROCEDURES**

Protein Preparation—A mutant of the αβγε subcomplex (αC193S, βHis-10 at N terminus, γS3C) and mutants of the ε subunit (134C or Q107C) of F₁ from thermophilic Bacillus PS3 were purified as described (11, 12). ATPase activity of reconstituted αβγε subcomplex (referred as F₁ hereafter) was measured as described previously (9). A molecular extinction coefficient of 154,000 at 280 nm was used for the determination of F₁ concentration.

Reconstitution of Fluorescent-labeled αβγε Complex—For FRET measurement, αβγε subcomplex and ε subunit (134C) were reacted with Cy3- and Cy5-maleimide (Amersham Biosciences, dye/protein molar ratio was 2) in PA5 buffer (50 mM HEPES-KOH, 5 mM MgCl₂, 100 mM KCl, pH 7.5) for 30 min at 25 °C, respectively, and unreacted dyes were removed by gel filtration. Labeling ratio of dye to protein was ~0.5 and >0.9 for αβγε (S3C-Cy3) and ε (134C-Cy5), respectively (judged by SDS-PAGE). For reconstitution of αβγε (S3C-Cy3) ε (134C-Cy5) subcomplex, αβγε (S3C-Cy3) and ε (134C-Cy5) were mixed (molar ratio of ε subunit to αβγε was 3) and incubated for 3 h at 25 °C, and excess ε (134C-Cy5) was removed by ultrafiltration. Reconstituted F₁ contained 0.66 mol of ADP and 0.26 mol of ATP/mol of F₁ and retained 76% steady-state ATPase activity as compared with that of wild type (134 ± 4.0 s⁻¹ measured at 50 °C). From this result we concluded that dye-
labeled F₁ used in this study was functional. Molecular extinction coefficients of 150,000 at 550 nm (Cy3) and 250,000 at 650 nm (Cy5) were used for determination of concentration.

**FRET Measurement**—FRET from the donor to the acceptor in the F₁ was monitored with a fluorescence spectrometer (FP-3000, Hitachi). Donor was selectively excited with the light of 532 nm (bandwidth was 3 nm). For time course measurements, fluorescence intensity of donor was measured at 565 nm (bandwidth was 10 nm). Measurements were carried out in PA5 buffer, and an ATP (GTP) regenerating system (5 mM phosphoenolpyruvate and 0.5 mg/ml pyruvate kinase) was added in some experiments as described. Contamination of ATP in the solutions of AMPPNP and GTP was less than 0.1% (estimated by high performance liquid chromatography). Steady-state fluorescence anisotropy of protein-conjugated Cy3 and Cy5 in the buffer PA5 was measured in a cuvette with a fluorescence spectrometer (FP-3000).

**Measurement of Affinity between Nucleotides and Isolated ε Subunit**—The Q107C mutant of the ε subunit was reacted with Cy3-maleimide (dye/protein molar ratio was 2), and unreacted dyes were removed by gel filtration. The labeling ratio of dye to protein was >0.9. Nucleotide was added to ε(Q107C-Cy3) in PA5 buffer, and fluorescence intensity was measured with a fluorescence spectrometer (FP-3000). Cy3 was excited with the light of 532 nm (bandwidth was 3 nm), and fluorescence intensity was measured at 565 nm (bandwidth was 10 nm). For the measurement of ADP affinity, hexokinase (6.7 units/ml) and glucose (200 mM) were supplemented to the buffer, and contaminated ATP was converted to ADP.

**RESULTS**

**Nucleotide-dependent, Reversible Conformational Change of the ε Subunit**—In a fluorescence spectrum of αβγε(S3C-Cy3)ε(134C-Cy5) obtained by excitation of donor dye (Cy3) at 532 nm (Fig. 2A, blue solid line), the peak of donor (Cy3, around 570 nm) decreased, as compared with the spectrum of αβγε(S3C-Cy3) (blue dashed line). Concomitantly the peak of acceptor (Cy5, around 670 nm) increased, indicating FRET from donor to acceptor with high efficiency. FRET was further confirmed by recovery of donor fluorescence intensity after photo-bleaching of acceptor by excitation at 670 nm (data not shown). When ATP (1 mM) was added, although the spectra of αβγε(S3C-Cy3) (red dashed line) and ε(134C-Cy5) (red dotted line) did not change, intensities of donor and acceptor fluorescence of αβγε(S3C-Cy3)ε(34C-Cy5) (red solid line) were increased and decreased, respectively. This ATP-induced transition from high FRET to low FRET primarily reflects the conformational change of the ε subunit because the γ and ε subunits rotate together as a body and the ε subunit does not undergo large conformational transition during catalysis. Hereafter, αβγε(S3C-Cy3)ε(134C-Cy5) subcomplex was referred as F₁ in this study and was used for FRET measurement.

Apparent FRET efficiency before and after addition of ATP, estimated from the spectra shown in Fig. 2A, was about 70 and 20%, respectively. It should be mentioned that the distance between donor and acceptor could not be estimated precisely from FRET efficiency in our experiments, since steady-state fluorescence anisotropy of Cy3 and Cy5 conjugated with γS3C and ε134C was high (>0.36). A high value of the anisotropy means that the mobility of the dye molecules conjugated with F₁ is highly restricted, and the estimation of the distance with high reliability is difficult. However, considering that the typical Förster distance (the distance which exhibits 50% FRET efficiency) is around 5 nm, the difference of about 1 nm in the estimated Förster distance of 134C-Cy5) and 134C-Cy5) is acceptable.

**Time course of change of FRET efficiency induced by nucleotides** were monitored by the change of the donor fluorescence intensity. As mentioned, 1 mM ATP increased donor fluorescence intensity (Fig. 2B, red line). On the contrary, ADP (1 mM) did not cause the change of donor fluorescence (Fig. 2B, blue line). AMPPNP (1 mM), which can bind to F₁, but is not hydrolyzed (13), also caused the increase in donor fluorescence, although the change was much slower than the case of ATP (Fig. 2B, light blue line). Therefore, ATP binding but not hydrolysis induces the conformational change from the extended form to the

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**FIGURE 1.** Probing conformational dynamics of the ε subunit by FRET. Structure of the γ (silver) and ε subunit (green) of F₁ from bovine mitochondria (5) (A), E. coli (6, 7) (B), and thermophilic Bacillus PS3 (C). Red and blue subunits are also shown (yellow). The red subunit of bovine mitochondrial F₁ is equivalent to the ε subunit of bacterial F₁ and is referred to as the ε here. Cy3 (donor, red circle) and Cy5 (acceptor, blue circle) were conjugated with the γS3C and ε134C of F₁ derived from thermophilic Bacillus PS3, respectively. The distances between two cysteine residues were estimated to be 8 nm (A), 2 nm (B), and 1 nm (C). Since the typical Förster distance is ~5 nm, a large change in the FRET efficiency according to the conformational change was expected.

**FIGURE 2.** Nucleotide dependence of FRET. A, fluorescence spectra of αβγε(S3C-Cy3) (dashed lines), ε(134C-Cy5) (dotted lines), and αβγε(S3C-Cy3)ε(134C-Cy5) (solid lines) before (blue) and after (red) addition of 1 mM ATP. Measurements were carried out at 35 °C, arbitrary unit. B, change in the donor fluorescence intensity after addition of various nucleotides. Nucleotides (1 mM) were added at time 0. In the case of ADP + NaN₃, NaN₃ (2 mM) was also added at time 0. In case of ADP + AlF₄⁻, AlCl₃ (1 mM) and KF (4 mM) were added at 300 s before the addition of ADP. All measurements were carried out at 50 °C. A and B, an ATP regenerating system was not included. C, effect of GTP on the donor fluorescence intensity. Measurements were carried out at 35 °C in the presence of an ATP (GTP) regenerating system. In all measurements in this figure, F₁ concentration was 10 nm.
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increased, recovery occurred more rapidly. Time courses of the recovery could be fitted by a single exponential function assuming transition between the active and inactive states (Fig. 3C, inset, green line). Apparent rate constants estimated from the fitting became larger as ATP concentration increased (Fig. 3D, circles).

The increase in donor fluorescence occurred in a time scale similar to ATPase recovery. However, the time courses could be better fitted by a double exponential function with comparable intensities than by a single exponential function (Fig. 3C, red line and green line, respectively). This suggests the presence of multiple pathways or intermediates toward low FRET state. Two (fast and slow) rate constants obtained from the double exponential function increased in a parallel manner as ATP concentrations increased. The fast rate constants showed a good match with the rate constants of recovery of ATPase activity at all ATP concentrations (Fig. 3D, triangles and circles, respectively). However, the lower one was several fold smaller (Fig. 3D, inverted triangles).

Temperature Dependence of Conformational Change—Physiological temperature of thermophilic Bacillus PS3 is about 65 °C, and F1 from this strain exhibits highest ATPase activity around this temperature (18). Next, temperature dependence of the rate of the conformational change of the ε subunit was investigated. An increase and decrease in donor fluorescence were induced by addition of ATP (1 mM) and by ATP depletion, respectively. Apparently, changes in both directions were strongly dependent on temperature. To reach a half-maximum fluorescence intensity, it took 95 s (increase) and 1300 s (decrease) at 30 °C, and 7.4 s (increase) and 130 s (decrease) at 45 °C.

Time courses for both increase and decrease were better fitted by the double exponential function at all temperatures. Values of four rate constants (fast and slow components for both directions) increased as temperature increased in almost parallel way and the Arrhenius plots were linear in the temperature range from 30 to 45 °C (Fig. 4, C and D). The Arrhenius activation energies ranged from 88 to 126 kJ/mol, and high Arrhenius activation energy is consistent with large conformational change of the ε subunit. Although measurement at 65 °C was difficult, since the ATP regenerating system and hexokinase were rapidly inactivated, if we assumed that the linearity of the Arrhenius plot was still retained at 65 °C, the extrapolated rate constants (faster one out of two rate constants) for the transitions reached to 2.7 s⁻¹ (extended-to-retracted) and 0.18 s⁻¹ (retracted-to-extended). These rates are much slower than that of ATPase turnover (>1000 s⁻¹).

Affinity of Nucleotides to the Isolated ε Subunit—Our previous study using gel chromatography showed that the isolated ε subunit binds ATP with relatively high affinity at 25 °C (the dissociation constant was expected to be lower than 10 μM) (12). However, quantitative estimation of the dissociation constant by gel chromatography was difficult. In this study, we applied fluorescence technique to detect nucleotide binding to the isolated ε subunit. When Cy3 was conjugated with ε(Q107C), a mutant that has a cysteine residue at the loop between two α-helices of the ε subunit, addition of a high concentration of nucleotide caused an increase in the fluorescence intensity of Cy3 several folds, presumably due to the environmental change around the fluorophore induced by nucleotide binding (Fig. 5A). If the change in the fluorescence was plotted against concentration of nucleotide, the plots were well fitted by the function assuming the simple binding reaction (Eq. 5B). The dissociation constants of ATP and ADP to the isolated ε subunit at 36 °C, estimated by the fitting, were 1.4 and 130 μM, respectively. The affinity of ATP was about 100 times higher than that of ADP. Affinity of GTP was much lower than that of ATP, and the apparent dissociation constant at 36 °C was 1.8 mM. Note that the value of dissociation constant for GTP

4 ADP-Mg inhibited state: the catalytic turnover of ATP hydrolysis by F1, is interrupted by occasional stable trapping of ADP-Mg at the catalytic site(s). The binding of ATP to the β subunits facilitates the release of ADP-Mg from the affected catalytic sites, thereby recovering the ATP hydrolysis activity. This ADP-Mg inhibited state is different from the product inhibition and is observed for nearly all F1s and ATP synthases from various sources.

FIGURE 3. Comparison between the rate of ATPase recovery of F1, and that of conformational change of the ε. A and B, increase in the ATPase activity (A) and increase in the donor fluorescence intensity (B) after the addition of ATP. ATP concentrations are 0.1 (blue), 0.3 (green), 1 (yellow), and 3 mM (red), respectively. In A and B, measurements were carried out in the presence of an ATP regenerating system at 35 °C. F1 concentration was 5 nM. C, the time courses shown in A and B (1 mM ATP) were fitted by a single exponential function assuming the reaction between two forms (green) or a following double exponential function (red): \[ a + b (\exp(-k_{\text{fast}} t)) + c (\exp(-k_{\text{slow}} t)) \] where a, b, and c are constants, and k_{\text{fast}} and k_{\text{slow}} are fast and slow components of the rate constant. D, rate constants plotted against ATP concentration. Rate constants of increase in the ATPase activity (circles) and fast (triangles) and slow (inverted triangles) components of the rate constant of increase in donor fluorescence were plotted.
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is a lower limit, since contaminated ATP in GTP (<0.1% in our sample) could bind to the ε subunit and increase the Cy3 fluorescence.

Temperature dependence of the affinity between ATP and the isolated ε subunit was also investigated. The dissociation constant between ATP and the isolated ε subunit increased greatly as temperature increased (Fig. 5C), and the logarithmic plot of dissociation constant against reciprocal temperature showed linear relationship (Fig. 5D). The dissociation constant extrapolated to the physiological temperature (65 °C) was 0.67 mM.

DISCUSSION

Conformational Change of the ε Subunit in F1 Is a Slow Process—Although a large conformational change of the ε subunit in F1 (and F0F1-ATP synthase) has been suggested from previous structural and biochemical studies (3–9,19), FRET measurement for the first time enabled us to observe it in real time. FRET measurement showed that even in the presence of a saturating concentrations of ATP, the change from high FRET to low FRET takes >10 s (at 35 °C, Fig. 3D). Thus, the ATP-induced conformational change of the ε subunit in F1 is much slower than the catalytic turnover (20), suggesting that it cannot be one of obligate steps in each cycle of the catalytic turnover but rather it exerts a switch-like or gear change function of the catalysis.

Conformational Change of the ε Subunit in F1 Could Occur at the 80° Substep Position—As we reported previously, ATP binding to the β subunit induces an 80° rotation of the γ subunit (20). Subsequently the hydrolysis of ATP and another catalytic event (probably the release of ADP or phosphate) occur in 2 ms at this 80° position (catalytic dwell) (11), and the next 40° rotation follows to complete a 120° rotation, a unit rotation driven by single turnover of ATP hydrolysis. Also it was shown that F1 lapsed into ADP-Mg inhibited state at the 80° position (21). We observed that not only ATP, but also AMPPNP, ADP + Na3, and ADP + AlF6− induced the transition from high FRET to low FRET (Fig. 2B). These nucleotide conditions are thought to mimic the ATP-bound state (13, 22), the ADP-inhibited state (14), and the catalytic intermediate (15), respectively, and stabilize the 80° position. Therefore, it is likely that transition of α-helices of the ε subunit from the extended form to the retracted form can take place when the γ subunit in F1 dwells at the 80° position.

Relation between Recovery from Inhibition and Conformational Change of the ε Subunit—Recovery of ATPase from the inhibition by the ε subunit and FRET change induced by ATP addition occurred in a similar time scale, but their time courses did not agree each other. The
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real reason of this disagreement is not known. One of possible reasons might be ADP-Mg inhibition. In an attempt to avoid ADP-Mg inhibition, we supplemented a detergent, lauryldodecylamine oxide, to the solution of ATPase assay and FRET measurement and repeated the experiments of Figs. 3 and 4 (supplemental Figs. S1 and S2). Lauryldodecylamine oxide has been thought to help F_{1} not to lapse into ADP-Mg inhibition (23). The FRET change became faster in the presence of lauryldodecylamine oxide, while the time course of recovery of ATPase was almost unchanged. However, the kinetics of ATPase recovery and FRET change still showed disagreement. We suspect that our F_{1} preparation could be heterogeneous, and some of the molecules that do not contain the ε subunit would be activated from the beginning. We expect that FRET observation at the single molecule level will reveal any heterogeneous population of F_{1} preparation.

ATP Binding to the ε Subunit May Stabilize the Folded-hairpin Form—The isolated ε subunit of F_{1} from thermophilic Bacillus PS3 binds ATP (12), and our recent crystal structure of the ε-ATP complex has revealed that ATP binds to the ε subunit in a retracted conformation with folded-hairpin helices. Therefore, we assume that ATP binding to the ε subunit may stabilize the folded-hairpin form of the ε subunit in F_{1} to shift the equilibrium from the extended form. In the absence of ATP, the ε subunit adopts the extended form in most of time and transient conformational change induced by thermal fluctuation might be rare. A likely scenario is that ATP binding to the β subunits enhances the chance more often for the ε subunit with the extended form to make conformational transition to the folded-hairpin form to which another ATP binds subsequently, and the folded-hairpin form is stabilized. Non-catalytic nucleotide binding sites on the α subunits are not involved in this change because their deficient mutant F_{1}(ΔANC) (24) also exhibited conformational change by ATP (data not shown). A model of conformational dynamics of the ε subunit at the physiological temperature of thermophilic Bacillus PS3 (65 °C) is shown in Fig. 6. It should be noted that relatively weak ATP binding affinity of the ε subunit at the physiological temperature (K_{d} = 0.67 mM at 65 °C) is suitable to sense the cellular ATP concentration that is in submillimolar range. This weak affinity can explain why the ATP binding to the ε subunit was detected only for thermophilic Bacillus PS3 but not for other species such as Escherichia coli (12). If the ε subunit from mesopholic organisms living below 40 °C would have similar weak affinity to ATP, the binding would not be detected by gel chromatography at room temperature.

The ε Subunit, an Inhibitory or a Coupling Factor?—Even at 0.1 mM ATP, almost all ε subunit molecules adopt the retracted form in a couple of min after initiation of ATP hydrolysis (Fig. 3B), and an inhibitory effect of the ε subunit was observed only for a short period (Fig. 3A). However, the situation may be different for F_{0}F_{1}-ATP synthase (F_{0}F_{1}) working in vivo. In our previous cross-linking experiment, a significant population of the ε subunit in F_{0}F_{1} adopted the fully extended form even in the presence of relatively high concentrations of ATP (for example, ~40% of the ε subunit was in the fully extended form at 1 mM ATP) (9). The population was further increased by the proton motive force across the membrane. Therefore, compared with F_{1}, the equilibrium of the conformation of the ε subunit in F_{0}F_{1} must be more shifted to the fully extended form in vivo, and the ε subunit likely inhibits ATP hydrolysis more effectively. Furthermore, it was shown recently that reconstitutition of the ε subunit into the α_{5}β_{3}γ subcomplex greatly improved the efficiency of ATP synthesis when F_{1} was rotated in the ATP synthesis direction with magnetic tweezers (25). This result indicates that the ε subunit acts not only as an endogenous inhibitor of ATP hydrolysis but also as a coupling factor for the ATP synthesis, as suggested before (26).

Although the importance of the ε subunit is evident, it is totally unknown how the proton motive force affects the conformation of the ε subunit and how the ε subunit improves the efficiency of ATP synthesis. Rotational direction of the central stalk consisting of the γεc_{10} complex, i.e. the direction of force applied to the ε subunit, may affect its conformation and function in non-equilibrium condition. Recent single molecule FRET measurements indicated that there was no conformational change in the N-terminal β-sandwich part of the ε subunit during ATP hydrolysis and synthesis reaction catalyzed by F_{0}F_{1} from E. coli (27). The difference in the conformational states and dynamics of the C-terminal α-helices of the ε subunit during ATP hydrolysis and synthesis also would be directly probed by the single molecule FRET measurement during the forced rotation of F_{0}F_{1}.

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5 ATP binding to the isolated ε subunit was not affected by lauryldodecylamine oxide (K_{d} = 1.4 μM at 35 °C).

6 N. Kajiwara, H. Akutsu, Y. Kato-Yamada, and M. Yoshida, unpublished data.