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Probing conformations of the β subunit of F_0F_1-ATP synthase in catalysis

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Abstract

A subcomplex of F_0F_1-ATP synthase (F_0F_1), $\alpha_3\beta_3\gamma$, was shown to undergo the conformation(s) during ATP hydrolysis in which two of the three β subunits have the "Closed" conformation simultaneously (CC conformation) [S.P. Tsunoda, E. Muneyuki, T. Amano, M. Yoshida, H. Noji, Cross-linking of two β subunits in the closed conformation in F_1-ATPase, J. Biol. Chem. 274 (1999) 5701–5706]. This was examined by the inter-subunit disulfide cross-linking between two mutant β(I386C)s that was formed readily only when the enzyme was in the CC conformation. Here, we adopted the same method for the holoenzyme F_0F_1 from *Bacillus* PS3 and found that the CC conformation was generated during ATP hydrolysis but barely during ATP synthesis. The experiments using F_0F_1 with the ε subunit lacking C-terminal helices further suggest that this difference is related to dynamic nature of the ε subunit and that ATP synthesis is accelerated when it takes the pathway involving the CC conformation.
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F_0F_1-ATP synthase is prevalent in plasma membranes of bacteria, inner mitochondrial membranes of eukaryotic cells, and chloroplast thylakoid membranes of plant cells. Its function, ATP synthesis at the catalytic subunit β is driven by proton flow down the gradient of electrochemical potential of proton across the membranes. F_1, consisting of $\alpha_3\beta_3\gamma\delta\varepsilon$, is easily and reversibly detached from F_0 part and can catalyze ATP hydrolysis. A subcomplex of F_1, $\alpha_3\beta_3\gamma$, also has ATPase activity comparable to that of F_1. In ATP hydrolysis, free energy liberated from hydrolysis of ATP at the catalytic subunit β is used for driving rotation of the central stalk subunits γ, ε, and c_{10-14} [1–3].

Different catalytic states of the β subunit denoted by β_T, β_D, and β_E were revealed in a crystal structure of $\alpha_3\beta_3\gamma$ [4] according to nucleotide binding states. They bind AMP–PNP, ADP, and no nucleotide, respectively, at the catalytic site. In that structure, β_D was assumed to have the "tight" catalytic state because of tight interactions with the α subunit. Moreover, characteristic features of β_D were shown in another crystal structure [5] : the guanidinium group of αR373 and the water molecule involved in catalysis are both placed closer to BeF_3^- (mimicking γ-phosphate) compared to β_T to enable hydrolysis of ATP.

In relation to these different states of the β subunit, the β subunit was in two conformations in the crystal structure [4]. One is "Closed form" (denoted by C) for β_T and β_D, in which the C terminal domain of the β subunit is lifted up toward the N terminal domain. The other is "Open form" (denoted by O) in which the catalytic site is empty

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(β_E) and C terminal domain of the β subunit is in a relaxed form away from the N terminal domain. Comparisons of these two conformations suggest that the conformational transition arises from the domain-motions of the β subunit, which have become a focus in solving the mechanism of driving rotation [6]. Close look at the crystal structure [4] revealed that when two of the three β subunits are in the Closed conformation (denoted "CC conformation" hereafter), a βI386 residue in a Closed β comes into contact with the same residue in another Closed β although Open β does not (as in Fig. 1 [7]). In the previous study of β(I386C) mutant of $\alpha_3\beta_3\gamma$ from a thermophilic *Bacillus* PS3 [8], simultaneous occurrence of two Closed β subunits during ATP hydrolysis was confirmed by the β–β dimer formation which resulted from inter-subunit cross-linking between I386C residues of the two β(I386C) subunits.

Illustration of conformational changes of the β subunit has become more complex as a new conformation of the β subunit in between open and closed conformations was found in the crystal structure whose three catalytic sites were all occupied by nucleotides [9]. The third conformation of β in this structure was named "Half-closed."

Moreover, conformations of the ε subunit must be taken into account in the case we discuss conformations of β in the whole F_1 and F_0F_1. The ε subunit was known to be involved in catalysis by changing binding affinities of nucleotides to the β subunits [10] and distinguishing between different conformations of the β subunit [11]. Recently, the C-terminal helices of the ε subunit have been found to undergo drastic conformational changes and regulate ATPase activity [12–20]. When the C-terminal helices of the ε subunit are in the extended form that is induced by increase in proton motive force and ADP concentration, hydrolysis activity is largely suppressed although ATP synthesis apparently remains [17,18]. Meanwhile, when the C-terminal helices of the ε subunit are in the hairpin-folded form, ATP hydrolysis activity is not attenuated [17,18]. Most recently, the ε subunit was shown to play an important role in efficient synthesis of ATP [21]. With the ε subunit in the F_1 complex, tight coupling of ATP synthesis, that is, three ATPs produced by a forced 360°-rotation of γ, was achieved.

Thus, behaviors of the ε subunit and its relationship with activities are partly disclosed, but their relationship with conformational changes of the β subunit during catalysis has not been clarified yet.

The previous studies of the $\alpha_3\beta(\mathrm{I386C})_3\gamma$ mutant gave understanding of conformations of β under limited conditions: during ATP hydrolysis and without ε subunit. In the present studies, conformations of the β subunit for both ATP synthesis and hydrolysis are illustrated by relating to conformational changes of ε. For this purpose, the β(I386C) mutation was introduced into $F_1(\alpha_3\beta_3\gamma\delta\varepsilon)$ and F_0F_1 from thermophilic *Bacillus* PS3. Cross-linking studies and measurements of ATP synthesis activities indicated that CC conformation of the β subunit does not appear during ATP synthesis due to extended conformation of ε, which attenuated ATP synthesis activity.

Materials and methods

Buffers. PA3, 10 mM HEPES–KOH, pH 7.5, containing 5 mM $MgCl_2$, and 10% glycerol; PA4, 10 mM HEPES–KOH, pH 7.5, containing 100 mM KCl, and 5 mM $MgCl_2$; KP_i buffer, 100 mM potassium phosphate, pH 7.0, 100 mM KCl, and 2 mM of 1,2-cyclohexanediaminetetraacetic acid monohydrate (CDTA).

Strains and plasmids. The pTABG3-β(I386C) plasmid [8] in *Escherichia coli* strain JM103*Δunc*B-D [22] from the previous work was used for expression of $\alpha_3\beta(\mathrm{I386C})_3\gamma$ mutant. This strain leaves *unc*C (ε subunit) on the chromosome. Nevertheless, incorporation of *E. coli* ε subunit into the expressed $\alpha_3\beta_3\gamma$ complex of PS3 did not occur. A plasmid pTR19-ASDS-β(I386C) carrying a gene for F_0F_1 from thermophilic *Bacillus* PS3 whose β subunit contains I386C mutation was also prepared. This plasmid was used for transformation of an *E. coli* strain DK8 [bglR, thi-1, rel-1, HfrPO1, *Δ*(uncB-uncC), ilv::Tn10] [23] that lacked the whole F_0F_1 genes. It was used for preparation of F_1(βI386C) and F_0F_1(βI386C). In addition, both wild type and β(I386C) mutant of F_1 and F_0F_1 lacking C-terminal helices of the ε subunit (denoted by $\varepsilon^{\Delta C}$) were prepared by introducing a stop codon and a *Pst*I site just after εAsp87.

Preparation of $\alpha_3\beta_3\gamma$, F_1 and inverted E. coli membrane vesicles containing F_0F_1 from thermophilic Bacillus PS3. The *E. coli* JM103*Δunc*B-D which contained pTABG3-β(I386C) plasmid was cultivated in the terrific broth containing 100 μg/ml of ampicillin for 16 h at 37 °C. The cells were harvested and the proteins were purified as described previously [24]. The steady-state ATPase activities of $\alpha_3\beta(\mathrm{I386C})_3\gamma$, F_1(βI386C), and $F_1(\beta\mathrm{I386C}/\varepsilon^{\Delta C})$ were 1.3 U/mg for $\alpha_3\beta(\mathrm{I386C})_3\gamma$, 2.2 U/mg for F_1(βI386C), and 3.2 U/mg for $F_1(\beta\mathrm{I386C}/\varepsilon^{\Delta C})$ at 45 °C in the presence of 2 mM ATP. The *E. coli* DK8 which harbors pTR19-ASDS plasmid [25] was cultivated for 17 h and treated as described previously [25] for preparation of F_0F_1 in membrane vesicles and purified F_1. The F_1 samples were stored in −80 °C, and the final purification was performed in the same procedures as that of the $\alpha_3\beta(\mathrm{I386C})_3\gamma$ subcomplex.

Expression of $\varepsilon^{\Delta C}$ subunit was confirmed by MALDI-TOF MS measurements (AXIMA CFR-Plus, Shimadzu, Kyoto). The average mass of ε of $F_1(\beta\mathrm{I386C}/\varepsilon^{\Delta C})$ was 9215.3, which was in good agreement with the expected average mass of 9210.5.

Inter-subunit Cross-linking of β(I386C) mutants. To compare cross-linking efficiencies, 1 μM of $\alpha_3\beta(\mathrm{I386C})_3\gamma$, F_1(βI386C), and $F_1(\beta\mathrm{I386C}/\varepsilon^{\Delta C})$ from final purification by gel filtration were each incubated with 10 μM $CuCl_2$ at 45 °C for 5 min. in PA4 buffer (containing 5 mM $MgCl_2$) in the presence or absence of 1 mM of nucleotides (Mg–ATP, Mg–ADP, Mg–AMP–PNP, and Mg–ATP–γS). ATP-regenerating system was concurrently added in the case of ATP (0.1 mg/ml pyruvate kinase and 2.5 mM phosphoenolpyruvate), and, in the case of ADP, hexokinase (10 U/ml) and glucose (30 mM) were added to eliminate contaminated ATP. As control experiments, incubations with 1 mM DTT and without any added reagents were also performed under the same conditions. The cross-linking reactions were terminated by addition of 10 mM of *N*-ethylmaleimide. They were then subjected to non-reducing polyacrylamide gel electrophoresis (SDS–PAGE). Formation of cross-linking was confirmed by appearance of new bands at the position of β–β dimer and decrease in density of bands of β monomer. The same protocols were applied to the cross-linking studies of membrane vesicles containing F_0F_1 except for the differences in the following conditions: 1 mg/ml membrane protein was used for cross-linking. Incubation was performed with 20 μM $CuCl_2$ for 10 min. The same experiments were also carried out using βI386C-less enzymes as controls and absence of cross-linking was confirmed.

Measurements of ATPase activity. Pretreatment of membrane vesicles was carried out as follows: in the case of investigating the effects of cross-linking on ATPase activity, 1 mg/ml of membrane vesicles was treated with either 1 mM DTT or 1 mM Mg–ATP plus 50 μM $CuCl_2$ in PA4 buffer at 45 °C for 10 min, which was terminated by addition of 1 mM EDTA. It also has an effect to chelate Cu^{2+} from membranes. In the case of a measurement of DCCD (*N*,*N*′-dicyclohexylcarbodiimide) sensitivity, 2 mg/ml of membrane vesicles was treated with the same volume of 200 μM DCCD in 100 mM Tris–HCl, pH 8.8. Incubation and termination

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