Cross-linking of Two $\beta$ Subunits in the Closed Conformation in F$_1$-ATPase

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The crystal structure of mitochondrial F$_1$-ATPase, two $\beta$ subunits with a bound Mg-nucleotide are in “closed” conformations, whereas the third $\beta$ subunit without bound nucleotide is in an “open” conformation. In this “CCO” ($\beta$-closed $\beta$-closed $\beta$-open) conformational state, Ile-390s of the two closed $\beta$ subunits, even though they are separated by an intervening $\alpha$ subunit, have a direct contact. We replaced the equivalent Ile of the $\alpha_3$$\beta_3$$\gamma$ subcomplex of thermophilic F$_1$-ATPase with Cys and observed the formation of the $\beta$-$\beta$ cross-link through a disulfide bond. The analysis of conditions required for the cross-link formation indicates that: (i) F$_1$-ATPase takes the CCO conformation when two catalytic sites are filled with Mg-nucleotide, (ii) intermediate(s) with the CCO conformation are generated during catalytic cycle, (iii) the Mg-ADP inhibited form is in the CCO conformation, and (iv) F$_1$-ATPase dwell in conformational state(s) other than CCO when only one (or none) of catalytic sites is filled by Mg-nucleotide or when catalytic sites are filled by Mg$^{2+}$-free nucleotide. The $\alpha_3$$\beta_3$$\gamma$ subcomplex containing the $\beta$-$\beta$ cross-link retained the activity of uni-site catalysis but lost that of multiple catalytic turnover, suggesting that open-closed transition of $\beta$ subunits is required for the rotation of $\gamma$ subunit but not for hydrolysis of a single ATP.

F$_1$, together with the membrane-embedded F$_0$ part, constitutes ATP synthase which couples a transmembrane proton flow to ATP synthesis/hydrolysis. F$_1$ is easily and reversibly separated from F$_0$ part as a water soluble ATPase and is called F$_1$-ATPase. The F$_1$-ATPase is composed of five subunits, $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ subunits, in a molar ratio of 3:3:1:1:1 (1–3). In the crystal structure of bovine mitochondrial F$_1$-ATPase (MF$_1$)$^1$ (4), three $\alpha$ and three $\beta$ subunits are arranged alternately like the segments of an orange around the central coiled-coil structure of the $\gamma$ subunit. F$_1$-ATPase contains six nucleotide binding sites; three of them are catalytic sites and mainly located on each $\beta$ subunit, whereas the other three do not bear a catalytic role and are mainly located on each $\alpha$ subunit (4). The $\alpha_3$$\beta_3$$\gamma$ subcomplex of F$_1$-ATPase has been recognized as a minimum ATPase-active complex with stability and characteristics similar to native F$_1$-ATPase (5–7).

F$_1$-ATPase shows a complicated kinetic behavior in ATP hydrolysis. Three catalytic sites in F$_1$-ATPase are not independent but are related to one another in a cooperative manner. Binding of ATP to the catalytic sites is governed by negative cooperativity, and inversely, hydrolysis of bound ATP at each catalytic site is governed by positive cooperativity. When stoichiometric amounts of ATP relative to the enzyme are added, ATP is hydrolyzed very slowly (uni-site catalysis) (8–10). This reaction is accelerated by chase-addition of an excess amount of ATP (chase-promotion) (8–10). As a whole, ATP hydrolysis reaction by F$_1$-ATPase usually exhibits negative cooperativity as a function of ATP concentration (11–16). These and other features had been unified into the binding change mechanism by Boyer (3). According to the mechanism, three catalytic sites interchange their roles alternately and sequentially during catalytic turnover accompanying the rotation of $\gamma$ subunit in the center of the enzyme. Consistent with this rotary mechanism, the $\alpha_3$$\beta_3$$\gamma$ subcomplex of thermophilic Bacillus PS3 F$_1$-ATPase (TF$_1$) containing two intact and one incompetent catalytic sites lost the ability to mediate catalytic turnover while it showed uni-site ATP hydrolysis and chase-promotion (17). The rotation of the $\gamma$ subunit was supported by various methods (18–20) and was directly proved by single molecule observation of the $\alpha_3$$\beta_3$$\gamma$ subcomplex of TF$_1$ (21).

Another complication of F$_1$-ATPase kinetics is caused by the “Mg-ADP inhibited form” (22–25). In general, F$_1$-ATPases from mitochondria, chloroplasts, and bacteria are prone to develop turnover-dependent inactivation; Mg-ADP trapped transiently in a catalytic site causes the slow transition from an active form to an inhibited form called as Mg-ADP inhibited form (26–28). The slow transition during catalysis is accelerated by the simultaneous occupation of two catalytic sites by Mg-nucleotides (25, 29). Mg-ATP bound to the noncatalytic nucleotide binding site promotes dissociation of inhibitory Mg-ADP from the affected catalytic site (30). Further, when the enzyme is preincubated with stoichiometric Mg-ADP, the Mg-ADP inhibited form is generated. An inhibitor of F$_1$-ATPase, azide stabilizes the Mg-ADP inhibited form (23–25), and an activator of F$_1$-ATPase, N,N-dimethyldecylamidine-N-oxide (LDAO) destabilizes it (31).

The crystal structure of MF$_1$ (4) revealed that three $\beta$ subunits in the MF$_1$ molecule are in different states; one $\beta$ ($\beta_{TP}$) has an ATP analog, Mg-AMP-PNP, at its catalytic site; another $\beta$ ($\beta_{DP}$) has Mg-ADP; the third $\beta$ ($\beta_{E}$) has none. The structures of $\beta_{TP}$ and $\beta_{DP}$ are very similar to each other, and they are in the “closed” conformation, in which the carbonyl-terminal domain is lifted close to the nucleotide binding domain. In contrast, $\beta_{E}$ adopts the “open” conformation, in which the crevice

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1 The abbreviations used are: MF$_1$, F$_1$-ATPase from mitochondria; TF$_1$, F$_1$-ATPase from thermophilic Bacillus strain PS3; PAGE, polyacrylamide gel electrophoresis; LDAO, N,N-dimethyldecylamidine-N-oxide; DTT, dithiothreitol; TNP-ATDP, 2’-3’-O-(2,4,6-trinitrophenyl) derivatives of ATP; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate; TNP-ATP, 2’(3’)-O-(2,4,6-trinitrophenyl)adenosine 5’-triphosphate.
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for substrate binding is open. This crystal structure, characterized by two closed and one open β subunits ("CCO" conformational state) has been assumed to be a "snapshot" of TF₁, cycling the catalytic turnover along the pathway predicted by the binding change mechanism. Whether this assumption is really the case has not been established by experiments. To address this question, we need a specific probe to detect the CCO conformational state of F₁-ATPase in solution. Looking at the structure of MF₁ carefully, we noticed that the two closed β subunits, even though separated by an intervening α subunit, have a direct contact at the position of Ile-390 of each β subunit (see Fig. 1, A and B). If this Ile is replaced with Cys, two closed β subunits in F₁-ATPase in the CCO conformation would be cross-linked by a disulfide bond, and it would fix two β subunits in the closed conformation. Indeed, when we examined this experiment using αβγCys subcomplex of TF₁, the cross-link was formed in an Mg-nucleotide-dependent, azide-facilitated manner with concomitant loss of the activity of catalytic turnover.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Preparation of Subcomplexes—Escherichia coli strains used were JM109 (32) for preparation of plasmids, C6328 (33) for generating uracil-containing single-stranded plasmids for site-directed mutagenesis, and JM103D (uncB-uncD) (34) for expression of the wild-type and mutant αβγCys subcomplexes of TF₁. Plasmid pTABG3 (6), which carried genes for the α, β, and γ subunit of TF₁, was used for mutagenesis and expression. The helper phage M13KO7 was obtained from Amersham Pharmacia Biotech, Tokyo. The expression plasmid for the mutant subcomplex was constructed as follows. The mutation (Ile → Cys at β-386) was introduced into pTABG3 (6) by using a synthetic oligonucleotide: 5′-TTGCTTCTATCGAAGCTATCTCCATCCCACCATGGGATGCTTGTACCCGAGTT-3′ (c changed bases are underlined) (33). The EcoRI-BglII fragment from the resultant plasmid was ligated into the EcoRI-BglII site of pKABG3 to produce the expression plasmid, pKABG3-αβγCys. Recombinant DNA procedures were performed as described in the manual (35). The wild-type and the mutant αβγCys subcomplexes were purified and stored as described previously except that 2 mM diithiothreitol (DTT) was added in all buffers (6, 17). Just before use, they were subjected to gel-filtration HPLC with a TSK-G3000SWXL column (Tosoh, Japan) equilibrated with 50 mM Tris-HCl (pH 7.0), 200 mM NaCl (Tris-NaCl buffer). Gel-filtrated preparation of the mutant subcomplex did not contain a detectable amount of endogenously bound adenine nucleotide (<0.1 mol/mol of subcomplex). In the presence of LDAO, the mutant subcomplex showed the steady-state ATPase activity of 15.2 μmol of ATP hydrolyzed/min/mg at 25 °C, which is ∼70% of that of the wild-type subcomplex (21.3 μmol of ATP hydrolyzed/min/mg). In the absence of LDAO, the activity was 4.6 (mutant subcomplex) and 7.7 (wild-type subcomplex) pmol of ATP hydrolyzed/min/mg. Disulfide Cross-link Formation of the Mutant Subcomplex—The subcomplexes were incubated at 25 °C in Tris-NaCl buffer containing dicarboxylated components. At the indicated time, an aliquot was taken out and analyzed. The activity was 4.6 (mutant subcomplex) and 7.7 (wild-type subcomplex) pmol of ATP hydrolyzed/min/mg at 25 °C, which is 4.6 (mutant subcomplex) and 7.7 (wild-type subcomplex) pmol of ATP hydrolyzed/min/mg. In the absence of LDAO, the activity was 4.6 (mutant subcomplex) and 7.7 (wild-type subcomplex) pmol of ATP hydrolyzed/min/mg.

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RESULTS

Two β Subunits in the Closed Form Have a Contact Site—In the crystal structure of MF₁, two β subunits with a bound nucleotide, βPₐ and βPₜ, take the closed conformation and contact each other at the carboxyl-terminal region near the central axis (Fig. 1, A and B, shown by an arrow). The residues in the contact position are Ile-390 of each β subunit (Fig. 1, A and B, shown by red). The distance between α carbons of the two Ile-390s is 0.79 nm. The distance between the nearest carbon atoms (C₃ of βPₜ-Ile-390 and C₃ of βPₐ-Ile-390) is 0.42 nm, and the two residues actually appear to have hydrophobic interaction. On the contrary, the distance between α carbons of βPₜ-Ile-390 and βPₐ-Ile-390 and that between βPₐ-Ile-390 and βPₜ-Ile-390 are 2.40 nm and 2.58 nm, respectively. This residue is located in a region just preceding the "DELSSEET sequence" (39) and is highly conserved in F₁-ATPases from various sources. The αβγCys subcomplex with Cys at the Contact Sites Formed β-β Cross-link in the Presence of Both Mg²⁺ and ATP—We generated a mutant αβγCys subcomplex of TF₁ in which β-386, an equivalent residue to MF₁-β-390, was replaced with Cys. ATPase activity of the mutant subcomplex was about 70% of that of the wild-type subcomplex (see "Experimental Procedures"). The cross-linked β dimer in the mutant subcomplex was detected by non-reducing SDS-PAGE after incubation at 25 °C for 2 h in the Tris-NaCl buffer containing 0.25 mM CuCl₂ and indicated compounds (Fig. 2). No new band other than three subunit bands, α, β, and γ was found in a reference sample incubated alone with DTT (Fig. 2, lane 1 and lane 2). When the solution contained Mg-ATP (lane 8), Mg-ADP (lane 9), or Mg-ADP + azide (lane 10), a 100-kDa protein band appeared. The exposure of the complex to a reducing reagent prior to electrophoresis eliminated the band (lane 11). It was identified to be a cross-linked β dimer because two-dimensional electrophoresis showed that the 100-kDa band in the first non-reducing SDS-PAGE was developed into a β subunit band in the second reducing SDS-PAGE (data not shown). Consistently, the staining intensities of the β subunit band in lanes 8–10 decreased in parallel with the increase of those of the β dimer band. Because the cross-link was formed in Mg-ADP, catalytic turnover of ATP hydrolysis is not absolutely required for the cross-linking. No cross-link was observed in AT(D)P + EDTA (lanes 5 and 6), ADP + azide + EDTA (lane 7), and Mg²⁺ alone (lane 11). Therefore, both AT(D)P and Mg²⁺ are neces-
sary to form the cross-link. The cross-linked β dimer was formed even under air oxygen without CuCl2, but the yield of the cross-linked β dimer is formed even under air oxygen without CuCl2 and essentially the same dependence of the cross-linking on Mg-nucleotide was observed. However, the time courses of the cross-linking under air oxygen varied in the experiments in different days although they were reproducible in one sequence of experiments in the same day. By this reason, unless otherwise stated, we included 0.25 μM CuCl2 in the reaction mixtures in which the cross-linking occurred at reproducible rates of the order of 10 min as described later. At high concentration of CuCl2 (100 μM), the cross-link was formed after a 2-h incubation in the absence of Mg-ATP, but the yield of the cross-linked β dimer was partial (lane 3). At 100 μM CuCl2 in the presence of Mg-ATP, the cross-link was formed instantaneously (see next paragraph). Prior treatment of the subcomplex with NEM prevented the cross-link (lane 4).

The Enzyme Containing the Cross-link Was Inactive in Catalytic Turnover—As mentioned above, cross-linking occurs in Mg-ATP where the enzyme is catalyzing hydrolysis of ATP. When we added 100 μM CuCl2 during continuous assay of ATPase, ATP hydrolysis stopped almost immediately (<15 s), but the full activity was recovered by addition of 150 mM DTT (Fig. 3A, trace a). It should be noted that the solutions contained LDAO so that generation of the Mg-ADP inhibited form was avoided. ATPase activity of the mutant subcomplex pre-treated with NEM (trace b) and that of the wild-type subcomplex (trace c) were not affected either by CuCl2 or DTT. This suggests that the cross-linking causes the loss of the activity to cycle the catalysis. Indeed, the time-course of the ATPase inactivation mirrored that of the yield of the cross-linked β dimer (Fig. 3B), and the analysis of their correlation showed that the subcomplex containing a cross-linked β dimer completely lost the ability to mediate catalytic turnover of ATP hydrolysis (Fig. 3C). The inactivated subcomplex recovered a full activity by incubation with a reducing reagent, for example 100 mM DTT (data not shown).

Cross-linking Occurred Most Rapidly in Mg-ADP + Azide—Rates of cross-linking were assessed by measuring the rates of ATPase inactivation in the presence of nucleotide and azide (Fig. 4). To ensure that the residual ATPase activities at each time point reflect correctly the fraction of the native subcomplexes without the cross-link, they were measured in the ATPase assay solutions containing LDAO in which subcomplexes showed uninhibited linear activities without an initial lag. Inactivation of ATPase activities proceeded with single exponential curves (solid lines) most rapidly in Mg-ADP + azide, next in Mg-ADP, Mg-ATP + azide, and most slowly in Mg-ATP. The half-decay times were 3.6, 5.8, 10.2, and 21.0 min under the described conditions. Because the Mg-ADP inhibited form is produced most efficiently when F1-ATPase is incubated with Mg-ADP and it is further facilitated by azide (23–25), the cross-linking of the Mg-ADP inhibited form appears to be favorable for the cross-linking.

Effect of Mg-Nucleotide Concentrations on the Cross-linking—To estimate how many nucleotides were required for the formation of the cross-link at 0.25 μM CuCl2, the mutant subcomplex was incubated with various concentrations of Mg-ADP and Mg-TNP-ADP, and the final yield of the cross-linked β dimer was measured. At low concentrations of nucleotides, cross-linking proceeded slowly and it was safe to wait for 24 h to reach the final, maximum yield (Fig. 5). Formation of the cross-link was saturated when the concentrations of Mg-nucleotide, expressed as a molar ratio Mg-nucleotide:subcomplex, reached 3:1. It should be noted that at molar ratio 1:1, cross-linked β dimer was formed in only a small fraction of the subcomplexes without the cross-link, they were measured in the ATPase assay solutions containing LDAO in which subcomplexes showed uninhibited linear activities without an initial lag. Inactivation of ATPase activities proceeded with single exponential curves (solid lines) most rapidly in Mg-ADP + azide, next in Mg-ADP, Mg-ATP + azide, and most slowly in Mg-ATP. The half-decay times were 3.6, 5.8, 10.2, and 21.0 min under the described conditions. Because the Mg-ADP inhibited form is produced most efficiently when F1-ATPase is incubated with Mg-ADP and it is further facilitated by azide (23–25), the cross-linking of the Mg-ADP inhibited form appears to be favorable for the cross-linking.

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catalytic sites need to be filled by Mg-nucleotides.

Subcomplex Containing the Cross-link Had Two Bound Nucleotides—The amount of bound nucleotide in the mutant subcomplex was measured after the cross-link was formed in Mg-ATP (Table I). Unbound nucleotide was removed by gel filtration, and nucleotide bound to the subcomplex in a stable manner was analyzed. For comparison, two samples, the wild-type subcomplex incubated with Mg-ATP and the mutant subcomplex incubated with Mg-ATP + DTT were also analyzed. The mutant subcomplex incubated with Mg-ATP + DTT had only a trace amount of bound nucleotide. The mutant subcomplex after formation of the cross-link retained about 2 mol/mol bound ADP (Mg$^{2+}$ was not analyzed). This result indicates that when the carbonyl-terminal domains of the two $\beta$ subunits are fixed in the closed conformation by the cross-link, two Mg-ADPs remain trapped in their catalytic sites. The wild-type subcomplex had about 1 mol/mol bound ADP (Table I). It can bind as much as 3 mol/mol Mg-ADP when analyzed with equilibrium dialysis (5), but 2 mol of Mg-ADP probably dissociated upon separation from unbound Mg-ADP during gel filtration. Related to our observation, it was reported that when a subunit and $\gamma$ (or $\epsilon$) subunit of E. coli F$_1$-ATPase were cross-linked, entrapped nucleotide (ATP or ADP) cannot be released (41)

The Enzyme Containing the Cross-link Catalyzed Uni-site Catalysis and Chase-promotion—Hydrolysis of a substoichiometric amount of substrate (uni-site catalysis) and its acceleration by chase-added ATP (chase-promotion) of the subcomplex with the cross-link were measured. To avoid confusion arising from bound Mg-ADP, nucleotide-free subcomplex with the cross-link was prepared in 100 $\mu$M CuCl$_2$ in the absence of nucleotide. The subcomplex thus prepared did not have the activity of steady-state ATP hydrolysis, but it retained the ability to catalyze uni-site hydrolysis of TNP-ATP (Fig. 6). The rates of uni-site catalysis of the subcomplexes with and without the cross-link were almost the same at 26 °C (Fig. 6, A and B, closed circles), and chase-promotion was also observed for both (open circles). This result indicates that a single high affinity catalytic site and its communication with the second catalytic site of the subcomplex are not lost by the cross-linking. However, the efficiency of the communication was diminished because at low temperature, 8 °C, chase-promotion observed for the subcomplex with the cross-link was very poor compared with that of the subcomplex without the cross-link (Fig. 6, A and B, insets).

**DISCUSSION**

$\beta$-$\beta$ Cross-linking as a Specific Probe to Detect the CCO Conformational State of F$_1$-ATPase—Intersubunit cross-linking has been proved to be a useful method in the study of F$_1$-ATPase to know relative location and motion of subunits (19, 41, 42). In the crystal structure of the $\alpha_3\beta_3$ subcomplex of TF$_i$, the whole structure is arranged in an exact three-fold
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Fig. 4. Effect of Mg-ATP(1)P and NaN₃ on the rates of ATPase inactivation caused by cross-linking. The reactions were initiated by addition of the mutant subcomplex into the following solutions: (final concentrations) 2 mM ATP and 2 mM MgCl₂ (●); 2 mM ATP, 2 mM MgCl₂, and 10 mM NaN₃ (○); 2 mM ADP and 2 mM MgCl₂ (●); or 2 mM ADP, 2 mM MgCl₂, and 10 mM NaN₃ (○). All the solutions contained 0.25 mM CuCl₂. The mixtures were incubated at 25°C. At every 5 min, aliquots were taken out, and residual ATPase activities were measured in the ATPase assay solutions containing 0.1% LDAO.

Fig. 5. Effect of Mg-nucleotide concentrations on the cross-linking. The 1 μM mutant subcomplex was incubated with ADP (●) or TNP-ADP (○) in the presence of 2 mM MgCl₂ at 25°C for 24 h, and the yield of the cross-linked β–β dimer was analyzed. The concentrations of nucleotides are expressed as molar ratio to the subcomplex.

Table I

| αβγ | Cross-linking (%) | Bound ADP |
|-----|-----------------|-----------|
| I386C | 0 | 0.1 |
| I386C | 100 | 2.0 |
| Wild type | — | 1.3 |

* From the non-reducing SDS-PAGE.
  * Average of two experiments.

Symmetry, and all three β subunits are in the open conformation (43). The distance between Ile-386s of the two β subunits in this subcomplex is 3.27 nm, and a disulfide bond would not be possible even if β-Ile-386s are replaced with Cys. By substituting the closed β subunit in the crystal structure of MF₁ with the open β subunit with the aid of a computer, we generated the structure of the αβγ subcomplex with one closed and two open β subunits (COO conformational state) (Fig. 1C) and that with three open β subunits (OOO conformational state) (Fig. 1D). β-Ile-390s in the COO conformational state are distant from each other. The distances between Ile390 of the closed β and that of each of the two open βs are 2.42 and 3.65 nm, too far for cross-linking. This is also the case for the OOO conformational state; β-Ile390s are far away from each other, just similar to αββ subcomplex of TF₁ (43). Only when two β subunits take the closed conformation (CCO conformational state) do their lifted carboxyl-terminal domains bring β-Ile-390s into the contact position (Fig. 1, A and B). One might think of a CCC conformational state, but when we generated CCC conformational state based on the MF₁ structure, γ subunit could not be accommodated in the center of the molecule without steric collision. Therefore, it is unlikely that all three β subunits take closed conformations. Thus, β–β cross-linking between the introduced Cys at the position β-386 of TF₁ is a specific means to identify the CCO conformational state of F₁-ATPase in solution. This rationale should be valid for F₁-ATPases from other sources.

F₁-ATPase Takes the CCO Conformation in Catalytic Cycle and in the Mg-ADP Inhibited Form—The cross-link was formed in the solution containing Mg-ATP and ATP regeneration system where catalysis was going on (Fig. 3A). Therefore, at least one of the intermediates in the catalytic cycle of the subcomplex takes the CCO conformation.²

Because azide stabilizes the Mg-ADP inhibited form (23–25), accelerated formation of cross-linking in Mg-ATP by azide (Fig. 4) indicates that the Mg-ADP inhibited form, which is probably derivatized from the catalytic intermediate(s) with a CCO conformation as mentioned above, also takes a CCO conformation. This contention is supported by the fact that cross-linking occurs even more efficiently in Mg-ADP and Mg-ADP + azide, conditions where the Mg-ADP inhibited form is produced efficiently. Requirement for 2 mol of Mg-nucleotide per mol of the subcomplex for the cross-linking (Fig. 5) is consistent with the observation that the rate of development of azide inhibition during ATP hydrolysis was saturated at an ATP concentration of about 10 μM, a concentration range of bi-site catalysis where the enzyme operates with two catalytic sites being occupied by substrates (22).

² One can argue that the Mg-ADP inhibited form, but not an active intermediate, is the molecular species in which cross-link is formed under the conditions in Fig. 3A because the Mg-ADP inhibited form, which also takes the CCO conformation, is generated more or less in a dynamic equilibrium during catalysis. However, this assumption may not be the case. The solution contained LDAO, an activator known to keep F₁-ATPases from falling into the Mg-ADP inhibited form during catalysis of about 10 μM Mg-nucleotide per mol of the subcomplex for the cross-linking (Fig. 5) is consistent with the observation that the rate of development of azide inhibition during ATP hydrolysis was saturated at an ATP concentration of about 10 μM, a concentration range of bi-site catalysis where the enzyme operates with two catalytic sites being occupied by substrates (22).
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Existence of COO (or OOO) Conformational State of F₁-ATPase Is Suggested—It has been known that Mg-free AT(D)P can bind to catalytic sites of F₁-ATPase but not to noncatalytic nucleotide binding site (44, 45). The αβγ′ subcomplex can bind 3 mol of Mg-free ADP per mol (5). Nonetheless, the cross-linked β dimer was not formed in Mg-free AT(D)P (Fig. 2). This indicates that subcomplex with bound Mg-free AT(D)P at catalytic site(s) dwells in a conformational state, probably either in a COO or OOO conformational state, in which two or three β subunits are in open conformations. In this respect, the ligand acting as a general base in ATP hydrolysis reaction (17), by dicyclohexylcarbodiimide was completely blocked when Mg²⁺ was liganded in a catalytic site (46).

γ Subunit Cannot Rotate without Open-Closed Transition of β Subunits—The mutant subcomplex cannot catalyze multiple catalytic turnover when the cross-link was formed (Fig. 3). Because multiple catalytic turnover of F₁-ATPase couples with the rotation of γ subunit, shifting the carboxyl-terminal domain from the open position to the closed one. Probably related to this, the profound effect of Mg²⁺ on the binding affinity of AT(D)P to the catalytic sites of F₁-ATPase was reported by Senior’s group (44). Also, we previously observed that chemical labeling of β-Glu-190, a catalytic residue acting as a general base in ATP hydrolysis reaction (17), by dicyclohexylcarbodiimide was completely blocked when Mg²⁺ was liganded in a catalytic site (46).

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