THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 9, Issue of February 26, pp. 5701–5706, 1999

Printed in U.S.A.

Cross-linking of Two β Subunits in the Closed Conformation in F_1-ATPase*

(Received for publication, September 21, 1998, and in revised form, November 10, 1998)

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In the crystal structure of mitochondrial F_1-ATPase, two β subunits with a bound Mg-nucleotide are in "closed" conformations, whereas the third β subunit without bound nucleotide is in an "open" conformation. In this "CCO" (β-closed β-closed β-open) conformational state, Ile-390s of the two closed β subunits, even though they are separated by an intervening α 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 β-β 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 dwells 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 β-β cross-link retained the activity of uni-site catalysis but lost that of multiple catalytic turnover, suggesting that open-closed transition of β subunits is required for the rotation of γ 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, α, β, γ, δ, and ε 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 α and three β subunits are arranged alternately like the segments of an orange around the central coiled-coil structure of the γ subunit. F_1-ATPase contains six nucleotide binding sites; three of them are catalytic sites and mainly located on each β subunit, whereas the other three do not bear a catalytic role and are mainly located on each α 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 substoichiometric 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 γ 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 γ 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*-dimethyldodecylamine-*N*-oxide (LDAO) destabilizes it (31).

The crystal structure of MF_1 (4) revealed that three β subunits in the MF_1 molecule are in different states; one β (β_{TP}) has an ATP analog, Mg-AMP-PNP, at its catalytic site; another β (β_{DP}) has Mg-ADP; the third β (β_E) has none. The structures of β_{TP} and β_{DP} are very similar to each other, and they are in the "closed" conformation, in which the carboxyl-terminal domain is lifted close to the nucleotide binding domain. In contrast, β_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*-dimethyldodecylamine-*N*-oxide; DTT, dithiothreitol; TNP-AT(D)P, 2′,3′-*O*-(2,4,6-trinitrophenyl) derivatives of AT(D)P; 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.

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 MF_1 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 F_1-ATPase in solution. Looking at the structure of MF_1 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 F_1-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 $\alpha_3\beta_3\gamma$ subcomplex of TF_1, 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, CJ236 (33) for generating uracil-containing single-stranded plasmid for site-directed mutagenesis, and JM103D (*uncB-uncD*) (34) for expression of the wild-type and mutant $\alpha_3\beta_3\gamma$ subcomplexes of TF_1. Plasmid pTABG3 (6), which carried genes for the α, β, and γ subunit of TF_1 was used for mutagenesis and expression. A 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′-TTGTCTTCATCCGACAGTTCATCCATCCCCAAGCATGCGATGATGTCTTGCAATTCTTTATAACGTTCGA-3′ (changed bases are underlined) (33). The *Eco*RI-*Bgl*II fragment from the resultant plasmid was ligated into the *Eco*RI-*Bgl*II site of pKABG3 to produce the expression plasmid, pKAG3-β-Ile386Cys. Recombinant DNA procedures were performed as described in the manual (35). The wild-type and the mutant $\alpha_3\beta_3\gamma$ subcomplexes were purified and stored as described previously except that 2 mM dithiothreitol (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) μmol 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 indicated components. At the indicated time, an aliquot was taken out and formation of the cross-link was analyzed with 10.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate without exposure to a reducing reagent (non-reducing SDS-PAGE) (36). To prevent further oxidation during electrophoresis, all sample solutions were treated with 10 mM *N*-ethylmaleimide (NEM) for 5 min prior to electrophoresis. The gels were stained with Coomassie Brilliant Blue R-250. The yields of the cross-linked product were determined from the staining intensity of bands with a photodensitometer (model SM3, Howtec Inc.) using the α subunit band as an internal standard.

Analysis of Bound Adenine Nucleotide—The wild-type and mutant subcomplexes were incubated in 20 μM ATP and 2 mM $MgCl_2$ for 2.5 h at 25 °C. The yield of cross-linked β dimer of the mutant subcomplex estimated from non-reducing SDS-PAGE was nearly 100% after this incubation. As a control, the samples treated with the same procedures, except that 10 mM DTT was included in the solution, were also analyzed. To remove free adenine nucleotide, the samples were passed through an HPLC column TSK-G3000SWXL. The protein fractions were recovered, and perchloric acid was added to precipitate proteins. The amounts of adenine nucleotides contained in the supernatant were measured with reversed-phase HPLC on a Cosmosil 5C18-AR-II (Nacarai Tesque, Japan), equilibrated, and eluted with 0.1 M sodium phosphate buffer (pH 6.9).

Other Assays—ATPase activity was measured at 25 °C in the presence of an ATP-regenerating system (37). Unless stated, a neutral detergent, LDAO, was included in the assay mixture to eliminate the effect of the initial lag phase caused by the Mg-ADP inhibition (31). The assay mixture contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2.5 mM phosphoenolpyruvate, 2.5 mM ATP, 2 mM $MgCl_2$, 0.2 mM NADH, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, and 0.1% LDAO. Uni-site ATPase activity and chase promotion were measured at 26 and 8 °C using TNP-ATP as a substrate as described previously (38). A reaction mixture (50 μl) containing 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 200 mM KCl, and 0.3 μM TNP-ATP was incubated at 26 °C. The reaction was initiated by the addition of an equal volume of 1 μM subcomplexes in 50 mM Tris-HCl (pH 8.0). The reaction was quenched by the addition of 7.5 μl of ice-cold 24% perchloric acid. In chase-promotion experiments, 10 μl of 30 mM ATP-Mg was added instead of perchloric acid. After 5 s, the reaction was quenched by the addition of 7.5 μl of ice-cold 24% perchloric acid. The amounts of TNP-ATP and TNP-ADP were measured by HPLC (38). For uni-site and chase-promotion experiments, the mutant subcomplex with the cross-link lacking bound nucleotide was prepared. The mutant subcomplex was treated with 100 μM $CuCl_2$ for 24 h at 25 °C, and the subcomplex was separated from $CuCl_2$ with gel filtration (Sephadex G-25 column, 2 cm × 5 cm). Formation of the cross-link at 100% yield was confirmed with SDS-PAGE before use. Protein concentration of $\alpha_3\beta_3\gamma$ subcomplex was determined by UV absorbance using the factor of 0.45 at 280 nm as 1.0 mg/ml.

RESULTS

Two β Subunits in the Closed Form Have a Contact Site—In the crystal structure of MF_1, two β subunits with a bound nucleotide, β_{TP} and β_{DP}, 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-390s of each of β subunits (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 (C_β of β_{TP}-Ile-390 and $C_{\gamma 2}$ of β_{DP}-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 β_{TP}-Ile-390 and β_E-Ile-390 and that between β_{DP}-Ile-390 and β_E-Ile-390 are 2.40 nm and 2.58 nm, respectively. This residue is located in a region just preceding the "DELSEED sequence" (39) and is highly conserved in F_1-ATPases from various sources.

The $\alpha_3\beta_3\gamma$ Subcomplex with Cys at the Contact Sites Formed β-β Cross-link in the Presence of Both Mg^{2+} and AT(D)P—We generated a mutant $\alpha_3\beta_3\gamma$ subcomplex of TF_1 in which β-Ile-386, an equivalent residue to MF_1 β-Ile-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 μM $CuCl_2$ 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 or with DTT (Fig. 2, *lanes 1* and *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 samples to a reducing reagent prior to electrophoresis eliminated the band (*lane 12*). 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 Mg^{2+} alone (*lane 11*). Therefore, both AT(D)P and Mg^{2+} are neces-

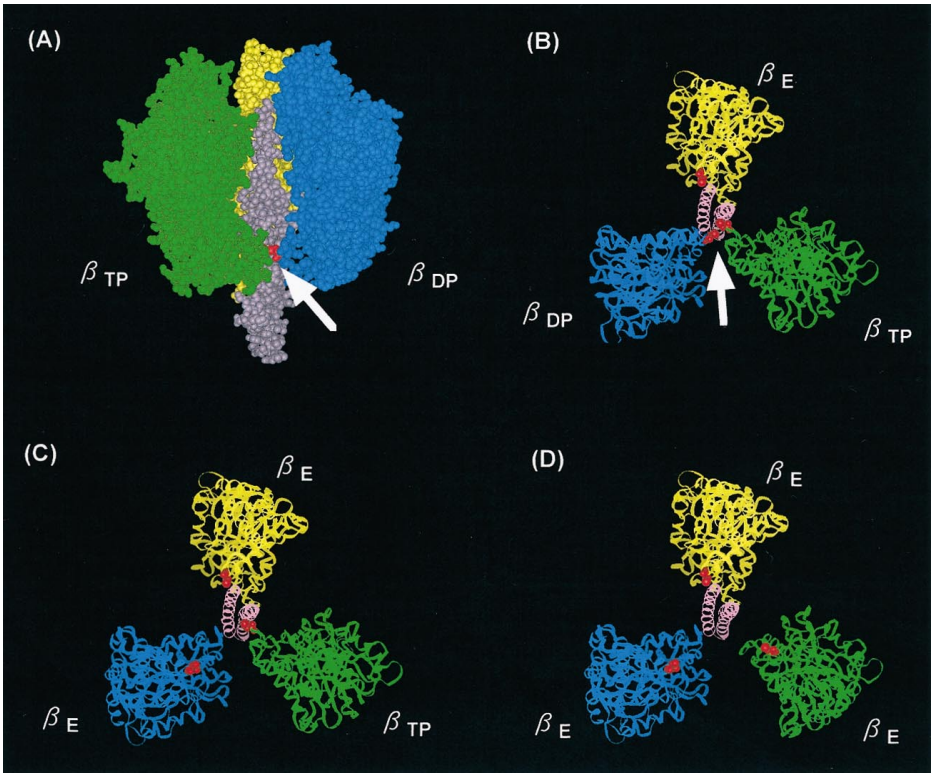

FIG. 1. **A contact point between two β subunits in a closed conformation in F_1-ATPase.** Illustrations are based on the structure of bovine MF_1 (4). Subunits β_{TP}, β_{DP}, and β_E represent the β subunits with their nucleotide binding sites occupied by Mg-AMP-PNP, Mg-ADP, and none, respectively. The contact point is indicated by an *arrow*. The α subunits are removed from the structure and only a part of the γ subunit (*A*, residues 1–45, 73–90, and 209–272; *B*, residues 1–45 and 215–272) is shown to make the contact point visible. Ile-390s are shown by *red space-filling atoms*. *A*, a side view; *B*, a view from the membrane (F_0) side. *C*, two β subunits are in the open conformation and one β subunit is in the closed conformation. *D*, three β subunits are all in the open conformation. *C* and *D* were generated by replacing one (β_{DP}) or two (β_{DP} and β_{TP}) β subunits in the crystal structure with the β subunit(s) in the open conformation (β_E). Replacement was carried out using Insight II computer program. The open β subunit was overlaid with the closed β subunit with maximum overlapping at the region of residues 9–150 and then the closed β subunit was erased.

sary to form the cross-link. The cross-linked β dimer was formed even under air oxygen without $CuCl_2$ and essentially the same dependence of the cross-linking on Mg-nucleotide were 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 $CuCl_2$ 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 $CuCl_2$ (100 μM), the cross-link was formed after a 2-h incubation in the absence of Mg-AT(D)P, but the yield of the cross-linked β dimer was partial (*lane 3*). At 100 μM $CuCl_2$ in the presence of Mg-AT(D)P, 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 $CuCl_2$ 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. 3*A*, *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 pretreated with NEM (*trace b*) and that of the wild-type subcomplex (*trace c*) were not affected either by $CuCl_2$ 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-β dimer (Fig. 3*B*), 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. 3*C*). 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 F_1-ATPase is incubated with Mg-ADP and it is further facilitated by azide (23–25), the conformation 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 $CuCl_2$, 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 subcomplex (20%, Mg-ADP; 3%, Mg-TNP-ADP) but at molar ratio 2:1, 50% or more populations of the subcomplexes had a cross-linked β dimer. Similar results were obtained when the subcomplex was incubated with Mg-ATP and Mg-TNP-ATP (not shown). It was demonstrated previously from difference absorption spectrum titration that, when added with 1:1 molar ratio at micromolar range, almost all Mg-TNP-ADP bound to a catalytic site of the subcomplex (7). Also, nearly all Mg-ADP added at 1:1 molar ratio was bound to the subcomplex (22, 40). Therefore, for cross-linking to occur, occupation of a single catalytic site by Mg-nucleotide is not sufficient but at least two

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 carboxyl-terminal domains of the two β 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 α subunit and γ (or ε) subunit of *E. coli* F_1-ATPase were cross-linked, entrapped nucleotide (ATP or ADP) cannot be released (41).

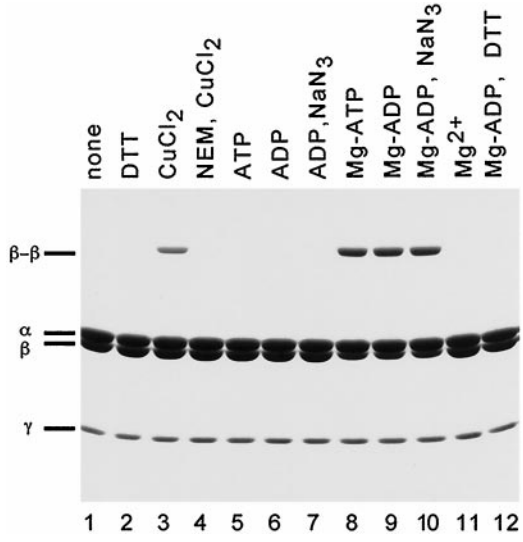

FIG. 2. **The formation of the cross-linked β dimer through a disulfide bond in the mutant $\alpha_3\beta_3\gamma$ subcomplex.** *Lanes 1-11.* The mutant subcomplex was incubated for 2 h at 25 °C with following reagents and analyzed on non-reducing SDS-PAGE: none (*lane 1*); 10 mM DTT and 0.25 μM $CuCl_2$ (*lane 2*); 100 μM $CuCl_2$ (*lane 3*); 2 mM NEM and 100 μM $CuCl_2$ (*lane 4*); 2 mM ATP, 1 mM EDTA, and 0.25 μM $CuCl_2$ (*lane 5*); 2 mM ADP, 1 mM EDTA, and 0.25 μM $CuCl_2$ (*lane 6*); 2 mM ADP, 10 mM NaN_3, 1 mM EDTA, and 0.25 μM $CuCl_2$ (*lane 7*); 2 mM ATP, 4 mM $MgCl_2$, and 0.25 μM $CuCl_2$ (*lane 8*); 2 mM ADP, 4 mM $MgCl_2$, and 0.25 μM $CuCl_2$ (*lane 9*); 2 mM ADP, 4 mM $MgCl_2$, 10 mM NaN_3, and 0.25 μM $CuCl_2$ (*lane 10*); and 4 mM $MgCl_2$ and 0.25 μM $CuCl_2$ (*lane 11*). *Lane 12*, the sample treated as *lane 9* was then incubated for 1 min with 10 mM DTT prior to SDS-PAGE.

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 μM $CuCl_2$ 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

β-β 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_1, the whole structure is arranged in an exact three-fold

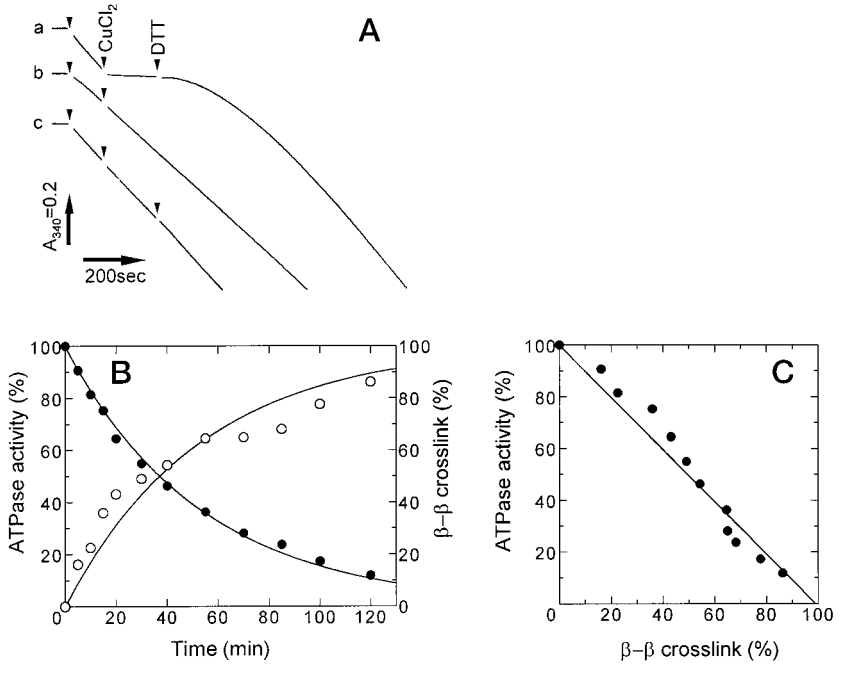

FIG. 3. **The cross-linking inactivates ATPase activity.** *A*, traces of ATP hydrolysis catalyzed by the subcomplexes. Hydrolysis was monitored by the oxidation of NADH with an ATP-regenerating system as described under "Experimental Procedures." The reaction mixture contained 0.1% LDAO to avoid the influence of the Mg-ADP inhibited form. The reactions were initiated by addition of the subcomplex at the time indicated by *arrowheads* (*a*, 1.1 μg of the mutant subcomplex; *b*, 1.1 μg of mutant subcomplex treated with 1 mM NEM for 20 min at 25 °C; *c*, 0.8 μg of wild type subcomplex). Then 100 μM $CuCl_2$ or 150 mM DTT were added at the time indicated by *arrowheads*, respectively. These high concentrations of $CuCl_2$ and DTT were used to ensure the rapid reactions. *B*, time courses of formation of the cross-linked β-β dimer and inactivation of ATPase activity. The mutant subcomplex was incubated at 25 °C with 2 mM ATP and 2 mM $MgCl_2$. At indicated times, aliquots were taken out, and ATPase activity (●) and the yield of the cross-linked β-β dimer (○) were analyzed. *C*, correlation between the cross-linked β-β dimer and residual ATPase activity.

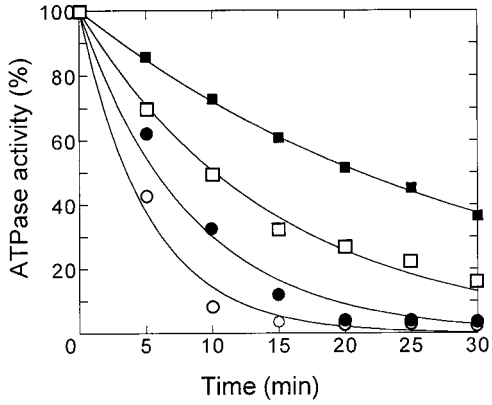

FIG. 4. **Effect of Mg-AT(D)P and NaN_3 on the rates of ATPase inactivation caused by cross-linking.** The reactions were initiated by addition of the mutant subcomplex into the following solutions: (final concentrations) 2 mM ATP and 2 mM $MgCl_2$ (■); 2 mM ATP, 2 mM $MgCl_2$ and 10 mM NaN_3 (□); 2 mM ADP and 2 mM $MgCl_2$ (●); or 2 mM ADP, 2 mM $MgCl_2$, and 10 mM NaN_3 (○). All the solutions contained 0.25 μM $CuCl_2$. 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.

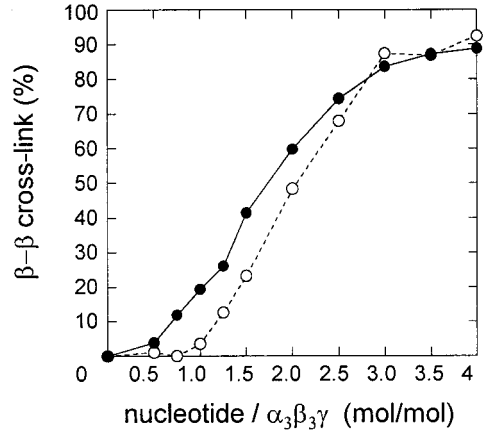

FIG. 5. **Effect of Mg-nucleotide concentrations on the cross-linking.** The 1 μM mutant subcomplex was incubated with ADP ● or TNP-ADP (○) in the presence of 2 mM $MgCl_2$ at 25 °C for 24 h, and the yield of the cross-linked β-β dimer was analyzed. The concentrations of nucleotides are expressed as molar ratio to the subcomplex.

TABLE I
Quantitative analysis of bound nucleotides

$\alpha_3\beta_3\gamma$	Cross-linking[a]	Bound ADP[b]
	%	*mol/mol*
I386C	0	0.1
I386C	100	2.0
Wild type	—	1.3

[a] From the non-reducing SDS-PAGE.
[b] 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 MF_1 with the open β_E subunit with the aid of a computer, we generated the structure of the $\alpha_3\beta_3\gamma$ subcomplex with one closed and two open β subunits (COO conformational state) (Fig. 1*C*) and that with three open β subunits (OOO conformational state) (Fig. 1*D*). 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 $\alpha_3\beta_3$ subcomplex of TF_1 (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 MF_1 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 TF_1 is a specific means to identify the CCO conformational state of F_1-ATPase in solution. This rationale should be valid for F_1-ATPases from other sources.

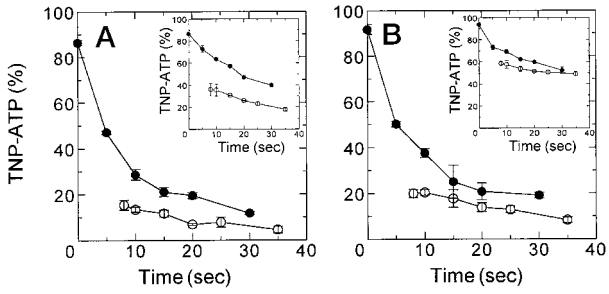

FIG. 6. **Uni-site hydrolysis of substoichiometric TNP-ATP and chase-promotion by the mutant subcomplex.** *A*, the mutant subcomplex without cross-link; *B*, the mutant subcomplex with the cross-link. The reaction mixture contained 0.5 μM mutant subcomplex and 0.13 μM TNP-ATP. Uni-site catalysis (●) and chase promotion (○) were assayed at 26 °C as described under "Experimental Procedures." The experimental data of chase promotion are shown at the time when the reactions were stopped. *Inset*, uni-site catalysis and chase promotion measured at 8 °C.

F_1-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. 3*A*). 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. 3*A* 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 argument may not be the case. The solution contained LDAO, an activator known to keep F_1-ATPases from falling into the Mg-ADP inhibited form during catalysis and ATP regenerating system to convert the free ADP into ATP. Therefore, the fraction of the Mg-ADP inhibited form should be minimal in the solution. The inactivation of ATPase activity, which was caused by cross-linking, occurred immediately when 100 μM $CuCl_2$ was added. If the cross-link had been formed only from the Mg-ADP inhibited form, the inactivation would have proceeded very slowly in the presence of LDAO.

Existence of COO (or OOO) Conformational State of F_1-ATPase Is Suggested—It has been known that Mg-free AT(D)P can bind to catalytic sites of F_1-ATPase but not to noncatalytic nucleotide binding site (44, 45). The $\alpha_3\beta_3\gamma$ 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 liganding of Mg^{2+} to nucleotide at the catalytic site has a great effect on the conformation of the β subunit; shifting the carboxyl-terminal domain from the open position to the closed one. Probably related to this, the profound effect of Mg^{2+} on the binding affinity of AT(D)P to the catalytic sites of F_1-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 Mg^{2+} 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 F_1-ATPase couples with rotation of γ subunit, indication of this result is that, when two β subunits are fixed in the closed conformation, the rotation of the γ subunit is blocked. The open-closed conformational transition of each of the β subunits and exchange of the CC (β-closed, β-closed) pair among three β subunits appear to be necessary for the rotation of the γ subunit and mechanical prevention of this transition by the cross-linking results in blocking the rotation and, thereby, turnover of ATPase cycle. Probably by the same reason, *E. coli* F_1-ATPase in which the residue β-381, an equivalent residue of TF_1 β-E391, is labeled by a bulky fluorescent dye is inactive in ATPase catalytic turnover (42). In a sense, the mechanism of inactivation caused by cross-linking may be similar to that of the Mg-ADP inhibition, blocking sequential transition of the intermediate states in catalysis of the enzyme. The difference is that the former blocks physically and the latter does kinetically. Once the cross-link is formed in the Mg-ADP inhibited form, the transition is now physically blocked.

Based on the experiments of the β-γ cross-linked *E. coli* F_1-ATPase, Capaldi's group has proposed that uni-site catalysis does not accompany the rotation (47). Our result, that the subcomplex with the β-β cross-link can catalyze uni-site TNP-ATP hydrolysis (Fig. 6), again reinforces this conclusion. Mere hydrolysis of a single ATP probably does not require the open-closed transition of the β subunits. Furthermore, because the uni-site catalysis by the subcomplex with the β-β cross-link was promoted by chase-added ATP (Fig. 6), communication between two β subunits in the enzyme is still possible without open-closed transition of the β subunits. Probably the open-closed transition of the β subunits is needed to drive the critical catalytic step, *i.e.* simultaneous exchange of the role of each catalytic site.

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