Oxidation of the \(\alpha_3(\beta D311C/R333C)\gamma\) Subcomplex of the Thermophilic Bacillus PS3 F1-ATPase Indicates That Only Two \(\beta\) Subunits Can Exist in the Closed Conformation Simultaneously*

(Received for publication, June 10, 1999, and in revised form, August 2, 1999)

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In the crystal structure of the bovine heart mitochondrial F1-ATPase (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628), the two liganded \(\beta\) subunits, one with MgAMP-PNP bound to the catalytic site (\(\beta_\alpha\)) and the other with MgADP bound (\(\beta_\beta\)), have closed conformations. The empty \(\beta\) subunit (\(\beta_\gamma\)) has an open conformation. In \(\beta_\alpha\) and \(\beta_\beta\), the distance between the carboxylate of \(\beta\text{-Asp}^{155}\) and the guanidinium of \(\beta\text{-Arg}^{262}\) is 3.0–4.0 Å. These side chains are at least 10 Å apart in \(\beta_\gamma\). The \(\alpha_3(\beta D311C/R333C)\gamma\) subcomplex of TF1, with the corresponding residues substituted with cysteine has very low ATPase activity unless it is reduced prior to assay or assayed in the presence of diithiothreitol. The reduced subcomplex hydrolyzes ATP at 50% of the rate of wild-type and is rapidly inactivated by oxidation with CuCl2 with or without magnesium nucleotides bound to catalytic sites. Titration of the subcomplex with iodo[14C]acetamide after prolonged treatment with CuCl2 in the presence or absence of 1 mM MgADP revealed nearly two free sulfhydryl groups/mol of enzyme. Therefore, one pair of introduced cysteines is located on a \(\beta\) subunit that exists in the open or partially open conformation even when catalytic sites are saturated with MgADP. Since \(V_{\text{max}}\) of ATP hydrolysis is attained when three catalytic sites of F1 are saturated, the catalytic site that binds ATP must be closing as the catalytic site that releases products is opening.

The proton-translocating F0F1-ATP synthases couple proton electrochemical gradients to condensation of ADP with Pi in energy-transducing membranes. The F0 moieties are a membrane-embedded protein complex that mediates proton translocation. F1 is a peripheral membrane protein complex composed of five different subunits with \(\alpha_3\beta_3\gamma\delta\epsilon\) stoichiometry. When removed from the membrane, F1 is an ATPase containing six nucleotide binding sites. Three are catalytic sites that are predominantly on \(\beta\) subunits at \(\alpha/\beta\) interfaces. The three other sites, called noncatalytic sites, are located predominantly on \(\alpha\) subunits at different \(\alpha/\beta\) interfaces (1–3).

The crystal structures of the bovine heart and rat liver mitochondrial F1-ATPases as well as that of the \(\alpha_3\beta_3\gamma\) subcomplex of the TF1-ATPase have been determined (4–6). In the crystals of the bovine heart enzyme (BH-MF1), which form in media containing AMP-PNP, ADP, Mg2+, and N7, noncatalytic sites are homogeneously liganded with MgAMP-PNP, whereas catalytic sites are heterogeneously liganded (4). One, designated \(\beta_\gamma\), contains MgAMP-PNP, another, designated \(\beta_\delta\), contains MgADP and the third catalytic site, designated \(\beta_\epsilon\), is empty. Except for small differences in the regions of the terminal phosphates of bound nucleotides, the arrangements of functional amino acid side chains in catalytic sites in \(\beta_\alpha\) and \(\beta_\beta\) are essentially identical. In contrast, these residues are arranged differently in \(\beta_\epsilon\). In the crystals of the \(\alpha_3\beta_3\gamma\) subcomplex of TF1 that form in media free of Mg2+ and nucleotides, the \(\alpha\) subunits are in closed conformations corresponding to the liganded \(\alpha\) subunits of the mitochondrial enzymes. The \(\beta\) subunits in the \(\alpha_3\beta_3\gamma\) structure are in open conformations corresponding to \(\beta_\epsilon\) of BH-MF1 (5).

In the crystals of the rat liver F1-ATPase (RL-MF1), which form in media containing ATP, Pi, and EDTA, noncatalytic sites are homogeneously occupied with MgATP. In contrast to the crystals of BH-MF1, all three catalytic sites of RL-MF1 contain bound nucleotides in the absence of Mg2+ (6). One catalytic site contains ADP alone, whereas the other two contain ADP and Pi. Since Mg2+ is not associated with ADP and Pi bound to catalytic sites of RL-MF1, side chains in catalytic sites that interact with the Mg2+ ion in the crystal structure of BH-MF1 are arranged differently in the crystal structure of RL-MF1. However, these differences are minor compared with the difference between the closed conformations of \(\beta_\beta\) and \(\beta_\epsilon\) and the open conformations of \(\beta_\gamma\) in BH-MF1. In the crystal structure of RL-MF1, the conformations of all three \(\beta\) subunits resemble the closed conformation of \(\beta_\alpha\) and \(\beta_\beta\) in the crystal structure of BH-MF1. Bianchet et al. (6) suggest that the open conformation of \(\beta_\epsilon\) in the crystal structure of BH-MF1 is the consequence of low concentrations of nucleotides in the crystallization medium. Extending this argument, they propose that the RL-MF1 structure with three closed \(\beta\) subunits plays a central role in catalysis, whereas the BH-MF1 structure with two closed \(\beta\) subunits and one open \(\beta\) subunit exists transiently during catalysis when products dissociate and substrates rebind.

To determine which of these structures is more consistent with properties of F1-ATPases in solution, we took advantage of pairs of amino acid residues in \(\beta\) subunits that do not contrib-

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* This work was supported by NIGMS, National Institutes of Health, Grant GM16974. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: TF1, BH-MF1, and RL-MF1, the F1-ATPases from the thermophilic Bacillus PS3, bovine heart mitochondria, and rat liver mitochondria, respectively; DTT, dithiothreitol; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; AMP-PNP, adenosine 5'-([\(\beta,\gamma\text{-imino}]triphosphate).
ute to catalytic sites in BH-MF₁ that have side chains within interaction distance in β₁ and β₂, but are considerably distant from each other in β₄. For example, the carboxylate of β-Asp³¹⁵ is within 3.0 and 3.7 Å of the guanidinium of β-Ala¹⁵⁸, in β₇ and β₉, respectively, whereas these side chains are 10.3 Å apart in β₅. Other pairs of amino acid residues that meet these criteria are β-Ala¹⁵⁸/β-Tyr³⁰⁷, β-Met²⁰⁷/β-Phe¹⁸₃, and β-Lys¹⁷⁵/β-Ser³⁰³. Double mutants of the α₀β₂γ subcomplex of TF₁ (7) were prepared in which residues corresponding to β-Ala¹⁵⁸, β-Met²⁰⁷/β-Phe¹⁸₃, and β-Lys¹⁷⁵/β-Ser³⁰³ of BH-MF₁ were substituted with cysteine.

The aim of preliminary studies was to find a double mutant that was stable and also could be converted reversibly between an oxidized, inactive form and a reduced, active form. Of the four double mutants generated, only the α₀β₂γ subcomplex corresponding to the β-Asp³¹⁵/β-Ala¹⁵⁸ mutant pair in BH-MF₁ met these criteria.

**EXPERIMENTAL PROCEDURES**

**Materials**—Biochemicals used in assays and buffer components were purchased from Sigma. Dithiothreitol, o-iodosobenzoic acid, iodoacetamide, dithiothreitol, DEAE-Sepharose, and Sephacryl S-300HR were also from Sigma. The radioactive reagents used were [³H]ADP from NEN Life Science Products; iodo[³H]acetate from American Radiolabeled Chemicals, and iodo[¹²⁵]I-lycetate from ICN. Aldrich supplied 5,5'-dithiobis-(2-nitrobenzoic acid) and sodium fluoride. Aluminum chloride was purchased from Fisher. Toronto Research Chemicals supplied [(1-trimethylammonium)methyl]methanethiosulfonate bromide. Toyopearl Butyl-650S resin was from Tosohaas. The oligonucleotides used for mutagenesis were purchased from Life Technology, Inc. The Escherichia coli strains and the plasmids used to prepare the wild-type and mutant α₀β₂γ subcomplexes were described by Matsui and Yoshida (7). The purified enzyme subcomplexes were stored as suspensions in 75% ammonium sulfate at 4 °C.

**Construction of Plasmids Containing Mutant Genes**—Plasmid pKK223-3, which carries genes encoding the α, β, and γ subunits of TF₁, was used for both mutagenesis and gene expression. Expression plasmids were constructed using polymerase chain reaction with the QuickChange™ site-directed mutagenesis kit from Stratagene. The plasmids were purified using the Wizard™ Plus mini-prep kit from Promega. The first mutation was introduced into wild-type pKK223-3 by polymerase chain reaction and confirmed by sequence analysis. The resulting mutant plasmid was used as template for the second polymerase chain reaction substitution, which was subsequently confirmed. The resulting pKK223-3 mutant plasmids were expressed in E. coli strain JM103 (unc). The primers used in the polymerase chain reactions are as follows with mismatched bases underlined: Y307C, 5'-CGATTTGCTGGATCCCGGACTAATGGACGAGC-3' ; 5'-GAGATCCAGCTGGGACGACATCGGGTACGTCGAC-3' ; Q177C, 5'-CACGATCCGGGCTGACGCTGGGACGACATCGGGTACGTCGAC-3' ; 5'-GAGATCCAGCTGGGACGACATCGGGTACGTCGAC-3' ; Q176C, 5'-GATTCTTATCGCGCGTTTATTCTGACGATGAAATG-3' ; F185C, 5'-GCGACTATACGGACC; R333C, 5'-CGAACGAACCTGGAGTGTAAGCTTGACGATGAAATG-3' ; D311C, 5'-CGAACGAACCTGGAGTGTAAGCTTGACGATGAAATG-3'.

**ATPase activity was determined spectrophotometrically with 2 mM ATP plus 3 mM Mg²⁺ using ATP regeneration with phosphonopyruvate and pyruvate kinase coupled to NADH oxidation by lactate dehydrogenase under conditions described earlier (10).**

**RESULTS**

**Comparison of the ATPase Activities of the α₀β₁α₀β₂γ Subcomplexes**—Table I shows the distances in the crystal structure of the α₀β₂γ subcomplex of TF₁ (5) between side chains of amino acid residues that were substituted with cysteine in the four double mutants examined in this study. The distances between the corresponding side chains in β₁, β₅, and β₇ in the crystal structure of MF₁ (4) are also tabulated.

The effects of DTT on the ATPase activities of the purified double mutant subcomplexes were determined. Each isolated enzyme subcomplex was treated with CDTA before assays were performed. This procedure removes endogenous MgADP from a catalytic site of the purified, wild-type α₀β₂γ subcomplex (8). The β₁α₀β₂γ subcomplex was isolated in an inactive form that could not be activated by treatment with DTT before assay or by including DTT in the assay medium. Lauryl dimethylamine oxide, which stimulates ATPase activity of the enzyme subcomplexes was determined by high pressure liquid chromatography using ion pairing with tetrabutyl ammonium hydrogen sulfate as described by Bullough et al. (12).

| Adjacent residues | β₁ | β₅ | β₇ |
|------------------|----|----|----|
| TF₁, β-Ala¹⁵⁸/β-Tyr³⁰⁷ | 7.9 |
| TF₁, β-Glu¹⁷⁷/β-Ser³⁰³ | 13.3 |
| TF₁, β-Gln²⁰⁷/β-Phe¹⁸₃ | 8.0 |
| TF₁, β-Lys¹⁷⁵/β-Ser³⁰³ | 14.7 |
| MF₁, β-Ala¹⁵⁸/β-Tyr³⁰⁷ | 3.6 | 4.2 | 12.3 |
| MF₁, β-Lys¹⁷⁵/β-Ser³⁰³ | 4.8 | 3.8 | 11.8 |
| MF₁, β-Met²⁰⁷/β-Phe¹⁸₃ | 4.8 | 4.8 | 10.6 |
| MF₁, β-Asp³¹⁵/β-Ala¹⁵⁸ | 3.0 | 3.7 | 10.3 |

**ATPase activity was determined spectrophotometrically with 2 mM ATP plus 3 mM Mg²⁺ using ATP regeneration with phosphonopyruvate and pyruvate kinase coupled to NADH oxidation by lactate dehydrogenase under conditions described earlier (10).**

**Other Analytical Methods**—Protein concentrations were determined by the method of Bradford (11). Endogenous nucleotides bound to the enzyme subcomplexes were determined by high pressure liquid chromatography using ion pairing with tetrabutyl ammonium hydrogen sulfate as described by Bullough et al. (12).

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It was inactivated much more slowly by 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The rate of inactivation of the $\alpha_\beta(\beta Q 169 C/F 185 C)_3$ subcomplex by o-iodosobenzoate or 5,5'-dithiobis-(2-nitrobenzoic acid) was attenuated by ADP or ATP with or without Mg$^{2+}$ present. After oxidation with o-iodosobenzoate, the $\alpha_\beta(\beta Q 169 C/F 185 C)_3$ subcomplex could not be reactivated by treatment with 10 mM DTT.

**Stabilization of the Reduced $\alpha_\beta(\beta D 311 C/R 333 C)_3$ Subcomplex during Assay—**Fig. 1A shows that the isolated $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex has negligible ATPase activity unless the assay medium is supplemented with DTT or another thiol. Whereas traces a and c of Fig. 1A were obtained in the absence of thios, trace b shows the time-dependent activation of ATPase activity observed when the assay medium contained 10 mM DTT. Fig. 1B shows that after reduction as described under “Experimental Procedures” the $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex was inactivated during assay unless DTT or 0.1 mM EDTA was included in the assay medium. Trace a in Fig. 1B illustrates assay of the enzyme in the absence of DTT or EDTA. Traces b and c of Fig. 1B represent assays conducted in the presence of 10 mM DTT or 100 mM EDTA, respectively. The dependence of protection against oxidation on EDTA concentration revealed that maximal protection was achieved with 50 mM EDTA. Other experiments indicated that a contaminant in the MgCl$_2$ was responsible for oxidation in the absence of EDTA. Therefore, 100 mM EDTA was included in the assay medium in experiments with the reduced $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex. Since the presence of EDTA in the assay medium does not affect ATPase activity of the isolated enzyme, illustrated by trace c of Fig. 1A, the isolated $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex exists in the oxidized form.

**Iodoacetate Inactivates the Reduced Form of the $\alpha_\beta(\beta D 311 C/R 333 C)_3$ Subcomplex—**Following reduction of the $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex with 1 mM DTT for 30 min, the addition of iodoacetate to 4 mM led to 80% inactivation of ATPase activity within 10 min followed by slower inactivation, illustrated in Fig. 2. The addition of increasing concentrations of ADP plus Mg$^{2+}$ to the reduced subcomplex prior to adding iodoacetate slowed the rate of inactivation in both phases. Fig. 3 correlates the mol of $[^{3}H]$acetate incorporated per mol of the $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex with the extent of inactivation observed during inactivation of the reduced, mutant enzyme with iodo$[^{3}H]$acetate. It is clear from Fig. 3 that multiple hits are required to cause full inactivation. To reconcile this unusual stoichiometry, it is possible that modification of a single cysteine in a given $\beta$ subunit wounds the enzyme, and modification of both Cys$^{311}$ and Cys$^{333}$ in the same $\beta$ subunit wounds the enzyme to a greater extent. The introduced cysteines appear to be the only residues that were appreciably carboxymethylated. The wild-type subcomplex was not inactivated when treated with iodo$[^{3}H]$acetate under the same conditions and incorporated no more than 0.3 mol of reagent/mol of enzyme in the absence of nucleotides and much less than that in the presence of MgADP. The wild-type subcomplex, like the mutant, contains a-Cys$^{193}$ that is resistant to modification by iodoacetate.

Complete carboxymethylation lowered the capacity of the $\alpha_\beta(\beta D 311 C/R 333 C)_3$ subcomplex to bind ADP in the presence of Mg$^{2+}$. This was established as follows. After carboxymethylation of the nucleotide-depleted mutant subcomplex by treatment with 4 mM iodoacetate in the presence of 1 mM DTT for 16 h, the modified enzyme was passed through a 1-ml centrifuge column of Sephadex G-50 equilibrated with 50 mM Tris-Cl, pH 8.0, to remove excess iodoacetate and DTT. The gel-filtered
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The reduced $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ subcomplex was prepared in 50 mM Tris-Cl, pH 8.0, containing 100 $\mu$M EDTA as described under "Experimental Procedures." The oxidized subcomplex was prepared by treating the reduced subcomplex at 1 mg/ml with 200 $\mu$M CuCl$_2$ in the presence or absence of 1 mM ADP plus 2 mM MgCl$_2$ for 2 h, at which time the reaction mixture was passed through a centrifuge column of Sephadex G-50 (9) that were equilibrated with 50 mM Tris-HCl, pH 8.0, containing 100 $\mu$M EDTA. ATPase activity was completely inactivated within 5 min of adding CuCl$_2$. Aliquots of the reduced and oxidized preparations at 1 mg/ml in Tris-Cl, pH 8.0, containing 100 $\mu$M EDTA were treated with 2 mM iodo$[^3H]$acetate or iodo$[^14C]$acetamide for 16 h to derivatize free sulphydryl groups. Unreacted iodo$[^3H]$acetate and iodo$[^14C]$acetamide were removed from the samples by passing them through two successive centrifuge columns of Sephadex G-50 equilibrated with Tris-Cl, pH 8.0, containing 100 $\mu$M EDTA. Samples (3 $\mu$l each of the second effluents) were taken to determine protein concentration (11), and 10 $\mu$l samples of the second effluents were submitted to liquid scintillation counting.

[Table II]

| Labeling reagent | Reduced mutant | Oxidized mutant | Oxidized mutant plus MgADP |
|------------------|----------------|-----------------|---------------------------|
| Iodo$[^3H]$acetate | 6.3 $\pm$ 0.2 | 1.5 $\pm$ 0.1 | 1.2 $\pm$ 0.1 |
| Iodo$[^14C]$acetamide | 6.1 $\pm$ 0.2 | 2.1 $\pm$ 0.1 | 1.7 $\pm$ 0.1 |

The reaction mixtures were then incubated at 23 °C for 30 min, at which time $[^3H]$iodoacetate (14 cmp/ml) was added to a final concentration of 4 mM. Samples (5 $\mu$l each) of the reaction mixture were assayed for residual ATPase activity at the times indicated in the presence of 10 mM DTT using the ATP regeneration system. At the same intervals, 50-$\mu$l samples of the reaction mixture were withdrawn and passed through 1-ml centrifuge columns of Sephadex G50 (9) that were equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA. The effluents were submitted to liquid scintillation counting to determine incorporation of $[^3H]$acetate.

The rate of conversion of the reduced mutant subcomplex to a more active form by iodoacetamide was not affected by MgADP. When the subcomplex was treated with 4 mM iodoacetamide in the presence of 1 mM DTT, 50% conversion was observed within 2 min. Maximal conversion occurred within 60 min. The addition of ADP with or without MgCl$_2$ during treatment with iodoacetamide did not affect the rate of conversion.

Prolonged Oxidation of the Reduced $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ Subcomplex Fails to Promote Formation of Disulfide Bonds in All Three $\beta$ Subunits—The reduced $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ subcomplex is rapidly inactivated in the presence of CuCl$_2$, tetra-chionate, o-iodosobenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid), or H$_2$O$_2$. To determine the number of $\beta$ subunits containing disulfide bonds on conversion of the reduced to the oxidized form, the reduced $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ subcomplex was inactivated with CuCl$_2$ in the presence or absence of MgADP. In each case, complete inactivation was observed within 5 min. The reaction mixtures were then incubated an additional 2 h to allow further oxidation. After removing CuCl$_2$, free ADP, and Mg$^{2+}$, inactivated enzyme was treated with iodo$[^3H]$acetate or iodo$[^14C]$acetamide for 16 h to derivatize free sulphydryl groups. These experiments are summarized in Table II. The results obtained when iodo$[^14C]$acetamide was used to monitor residual free sulphydryl groups, strongly indicate that disulfide bonds are formed in only two $\beta$ subunits during inactivation. In this case, nearly two sulphydryl groups were derivatized after inactivating the enzyme with CuCl$_2$. The slightly lower values of free sulphydryl groups titrated with iodo$[^3H]$acetate may reflect charge repulsion encountered during carboxymethylation of Cys$^{311}$ and Cys$^{333}$ in the same $\beta$ subunit that does not occur during carboxamidomethylation with iodoacetamide.

Formation of MgADP-Fluoroaluminate Complexes in the Reduced and Oxidized $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ Subcomplex—Previous studies have shown that the MgADP-fluoroaluminate complex forms slowly when Al$^{3+}$ and F$^-$ were added to wild-type and mutant $\alpha_3\beta_2\gamma$ subcomplexes of TF$_1$, after loading a single catalytic site with MgADP (13, 14). In contrast, when MgADP was loaded onto two catalytic sites or when the subcomplex was incubated with excess ADP plus Mg$^{2+}$, MgADP-fluoroaluminate complexes formed rapidly in two catalytic sites after the addition of Al$^{3+}$ and F$^-$. This suggests that MgADP-fluoroaluminate complexes are formed cooperatively in two catalytic sites. Table III compares the rates of formation of MgADP-fluoroaluminate complexes in catalytic sites of the reduced and oxidized $\alpha_3(\beta D 311 C/R 333 C)_3\gamma$ or wild-type $\alpha_3\beta_2\gamma$ subcomplexes under various conditions.
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**Table III**

Rates of formation of MgADP-fluoroaluminate complexes in active sites of the oxidized and reduced $\alpha_3(\beta D311C/R333C)_3\gamma$ subcomplexes under various conditions

| Subcomplex | 1:1 ADP $k_{\text{term}}$ | 1:1 ADP $+\gamma$ | 200 $\mu$M ADP $k_{\text{term}}$ | 200 $\mu$M ADP $+\gamma$ |
|------------|----------------|-----------------|----------------|----------------|
| $((\beta D311C/R333C))_{3\text{ox}}$ |  $1.1 \times 10^{-2}$ | $3.8 \times 10^{-3}$ | $1.2 \times 10^{-1}$ | $4.6 \times 10^{-1}$ |
| $((\beta D311C/R333C))_{3\text{red}}$ |  $0.6 \times 10^{-2}$ | $0.6 \times 10^{-3}$ | $0.6 \times 10^{-2}$ | $0.7 \times 10^{-3}$ |
| Wild type |  $0.7 \times 10^{-2}$ | <$10^{-3}$ | $0.6 \times 10^{-1}$ | $1.7 \times 10^{-1}$ |

The rates of formation ($k_{\text{term}}$) are the pseudo-first order rate constants for irreversible inactivation observed after adding $\text{Al}^{3+}$ and $\text{F}^-$ to the TF$_1$ subcomplexes in the presence of 200 $\mu$M ADP plus 2 mM Mg$^{2+}$ or with stoichiometric MgADP bound to a catalytic site as described previously (13, 14). To load a single catalytic site with MgADP, the isolated mutant subcomplex at 1 mg/ml in 50 mM Tris-Cl, pH 8.0, was incubated with stoichiometric ADP in the presence of 1 mM Mg$^{2+}$ for 30 min, at which time the solution was passed through a centrifuge column of Sephadex G-50 (9) that was equilibrated with the same buffer. The reduced $\alpha_3(\beta D311C/R333C)_3\gamma$ subcomplex used in these experiments was prepared by incubating the isolated enzyme with or without MgADP bound to a single catalytic site with 5 mM DTT for 30 min before making subsequent additions. The oxidized $\alpha_3(\beta D311C/R333C)_3\gamma$ subcomplex was prepared by treating the isolated enzyme with 1 mM CDTA for 30 min and passing it through a Sephadex G-50 centrifuge column (9) that was equilibrated with 50 mM Tris-Cl, pH 8.0. Assays were conducted in the presence of 10 mM DTT.

DISCUSSION

The proximity of the side chains of $\beta$-Asp$^{315}$ and $\beta$-Arg$^{337}$ in $\beta_T$ and $\beta_P$, but not in $\beta_N$, in the crystal structure of BH-MF$_1$ implies that these residues may participate in functionally important electrostatic interactions during catalysis. However, substitution of the corresponding residues in the $\alpha_3\beta_3\gamma$ subcomplex of TF$_1$ with cysteine does not severely impair ATP hydrolysis. The reduced $\alpha_3(\beta D311C/R333C)^{\gamma}$ subcomplex hydrolyzes 2 mM ATP at about 50% the rate exhibited by the wild-type subcomplex. Furthermore, carboxamidomethylation of the introduced cysteines slightly enhances ATP hydrolysis. Nevertheless, it is clear that the side chains of these residues must be free to change position during catalysis. The mutant subcomplex is rapidly inactivated by several reagents that promote oxidation of adjacent cysteine side chains in proteins to disulfide bonds. This presumably locks affected $\beta$ subunits in the closed conformation. Perception of the consequences of locking a $\beta$ subunit in the disulfide form can be realized from inspection of Fig. 4. The different positions of the side chains of $\beta$-Asp$^{315}$ and $\beta$-Arg$^{337}$ in the nucleotide binding domain of $\beta_T$ and $\beta_P$ in the crystal structure of BH-MF$_1$ are illustrated. It is clear that the nucleotide binding domains, shown in yellow, are folded very differently in $\beta_T$ and $\beta_P$. To highlight the different conformations of $\beta_T$ and $\beta_P$, helices B and C, which contain components of catalytic sites, are illustrated in cyan and magenta, respectively. Transition from the closed conformation of $\beta_T$ to the open conformation of $\beta_E$ moves the carboxylate of $\beta$-Asp$^{315}$ from the guanidinium of $\beta$-Arg$^{337}$ by more than 7 Å.

The finding that nearly two of the introduced cysteines can be derivatized with iodoacetamide after prolonged exposure of the $\alpha_3(\beta D311C/R333C)^{\gamma}$ subcomplex to oxidizing conditions in the presence or absence of saturating MgADP suggests that disulfide bonds are only formed in two of the three $\beta$ subunits. This is consistent with the results of a study recently reported by Tsunoda et al. (15) who used computer modeling of the crystal structure of BH-MF$_1$ to show that it is not possible for three $\beta$ subunits to exist in the closed conformation simultaneously.

It is interesting that carboxamidomethylation of the introduced cysteines of the $\alpha_3(\beta D311C/R333C)^{\gamma}$ double mutant inactivates, rather than stimulates, ATPase activity as observed when they are carboxamidomethylated. Maximal inactivation is observed only after carboxamidomethylation of all six of the introduced sulphydryl groups. This suggests that the diminution of ATPase activity that increases with increasing carboxamidomethylation is caused by charge repulsion of carboxamidomethylated cysteines on the same $\beta$ subunit. The observation that bound MgADP protects against carboxamidomethylation with...
iodoacetate, whereas it has no effect on carboxamidomethylation with iodoacetamide, is consistent with this premise. Liganding of catalytic sites forces the introduced cysteine side chains together in a given β subunit, thus making it more difficult to modify both of them with the negatively charged carboxymethyl group.

In the crystal of the (αβ)3 subcomplex of TF1 deduced by Shirakihara et al. (5), the three β subunits have identical, open conformations. As shown here, treatment of the αβ(βD311C/R333C)3γ double mutant with CuCl2 in the absence of bound nucleotides leads to disulfides in two β subunits. Therefore, two β subunits exist in closed or partly closed conformations in the absence of liganding catalytic sites with nucleotides in the presence of Mg2+. This asymmetry must be induced by the coiled-coil composed of the amino and carboxyl termini of the γ subunit within the central cavity of the (αβ)3 hexamer. That two β subunits can exist in the closed conformation in the absence of bound nucleotides was also demonstrated by Tsunoda et al. (15). In the crystal structure of BH-MF1, the side chain of Ile390 in βT is in contact with the side chain of Ile390 in βD. Computer modeling reported by Tsunoda et al. (15) showed that this contact only occurs when two β subunits are in the closed conformation. The residue in TF1 corresponding to β-Ile390 of BH-MF1 is β-Ile386. Treatment of the αβ(β386C)3γ subcomplex of TF1, with 0.25 μM CuCl2 in the presence of MgATP, MgADP, or MgADP and NH4 inactivated the enzyme and was accompanied by cross-linking of two β subunits. In the absence of nucleotides plus Mg2+, inactivation and cross-linking occurred more slowly. This indicates that two β subunits are at least partly closed in the absence of ligation of catalytic sites with MgATP or MgADP.

The demonstration that disulfide bonds are formed in two β subunits upon oxidation of the αβ(βD311C/R333C)3γ double mutant in the absence of bound nucleotides and that two β subunits are cross-linked on oxidation of the αβ(β386C)3γ subcomplex in the absence of bound nucleotides has important mechanistic implications. These findings are consistent with earlier observations, suggesting that the heterogeneous affinities of catalytic sites of F1-ATPases for MgATP reflect intrinsic asymmetry of catalytic sites dictated by the position of the coiled-coil of the γ subunit within the central cavity of the (αβ)3 hexamer rather than negative cooperativity of binding (16, 17). Three 3Kc values of about 2 nM, 0.2 μM, and 34 μM were detected when quenching of the tryptophan fluorescence of the αβ(βY341W)3γ subcomplex was titrated with MgADP (17). According to the results presented here, the two low 3Kc values represent binding of MgADP to β subunits in closed conformations, whereas the 3Kc of 34 μM represents binding of MgADP to the open catalytic site.

The findings reported here and those reported by Tsunoda et al. (15) demonstrating that only two β subunits exist in the closed conformation simultaneously in the presence of MgADP or MgATP are consistent with the crystal structure of BH-MF1 reported by Abrahams et al. (4). However, they are inconsistent with the crystal structure of RL-MF1 reported by Bianchet et al. (6) that indicates that all three β subunits are in the closed conformation in the absence of Mg2+ with two of them liganded with ADP and P, and the third liganded with ADP only.

Evidence indicating that only two β subunits can exist in closed conformations simultaneously is also inconsistent with models proposed for ATP hydrolysis and synthesis that include conformational states of F1, in which three catalytic sites are closed (1, 6). This does not mean that maximal rates of ATP hydrolysis are achieved when only two catalytic sites are saturated. Weber et al. have convincingly demonstrated that ATP hydrolysis catalyzed by E. coli F1 achieves maximal velocity when three catalytic sites are saturated (18). The model illustrated in Fig. 5 is proposed to accommodate this finding with the observations presented here and by Tsunoda et al. (15), indicating that three β subunits of F1 cannot exist in the closed conformation simultaneously.

The model, which is modified from a scheme in Ref. 3, depicts a round of trisite ATP hydrolysis in which one β subunit (βE) is transiently in the open conformation and the two others (βD and βT) are in the closed conformation and liganded with MgATP. Binding of MgATP to βE causes 1) simultaneous movement of the γ subunit in a counterclockwise direction; 2) propagation of a conformational signal from the catalytic site of βE to the catalytic site of βD (indicated by the curved arrow between βE and βD), and 3) opening of the catalytic site of βD, which is accompanied by ATP hydrolysis. The transition state for ATP hydrolysis is represented by [ATP]. During this process, the γ subunit rotates 120°. This is necessary to overcome steric restrictions imposed by the γ subunit pointed out by Tsunoda et al. (15) that prevent closing of three β subunits simultaneously. From computer modeling, they showed that the coiled-coil of the γ subunit within the central cavity of the (αβ)3 hexamer allows simultaneous closing of only two β subunits. Therefore, in order to proceed from the initial state illustrated in Fig. 5, where βD and βT are in closed conformations, to the final state, where βT and βE are in closed conformations, the position of the coiled-coil of the γ subunit within the central cavity must change as βE closes and βD opens. To be consistent with the parameters of rotational catalysis deduced by Noji et al. (19) and Yasuda et al. (20), the coiled-coil must rotate 120° in the counterclockwise direction in this process.

**Fig. 5. A model for trisite ATP hydrolysis with simultaneous closing of one catalytic site and opening of another.** The stippled circles represent α subunits liganded with MgATP. The hexagons represent unliganded β subunits in the open conformation. The ellipses represent β subunits that are in the process of converting between open and closed conformations. In the concerted process, βE is converting from the open to the closed conformation, whereas βT is converting from the closed to the open conformation.

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