Structural Analysis of the Regulatory Dithiol-containing Domain of the Chloroplast ATP Synthase γ Subunit*

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The γ subunit of the F1 portion of the chloroplast ATP synthase contains a critically placed dithiol that provides a redox switch converting the enzyme from a latent to an active ATPase. The switch prevents depletion of intracellular ATP pools in the dark when photophosphorylation is inactive. The dithiol is located in a special regulatory segment of about 40 amino acids that is absent from the γ subunits of the eubacterial and mitochondrial enzymes. Site-directed mutagenesis was used to probe the relationship between the structure of the γ regulatory segment and its function in ATPase regulation via its interaction with the inhibitory ε subunit. Mutations were designed using a homology model of the chloroplast γ subunit based on the analogous structures of the bacterial and mitochondrial homologues. The mutations included (a) substituting both of the disulfide-forming cysteines (Cys199 and Cys205) for alanines, (b) deleting nine residues containing the dithiol, (c) deleting the region distal to the dithiol (residues 224–240), and (d) deleting the entire segment between residues 196 and 241 with the exception of a small spacer element, and (e) deleting pieces from a small loop segment predicted by the model to interact with the dithiol domain. Deletions within the dithiol domain and within parts of the loop segment resulted in loss of redox control of the ATPase activity of the F1 enzyme. Deleting the distal segment, the whole regulatory domain, or parts of the loop segment had the additional effect of reducing the maximum extent of inhibition obtained upon adding the ε subunit but did not abolish ε binding. The results suggest a mechanism by which the γ and ε subunits interact with each other to induce the latent state of the enzyme.

The ATP synthase enzymes of the inner membranes of mitochondria and chloroplasts and of the bacterial cytoplasmic membrane couple the energy of a transmembrane electrochemical proton gradient to the synthesis of ATP from ADP and inorganic phosphate. The general structural features of the enzyme are highly conserved from one organism to another. It is composed of an integral membrane-spanning H+-translocating segment (F0 or factor O) and a peripheral membrane segment (F1 or factor 1), which contains the catalytic sites for ATP synthesis and hydrolysis. The F1 segment is composed of five different single polypeptide subunits designated α to ε in order of decreasing molecular weight. The subunit stoichiometry is α3β3γδε. There are six nucleotide binding sites, one at each of the six interfaces between α and β subunits (1–3). At least three of these sites are considered catalytically competent (reviewed in Ref. 4).

The γ and ε subunits are both closely involved in regulating CF13 activity and in coupling the movement of protons across the membrane via CF0 to conformational changes at the catalytic sites on CF1 during catalysis. Freshly isolated CF1 is a latent ATPase and can be activated in two ways. First, reduction of the only disulfide bond in the enzyme, formed between Cys199 and Cys205 of the γ subunit, gives rise to partial activation of CF1 (5). Second, removal of the ε subunit also results in partial activation of the enzyme (6). These two effects are additive (7).

Either removing ε from CF1 or reducing the γ disulfide results in exposure of several sites on γ within close proximity to the regulatory dithiol domain that are hypersensitive to trypsin. Concurrent with tryptic cleavage of γ at these sites is the complete loss of inhibition by the ε subunit (8, 9). A close physical proximity between the γ and ε subunits has also been shown (8, 10), consistent with the current view that these two subunits form part of a rotating spindle (11, 12). Rotation of the γ subunit of CF1 has been directly observed (13, 14).

On the membrane, the γ and ε subunits of CF1,F1 both undergo significant conformational changes in response to an imposed transmembrane potential (15–18). In the presence of ATP and reducing agents such as dithiothreitol, the imposed potential results in activation of the latent ATPase activity of the enzyme. The conformational state of CF1 under these activating conditions closely resembles the ε-deficient state of the soluble enzyme. For example, the same trypsin-sensitive sites on γ as those exposed upon removal of ε from soluble CF1 become exposed (9, 18). At the same time, epitopes on the C terminus of the ε subunit become exposed to antibodies present in the medium (16, 19, 20), and Lys159 on ε becomes solvent-exposed (17). Trypsin cleavage of γ in light-energized CF1

3 The abbreviations used are: CF1, the catalytic chloroplast coupling factor 1; CF, (-ε), CF, deficient in the δ and ε subunits; CF0, the proton transporting chloroplast coupling factor O; EcF1, the coupling factor 1 from E. coli; MF1, the coupling factor 1 from mitochondria; Tricine, N-(2-hydroxy-1-bis(hydroxymethyl)ethyl)glycine; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.
results in uncoupling of ATP synthesis from electron transport (21). The cleaved enzyme, when isolated from thylakoids, is partially deficient in the ɛ subunit, indicating that ɛ binding is weakened (22). These studies have led to the hypothesis that the ɛ subunit may bind in the immediate vicinity of the disulfide bridge on γ where its binding masks the disulfide bridge and the trypsin-sensitive sites on γ (9).

To obtain a clearer picture of the relationship between ɛ and γ in regulation and coupling in CF1, we developed methods to reconstitute structurally altered γ subunits with isolated αβ3 hexamers resulting in functional αβ3γ core enzyme assemblies (23, 24). In this study we have utilized this system to examine mutant γ subunits with structural changes within the dithiol-containing regulatory domain and within an additional loop segment (the extra loop) predicted by modeling studies to interact directly with the dithiol domain. The results identify potential structural requirements for dithiol regulation and provide new insight into how the ɛ subunit simultaneously couples proton movement to γ rotation and ATP synthesis while blocking the reverse reaction driven by ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

Materials—CF1 and CF1 deficient in the δ and ɛ subunits, CF1(-δɛ), were prepared from fresh market spinach as described previously (6, 25) and stored as ammonium sulfate precipitates. Prior to use the proteins were desalted on Sephadex G-50 centrifuge columns (26). An αβ3 complex devoid of the γ subunit was isolated from CF1(-δɛ) as described previously (23). The αβ3 subunit complex was recycled through the isolation procedure to ensure that trace amounts of contaminating γ subunit were removed.

ATP (grade II) and antibiotics (ampicillin and tetracycline) were purchased from Sigma. Tryptone and yeast extract were obtained from Difco. Urea (ultrapure) was purchased from Fluka and hydroxypropylate from Bio-Rad. All other chemicals were of the highest quality reagent grade available.

Production and Assembly of γ and ɛ Subunits—The atpG and atpE genes encoding the full-length γ and ɛ subunits, respectively, were cloned into PET expression vectors as described previously (27–29), and the constructs were used to transform *Escherichia coli* BL21 host cells for overexpression of the ɛ subunit. Protein overexpression was induced by addition of isopropylthiogalactoside (28). Inclusion bodies containing the γ protein were solubilized with urea, and the protein folded during slow dialysis and assembled with the isolated, native αβ3 complex as described previously (24). Unreconstituted subunits were separated from the reconstituted αβ3γ by anion-exchange chromatography (23). Similarly, the ɛ protein was solubilized from inclusion bodies in 8 M urea and folded by the dilution method of Cruz and McCarty (28). The cloned ɛ subunit was reconstituted with the αβ3γ complex using the same procedure for reconstituting the native ɛ subunit with CF1(-ɛ) (6).

Generation of atpG Mutants—Mutant γ subunits were constructed by enzymatic amplification of the expression plasmid pET8c-γ·BB1 (24) using a pair of “inverse” primers with abutting 5’-ends. Deletions were generated with primers whose 5’ termini defined the endpoints of the deletion. Oligonucleotides were generated with the aid of the Primer Design program (SciEd Software). Primers (obtained from Macromolecular Resources, Colorado State University) were 24–31 nucleotides long and were chemically phosphorylated at the 5’ termini. A mutant containing a deletion of γ amino acid residues 197–205 (γΔ197–205) was constructed using a forward primer with the base sequence corresponding to bases +616 to +640 and a reverse primer with the base sequence corresponding to bases +564 to +568 of the wild-type atpG gene. A second mutant containing alanine in place of both of the cysteines at positions 199 and 205 was prepared by substituting GCT in the forward primer for the two Cys codons TGC and TCT starting at positions +622 and +637, respectively. The reverse primer contained the sequence +591 to +615 of the atpC coding sequence.

A third γ mutant containing a deletion of amino acid residues from 224 to 240 (γΔ224–240) was constructed with the forward primer, 5’-pTA CGA ATT CGA ACA AGA TCC TGC TC (bases from +721 to +745 with respect to the atpC coding sequence), and the reverse primer, 5’-pTA GCT TAC CTT CGG GTT ATG TTA TTC GAA CAA GAT (bases from +643 to +669 with respect to the atpC coding sequence). In this mutant, 51 bp (17 amino acid residues) were deleted relative to the wild-type template (pET8c-γ·BB1), and at the same time γLeu-241 was mutated to Tyr241. A fourth mutant with the entire region between residues 196 and 241 deleted (γΔ197–240) was constructed using the forward primer: 5’-pATG ATG TAC TAA TTC GAA CAA GAT (bases +715 to +739 with respect to the atpG coding sequence) and the reverse primer: 5’-pTT CGG CGG ATC TCT TTA CGG ATC (bases +572 to +596 with respect to the atpG coding sequence). In this mutant 114 bp (38 amino acid residues) were deleted relative to the wild-type γ template and at the same time γGlu197-Ile198-Cys199 and Pro208–Ile240-Leu241 were mutated to Ser197-Ala198-Glu199 and Met209, Ser240-Tyr241, respectively. An N-terminal γ fragment containing residues 1–196 and a C-terminal fragment containing amino acid residues 206–322 were generated by restriction nuclease cleavage of pET8c-γ·BB1 using Ncol and Sall (the C-terminal fragment) and EcoR1 (the N-terminal fragment). In a series of additional mutants, residues 65–68, 65–73, 68–73, and 74–78 and the entire region 65–78 were deleted using abutting primers of 24 nucleotides in length.

Plasmid DNA for PCR was prepared either by ethanol precipitation after phenol/chloroform extraction (30) or using the Qiagen spin Miniprep kit (Qiagen, Valencia, CA). PCR was carried out in 50 μl of cloned *Pfu* DNA polymerase reaction buffer also containing 60 ng of the pET8c-γ·BB1 plasmid as described in detail elsewhere (30, 31). PCR products were purified using the Qiagen gel-extraction kit (Qiagen). The purified DNA was precipitated with ethanol and circularized by incubating 100–200 ng of the DNA with 3 units of T4 DNA ligase (Promega, Madison, WI) in T4 DNA ligase buffer overnight at room temperature. The resulting plasmid was transformed into competent *E. coli* XL1-blue cells. Cloned plasmid was isolated and transformed into the expression host *E. coli* BL21(DE3)/pLysS (30). The entire sequence of each mutant γ gene was confirmed using the fluorescent dyeoxy method (32).
RESULTS

Mutations within the Dithiol Domain of the γ Subunit—The recently published (11) modeled structure of the CF₁ γ subunit shown in Fig. 1 was used as the basis for preparing several mutant forms of the γ subunit (Table 1), partially to examine the structural requirements for the regulatory function of this region of γ, and partially to probe the structural requirement for ε binding and inhibition. There are three recognizable subdomains within the γ subunit as indicated in Fig. 1, a twisted helical domain formed by the extreme N- and C-terminal elements shown in blue, a central domain that links the two helical elements forming the twisted helical domain shown in green, and the regulatory dithiol-containing domain shown in red. A small loop structure, that is present in the chloroplast γ but not in the homologous mitochondrial subunit, is highlighted in cyan. Molecular dynamic studies predicted that the dithiol domain exists in a range of conformations between two extreme states; the fully open state, shown in Fig. 1, with the dithiol reduced, and a fully closed state with the dithiol oxidized to form a disulfide bridge and in which the regulatory domain has become more compact and has moved downward to come into close contact with the central domain (11). Thus, the open conformation is predicted to represent the activated state and the closed conformation the inactivated state (11). The model further predicts a close interaction between the dithiol domain and the extra loop in the closed state.

In one mutant (γ<sup>C199A/C205A</sup>) alanine was substituted for each of the two dithiol-forming cysteine residues at positions 199 and 205. In a second mutant (γ<sup>C199A/C205A/C224A/C240A</sup>), a small stretch of nine amino acids containing Glu<sup>197</sup> through Cys<sup>205</sup> was deleted. This sequence, containing the two dithiol-forming sulfhydryls, is unique to chloroplast F₁ and is highly conserved among higher plant species. Both of these mutants were expected to result in permanent activation of the enzyme with loss of oxidative regulation, without compromising the functional integrity of the γ subunit.

In a third mutant (γ<sup>C224A/C240A</sup>), the 17-residue section containing Thr<sup>224</sup> through Ile<sup>240</sup> was deleted while at the same time Leu<sup>241</sup> was changed to tyrosine. The rationale for the substitution came from an amino acid sequence alignment that indicated that organisms naturally missing the 17 amino acids have tyrosine in the equivalent position to Lys<sup>241</sup> in CF₁ γ. Thus the deletion mutant recreated a natural variant of the F γ subunit with respect to the central domain. Residues 224–240, which flank the dithiol-containing segment, are present in both higher plants and cyanobacteria but are missing in other organisms. In the fourth mutant, the entire regulatory domain, including Glu<sup>197</sup> through Ile<sup>240</sup>, was deleted. At the same time, a short segment of six amino acids, Ser-Ala-Glu-Ser-Met-Ser, was incorporated back into the deleted region. The construct thus exactly matched the central section sequence of bovine MF₁ γ in which the six amino acid segment acts as a connecting loop between the twisted helical domain and the central domain (3). In addition, two DNA fragments, one (γ<sup>2</sup>) encoding the N-terminal residues of γ from 1 to 196, the other (γ<sup>3</sup>) the C-terminal residues from 207 to 323, were constructed independently and individually overexpressed in the E. coli host.
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#### TABLE 1
Recombinant CF1 γ subunit constructs

| γ Structural element | Mutant γ subunit | Assembly competence |
|----------------------|------------------|---------------------|
| Dithiol domain       | C199A/C205A      | +                   |
|                      | γ197–205         | +                   |
|                      | γ197–205         | +                   |
|                      | γ224–240         | +                   |
|                      | γ197–240         | +                   |
| Subfragments         | γN               | -                   |
|                      | γ197–224         | +                   |
|                      | γ224–240         | +                   |
|                      | γ24–78           | +                   |
|                      | γ65–68           | +                   |
|                      | γ65–73           | +                   |
|                      | γ66–73           | +                   |
| Extra loop           | γN               | -                   |
|                      | γ197–224         | +                   |
|                      | γ224–240         | +                   |
|                      | γ24–78           | +                   |
|                      | γ65–68           | +                   |
|                      | γ65–73           | +                   |
|                      | γ66–73           | +                   |

- Recombinant γ mutants were reconstituted with native αβ3 and the assemblies purified as described under “Experimental Procedures.” In the γ199A/C205A mutant, both of the dithiol cysteines were substituted for alanines. In other mutants, the Δ symbol indicates that the residues indicated were deleted from the γ subunit. γ199A/C205A is the N-terminal γ fragment composed of residues 1–197; γC is the C-terminal γ fragment composed of residues 206–232.

- Assembly competence was judged by the yield of purified assembly relative to wild-type: +, normal yield; +/−, low yield; −, failed to assemble.

The mutant γ subunits were tested for their ability to assemble with the αβ3 complex to reconstitute a functional ATPase. All of the constructs, including the combined N- and C-terminal fragments, successfully assembled with the α and β subunits to produce stable complexes, which could be purified by anion-exchange chromatography under identical conditions to those used to purify the assembly containing the wild-type γ subunit (Table 1). The gel-electrophoresis patterns of representative mutant γ assemblies are shown in Fig. 2. The relative staining densities qualitatively indicated that the mutant γ subunits had been assembled in the same ratio as the wild-type subunit with respect to the α and β subunits. Neither the N-terminal nor the C-terminal fragment of γ alone could assemble with the α and β subunits to form an active enzyme. When un-reconstituted subunits were separated from stable αβ3γ complexes by anion-exchange chromatography (23), we found no evidence of an αβ3γ assembly formed in the presence of either γ fragment alone. However, when both N- and C-terminal fragments of γ were added together to the reconstitution mixture, a stable quaternary complex was produced (Fig. 2, lane 5), which eluted from DEAE-cellulose at the same salt concentration as the normal αβ3γ complex.

The Ca2+- and the Mg2+-dependent ATPase activities of the mutant assemblies, immediately following isolation, were comparable to those of the wild-type assembly with the exception of the γ199A/C205A and γ197–205 mutants, both of which were ~60% higher than wild-type (Table 2). The assemblies were exposed to conditions that either promote oxidation (100 μM CuCl2, 30 min, room temperature) or promote reduction (10 mM DTT, 30 min at room temperature) of the γ disulfide (8). The enzyme containing the wild-type γ subunit, possessing the two disulfide-forming thiols, was significantly activated by reduction with DTT as shown in Table 3. In contrast, the γ199A/C205A mutant and the γ197–205 mutant both exhibited maximal rates of ATPase activity without further activation by DTT, which is the expected consequence of permanently blocking formation of the γ disulfide bond. Interestingly, the Mg-ATPase activity of the γ197–205 mutant consistently showed a decrease (16 to 30% in different experiments) in activity following exposure to DTT, whereas the γ199A/C205A mutant did not. Exposure of the γ197–205 mutant to the alkylating agent N-ethylmaleimide, which would be expected to...
selectively alkylate the C-terminal Cys322 of γ under these conditions (7), blocked the DTT-induced inhibition (Table 3). The reason for this effect is unclear. The activity of γ224–240 mutant under oxidizing conditions was similar to that of the reduced, fully activated wild-type enzyme and was not further enhanced by thiol-reducing conditions. Similarly, removal of the entire regulatory domain in the γ197–240 mutant resulted in an assembly with 90% of the activity of the reduced wild-type enzyme and was insensitive to thiol reducing conditions (Tables 2 and 3).

Epsilon Binding to Mutant αβ3γ Assemblies Containing Substitutions or Deletions within the Dithiol Domain—To assess the role of the modified or deleted segments of the γ subunit in mediating ATPase inhibition by the ε subunit, reconstituted αβ3γ complexes were titrated with purified recombinant ε subunit. Typical titration data are shown in Figs. 3 and 4. The experimental conditions needed for ε reconstitution involve diluting the ε preparations from the ethanol/glycerol mixture in which they were isolated and stored, ~1 part with 2 parts of the assay buffer such that the final concentrations of ethanol and glycerol can maintain ε solubility while allowing ε to rebind to αβ3γ. Under these poised conditions, it is possible to obtain a meaningful measure of the dissociation constant for ε binding. The concentration dependence for ε binding does, however, afford a qualitative measure of the relative affinity of ε for the different γ constructs.

The αβ3γ complex, which was assembled with the recombinant wild-type γ subunit, exhibited a concentration-dependent response to ε essentially identical to that of the native αβ3γ complex, both being fully inhibited at the higher ε concentrations (Fig. 3 and Ref. 24). It was consistently observed that a 2- to 3-fold higher concentration of ε was required for 50% inhibition of the reduced αβ3γwild-type enzyme (closed circles) compared with the oxidized αβ3γwild-type enzyme (squares). This indicated that reducing the disulfide bond resulted in a decreased affinity for ε binding, which is consistent with the results of earlier studies (22). The pattern of inhibition of the αβ3γC199A/C205A mutant closely resembled that of the reduced αβ3γwild-type enzyme, an expected consequence of blocking formation of the disulfide bond. Similarly, the pattern of ε inhibition of the αβ3γ197–205 assembly in which the disulfide bond has been deleted resembled that of the reduced αβ3γwild-type assembly.

Fig. 4 compares the sensitivities to added ε subunit of the enzyme assemblies containing the larger γ deletions and the combined N- and C-terminal fragments of the γ subunit. All of the mutant αβ3γ assemblies, including the enzyme assembled with the two separate γ fragments (αβ3γN+γC), were maximally inhibited by ε to ~40%, significantly less than the wild-type, which approached 100% inhibition. Therefore, the ε subunit still binds, even following deletion of the entire regulatory segment. Neither the concentration dependence on ε nor the extent of inhibition changed in response to addition of CuCl2 or DTT (not shown) indicating that the mutants were insensitive to thiol-oxidizing or -reducing conditions. This was true also of the γ224–240 mutant, which still contained the disulfide bond but lacked the N-terminal flanking region, consistent with the lack of response of the ATPase activity of this mutant to DTT (Table 3).

Deletions within the Extra Loop on the γ Subunit—The γ model predicts a close interaction between the dithiol domain and the extra loop when the dithiol domain is in the closed conformation. This interaction is proposed to result in inhibition of catalytic function. To examine this aspect of the structure, several mutant γ subunits were prepared in which fragments of the putative loop structure were deleted as summarized in Table 1. The mutant γ subunit in which the entire loop between residues 65 and 78 was deleted failed to assemble with the α and β subunits after multiple attempts. This suggested that the mutation affected folding and/or
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assembly of the complex. Each of the smaller deletion mutants, however, assembled to give normal yields of purified complexes with subunit stoichiometries qualitatively identical to that of the wild-type complex (not shown). The catalytic activities of the mutant assemblies at saturating substrate concentrations, together with the effects of oxidizing and reducing conditions on the activities, are shown in Table 4. All but the γ^274–78 mutant assembly exhibited Ca-ATPase and Mg-ATPase activities similar to those of the wild-type construct. The activity of the γ^274–78 mutant was significantly lower than those of the other assemblies indicating that removing these residues impairs catalytic function. All four mutants showed decreased responses to dithiol oxidation compared with the wild-type assembly. The effect was most pronounced in the responses to dithiol oxidation compared with the wild-type

**DISCUSSION**

**Structural Requirements for Dithiol Regulation**—Deletion of the nine residues surrounding the two disulfide-forming cysteines, or substituting the disulfide-forming cysteines with alanines, had the obvious effect of rendering the catalytic activity of the enzyme insensitive to thiol-oxidizing or -reducing conditions. Interestingly, removing residues 224–240, which flank the dithiol-containing segment on the C-terminal side, had the same effect, indicating that the conformation of the entire regulatory domain is important for the redox response. This result is consistent with the modeled structure of the CF₁ γ subunit, which indicated that residues 224–240 closely associate with the central domain of γ when the disulfide is formed, resulting in inhibition of catalysis (11).

It was shown previously that exposure of CF₁ to a small amount of trypsin resulted in cleavage of the γ subunit at Lys^204 and Lys^219 with release of the intervening 15-amino acid fragment (9, 18, 35). At the same time about 20 residues were cleaved from the N terminus of the α subunit, and the δ and ε subunits were cleaved to different extents (35). Following cleavage, CF₁ lost its latency and became fully activated. Activation correlated with α and γ cleavage making it difficult to determine which subunit was involved (9, 35). The results obtained with the assembled γ fragments nicely confirm the fact that cleavage of γ within the region of the dithiol results in permanent activation of the enzyme.

Mutations within the extra loop segment of the γ subunit also exhibited a reduced response to dithiol oxidizing conditions. The effect was most pronounced in the αᵦβᵦγ^68–73 mutant suggesting the importance of residues 68–73 for disulfide regulation. The mutant in which the entire loop, residues 65–78, was deleted failed to reconstitute a stable enzyme complex. The mutant in which residues 74–78 were cleaved remained intact during purification by ion-exchange and gel-filtration chromatography, the reduced activity did not appear to be the result of a decreased stability of the assembly. Comparison of the amino acid sequences of the spinach chloroplast, *E. coli*, and bovine mitochondrial F₁ γ subunits indicated that loop residues 65–75 are naturally missing from the bovine mitochondrial enzyme but not residues 76–78. These latter residues are immediately adjacent to one of the most highly conserved regions of the γ subunit, composed of residues 79–100, and therefore may play an important role, possibly in maintaining structure within the central domain of the γ subunit.

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**TABLE 4**

| Assemblya | ATPase activity | Reduced/oxidizedb |
|-----------|-----------------|------------------|
|           | Mg²⁺/Ca²⁺       | µmol per min per mg protein | % Max. ATPase Activity |
| αᵦβᵦγ^204–206 | 34.8 ± 3.7 | 19.8 ± 0.3 | 1.50 |
| αᵦβᵦγ^65–73 | 62.9 ± 2.0 | 37.6 ± 0.1 | 1.27 |
| αᵦβᵦγ^274–278 | 49.7 ± 3.2 | 32.8 ± 0.4 | 1.08 |
| αᵦβᵦγ^68–73 | 56.0 ± 1.0 | 24.3 ± 0.2 | 1.03 |
| αᵦβᵦγ^74–78 | 10.2 ± 1.3 | 11.7 ± 0.6 | 1.20 |

a Core CF₁ assembled with the wild-type γ subunit or with the indicated γ deletion mutants were purified, pre-treated with 100 μM CuCl₂ for 30 min and assayed as described under “Experimental Procedures.”

b The ratio of Ca-ATPase activity of the reduced assembly to that of the oxidized assembly. The enzyme assembly was pre-treated with either 10 mM dithiothreitol or 100 mM CuCl₂ prior to assay. Errors are expressed as standard deviations with n = 4.

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**FIGURE 5.** Epsilon titration of reconstituted αᵦβᵦγ complexes containing recombinant γ subunits with deletions in the extra loop. Purified assemblies were pre-treated with 100 μM CuCl₂ to promote disulfide formation and reconstituted with the ε subunit, and calcium-dependent ATPase activity was measured as described in the legend to Fig. 3. Filled circles, αᵦβᵦγ assembly containing the recombinant wild-type γ subunit; filled triangles, αᵦβᵦγ^68–73; open triangles, αᵦβᵦγ^65–73; filled squares, αᵦβᵦγ^68–73; and open circles, αᵦβᵦγ^74–78.
Structural Requirements for Epsilon Inhibition—The reduced affinity of CF₁ for the ε subunit that resulted from deletions within the dithiol domain and the extra loop is consistent with a previous proposal (9), based on observed ε-dependent changes in exposure of the dithiol domain to DTT and trypsin, that ε binds in the immediate vicinity of the dithiol domain. In addition, fluorescence mapping studies (8, 29) have placed two amino acid residues on the ε subunit, the single cysteine residue at position 6 and tryptophan at position 57, within 20 Å of the γ dithiols. The possibility that ε binds in the immediate vicinity of the regulatory domain is further strengthened by the fact that tryptic cleavage of γ close to the dithiol results in loss of ε inhibition (8, 9), as well as by the apparent reduced inhibitory efficacy of ε in the F₁ assembled with the γN and γC fragments as shown in this study (Fig. 4). These latter observations indicate that the regulatory domain must be intact both for optimal ε inhibition and for modulation of activity by dithiol oxidation/reduction.

The three-dimensional structures of the bovine heart MF₁ and EcF₁ ε subunits have been determined (36–38) and shown to consist of two domains, an N-terminal β-barrel domain and a C-terminal helix-turn-helix domain. In the solved structure of an EcF₁ γε complex (Fig. 6, left), the β-barrel domain interacts closely with one end of the twisted helical pair formed by the N and C termini of the γ subunit where it would also interact with the ring of c subunits in the FO segment. The C-terminal domain of ε is composed of a helix-turn-helix motif that wraps around the central domain of the γ subunit, the extreme C-terminal tip extending down toward the center of the α₂β₃ hexamer, possibly interacting directly with the α and β subunits (39, 40). The structure is dynamic, as indicated by cross-linking studies that show that the C-terminal arm of ε undergoes a significant conformational change in response to occupancy of catalytic sites with different nucleotides (41, 42). A number of studies with CF₁ have indicated that the ε subunit is likely to exist in conformations similar to those of the EcF₁ enzyme (19, 20, 28, 43–45). We therefore propose that the inhibitory action of CF₁ ε results from a direct binding interaction between the C-terminal arm of ε and the central domain of the γ subunit, in particular residues within the regulatory domain and the extra loop segment as indicated by these studies. Alternative interpretations are possible, for example, mutations within the regulatory domain and extra loop may induce conformational changes that influence activity and ε binding at a distance without involving a direct interaction between these structural elements. Cross-linking studies are currently in progress to identify the site(s) of interaction between the C-terminal arm of ε and the γ subunit in CF₁.

Studies with thermophilic bacteria have indicated that the C terminus of the ε subunit may be in an even more extreme position than that shown in EcF₁, being fully extended with the C-terminal tip extending well into the α₁β₃ hexamer (46, 47). In addition, Hisabori et al. (48) assembled an α₁β₂γ complex using recombinant α and β subunits from the thermophilic bacterium PS3 and the recombinant spinach chloroplast γ subunit. The hybrid enzyme exhibited the expected response to the ox-
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dation state of the γ dithiol, however, the hybrid enzyme containing the reduced γ, or γ with the dithiol removed, bound ε with a higher rather than lower affinity. In addition, a mutant chloroplast γ subunit with the entire section between 194 and 230 deleted was not inhibited by the ε subunit, in contrast to the results of this study in which a similar mutant retained partial sensitivity to ε binding. These differences indicate that the conformation assumed by the γ subunit in the bacteria/chloroplast hybrid may not be physiologically relevant.

In marked contrast to the EcF1 γε complex as shown in Fig. 6, the C-terminal arm of the bovine MF1 δ subunit (equivalent to the EcF1, and CF1 ε subunits) is rotated ~180° such that it abuts the β-barrel domain of ε and does not interact directly with the γ subunit or with the α2β3 hexamer (36). One possible explanation for the difference is that the two structures represent two different conformational states of the ε subunit that occur during the catalytic cycle or as part of an activation/inactivation process. These observations led to the idea that the ε subunit may act as a ratchet, oscillating between the two conformational states as part of a mechanism designed to prevent or reduce the rate of ATP hydrolysis by the enzyme (41, 42).

Light-driven Activation of CF1, a Potential Mechanism—The ε subunit of CF1 undergoes a significant conformational change in response to formation of a light-driven membrane potential. This change was first detected using a polyclonal antibody against the whole ε subunit (16) and later using an antibody specific for the C-terminal arm of ε (28). In both cases, epitopes on ε were only recognized following imposition of the membrane potential. The existence of a light-driven change in the conformation of the C-terminal arm of ε is further supported by the observation that the apparent pK of Lys in the small loop connecting the two helical elements of the C-terminal arm of ε, changes upon light activation of CF1 (17). Moreover, formation of a light-induced membrane potential exposed the γ subunit to trypsin at the same sites that became exposed in soluble CF1 when ε was removed (8, 18), again pointing to the dissociation of the C-terminal arm of ε from the central domain of γ upon light activation.

Light-driven conformational changes also occur in the γ subunit. Cysteine 89 of CF1 γ, shown in yellow and space-filled in Fig. 6 (right side), is sandwiched between the twisted helical element and the central domain of γ. This residue is normally buried within the structure but becomes solvent-exposed upon forming a light-driven membrane potential (49). Moving Cys in from a buried to an exposed environment suggests a relative movement between the central domain and the twisted helical element of γ. It has been suggested that such a movement is essential, either for activation or for catalytic turnover, or both, and that it is impeded by oxidizing the γ dithiol (11, 50). The results of this study indicate that CF1 ε binds in a similar manner to that of the EcF1 ε, with the β-barrel domain of ε binding to the twisted helical element of γ and the helical C-terminal domain of ε binding to the central domain of the γ subunit. The dithiol domain, which is present in CF1 γ but absent from EcF1 γ, may be responsible for the observed higher binding affinity of CF1 than EcF1 for their respective ε subunits (8, 51). By binding to the central domain of γ, the C-terminal arm of ε would likely stabilize the closed, inhibited conformation of the γ regulatory domain (11). On the membrane, formation of a membrane potential would, via an interaction with the subunit III ring and the β-barrel domain of ε, induce a partial rotation of the ε subunit leading to release of the C-terminal arm of ε from its tight binding interaction, thus exposing the γ disulfide to the medium, favoring reduction and activation. This possible mechanism remains to be tested.

In conclusion, the results of this study have identified structural elements within the γ subunit of CF1 that are important for the regulatory interplay between this subunit and the inhibitory ε subunit. They have also helped to establish a testable mechanism for activation of the latent ATPase activity of the enzyme; a process that must be tightly regulated to prevent the futile depletion of ATP pools during dark periods when chloroplast electron transport is inactive.

REFERENCES

1. Girault, G., Berger, G., Galmiche, J. M., and Andre, F. (1988) J. Biol. Chem. 263, 14690–14695
2. Shapiro, A., Gibson, K. D., Scheraga, H., and McCarty, R. E. (1991) J. Biol. Chem. 266, 17276–17285
3. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
4. Richter, M. L., and Mills, D. A. (1996) in Advances in Photosynthesis (Yocum, C., and Ort, D., eds) Vol. IV, pp. 453–468, Elsevier, Amsterdam
5. Nalin, C. M., Belliveau, R., and McCarty, R. E. (1983) J. Biol. Chem. 258, 3376–3381
6. Richter, M. L., Patrie, W. J., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7371–7373
7. Richter, M. L., Lampton, J. D., and McCarty, R. E. (1987) in Progress in Photosynthesis Research (Biggins, J., ed) Vol III, pp. 57–60, Martinus Nijhoff, Dordrecht, The Netherlands
8. Richter, M. L., Snyder, B., McCarty, R. E., and Hammes, G. G. (1985) Biochemistry 24, 5755–5763
9. Hightower, K. E., and McCarty, R. E. (1996) Biochemistry 35, 4887–4851
10. Schuilenberg, B., Wellmer, F., Lil, H., Junge, W., and Engelbrecht, S. (1997) Eur. J. Biochem. 249, 134–141
11. Richter, M. L., Samra, H., He, F., Giessler, A., and Kuczeru, K. (2005) J. Biomed. Biophys. 37, 467–473
12. Oster, G., and Wang, H. (2000) Biochim. Biophys. Acta 1458, 482–510
13. Hisabori, T., Kondoh, A., and Yoshida, M. (1999) J. Biol. Chem. 274, 15130–15134
14. Richter, M. L., and McCarty, R. E. (1992) Biochemistry 31, 7371–7373
15. Ketcham, S. L., Davenport, J. W., Warnke, K., and McCarty, R. E. (1992) J. Biol. Chem. 267, 5910–5914
16. Sokolov, M., Lu, L., Tucker, W., Gao, F., Gegenheimer, P. A., and Richter, M. L. (1999) J. Biol. Chem. 274, 15324–15329
17. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899
18. Chen, Z., and McCarty, R. E. (1992) FEBS Lett. 298, 69–73
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