Four double mutants in the ε subunit were generated, each containing two cysteines, which, based on the NMR structure of this subunit, should form internal disulfide bonds. Two of these were designed to generate interdomain cross-links that lock the C-terminal α-helices (εM49C/A126C and εF61C/V130C). The second set should give cross-linking between the two C-terminal α-helices (εA94C/L128C and εA101C/L121C). All four mutants cross-linked with 90–100% efficiency upon CuCl₂ treatment in isolated Escherichia coli ATP synthase. This shows that the structure obtained for isolated ε is essentially the same as in the assembled complex.

Functional studies revealed increased ATP hydrolysis after cross-linking between the two domains of the subunit but not after cross-linking between the C-terminal α-helices. None of the cross-links had any effect on proton pumping-coupled ATP hydrolysis, on DCCD sensitivity of this activity, or on ATP synthesis rates. Therefore, big conformational changes within this activity, or on ATP synthesis rates. Therefor, can be ruled out as a part of the enzyme function. Protease digestion studies, however, showed that subtle changes do occur, since the ε subunit could be locked in an ADP or 5'-adenyl-β,γ-imidodiphosphate conformation by the cross-linking with resulting differences in cleavage rates.

A proton translocating F₁F₀ type ATP synthase can be found in the periplasmic membrane of bacteria, the thylakoid membrane of chloroplasts, and the cristae membranes of mitochondria. This enzyme can use a proton gradient to synthesize ATP, a process that is reversible in bacteria where the hydrolysis of ATP is used to generate a proton motive force for substrate and ion transport (1, 2). The best characterized F₁F₀ type ATP synthase is from Escherichia coli. It is composed of two parts: a membrane-embedded F₀ part containing three different subunits (a, b₂, c₁₁) (3–5) and a water-soluble ECF₁ part with five different subunits (α, β₂, γ, δ, ε). The F₀ part forms the proton channel (6, 7). The F₁ part contains the three catalytic sites, each predominantly on a β subunit, and located at the interfaces with the α subunits. Recent electron microscopy studies show that the F₁ and F₀ parts are connected by two stalks (8, 9), a rotating central stalk involving the γ and ε subunits (10, 11) and an outer stalk of the b and δ subunits (12, 13). This more peripheral stalk is thought to act as a stator that holds the α-β hexagon in position, while the central stalk is rotating inside the α-β subunits (14–16).

The structures of the α and β subunits are known in detail from x-ray crystallography of both bovine heart and rat liver mitochondrial F₁ (17, 18). Part of the γ subunit was resolved in the bovine heart F₁, but neither of these structures provided information about the ε subunit (δ subunit of mammalian F₁). However, the structure of the ε subunit when isolated from the E. coli enzyme has recently been solved by both NMR and x-ray crystallography (19–21).

This subunit is a two-domain protein with an N-terminal part arranged in a β-sandwich structure and the C-terminal portion in a helix-loop-helix motif. To understand how the ε subunit functions in the intact ECF₁F₀, it is necessary to know whether the structure of ε is the same in the assembled complex as when isolated. To examine this question, we have created four different mutants, each containing two cysteines, that should form disulfide bridges if the structure of the ε subunit is the same as in solution. The effects of generating disulfide bridges within the ε subunit on the functioning of ECF₁F₀ have also been examined.

MATERIALS AND METHODS

Site-directed Mutagenesis—Site-directed mutagenesis was carried out according to Kunkel et al. (22) using C/J236 (New England Biolabs). For routine cloning procedures, the strain XLI-Blue (Stratagene) was used (23, 24).

The double mutations in ε were created using two oligonucleotides (see Table I) with M13mp18 that contained the wild-type ε gene. Screening for mutants was done using the appropriate restriction enzymes. Subcloning of the mutated ε gene into pPH100 was performed as described by Agedger et al. (25).

Restriction enzymes used were purchased from Roche Molecular Biochemicals or New England Biolabs.

Determination of ε Internal Cross-links: Labeling of Noncross-linked Cysteines—Reconstituted vesicles were washed in DTT-free buffer A (25 mM MOPS/HC1, 5 mM MgCl₂, 10% glycerol, pH 7.0) before inducing cross-linking with varying concentrations of CuCl₂ (1 h, room temperature). The reaction was stopped with 10 mM EDTA (5 min) before 25 μM E₆M was added (5 min, room temperature, in the dark). The reaction was quenched by saturating the unreacted cysteines with 10 mM NEM (5 min, room temperature). As a zero control, washed, reconstituted vesicles were reacted with E₆M directly. A second control was incubated with 10 mM DTT (1 h, room temperature) and then quenched by adding 20 mM NEM. To all samples, DTT-free dissociation buffer was added and then subjected to 10–20% SDS-polyacrylamide gel electrophoresis. The same procedure was applied to inner membranes where necessary.

Labeling of Cross-linked Cysteines—Reconstituted ECF₁F₀ was washed and CuCl₂-treated as described above. The then cross-linked cysteines were reacted with NEM (10 mM) for 10 min at room temperature, and the volume was raised to 1 ml with buffer A, followed by centrifugation at 60,000 rpm, 20 min, 4 °C in a Beckman TL100.2 rotor. This procedure was repeated once before adding 5 mM DTT.
min, room temperature) to open the disulfide bridges in the \( \epsilon \) subunit.

After repeating the washing step twice, free cysteines were reacted with 25 \( \mu \)M E\(_{\text{M}}\) for 10 min at room temperature in the dark, and the reaction was quenched with 10 mM NEM. Noncross-linked ECF\(_{1F0}\) was used as a control. All samples were applied on a 10–20% polyacrylamide gel as before.

**Protease Digestion**—Preweighed trypsin was purchased from Roche Molecular Biochemicals. Reconstituted vesicles were washed in 25 mM Tris/HCl, 5 mM MgCl\(_2\), 10% glycerol, pH 7.5, to remove DTT and resuspended in the same buffer at 1 mg/ml. Samples were preincubated for 5 min at room temperature in the presence or absence of 5 mM AMP-PNP. Cross-linking was done for 1 h at room temperature in the presence of 75 \( \mu \)M CuCl\(_2\). The reaction was stopped with 7 mM EDTA, and Mg\(^{2+}\) AMP-PNP was added to a final concentration of 5 mM (5 min, room temperature) before the trypsinization was started (ECF\(_{1F0}\)-tryptic, 50:1). The cleavage was stopped at different times by adding 2 \( \mu \)M phenylmethylsulfonyl fluoride. The same procedure was applied to noncross-linked ECF\(_{1F0}\). Samples were applied on a 10–20% polyacrylamide gel either in the presence or absence of DTT.

**ACMA Quenching**—50 \( \mu \)l (1 mg/ml) of inner membranes were diluted 10-fold in buffer B (10 mM Hepes, 5 mM MgCl\(_2\), 100 mM KCl, pH 7.0), and then 1 \( \mu \)l of valinomycin (1.8 mM), 5 \( \mu \)l of ACMA (0.1 mM), 5 \( \mu \)l of NADH (50 mM), and 5 \( \mu \)l of KCN (200 mM) were added. The fluorescence at 480 nm was measured for a short time before adding 5 \( \mu \)l of ATP (200 mM). After the signal reached a plateau, 1 \( \mu \)l of nigericin (1.8 mM) was added to uncouple the system. The excitation wavelength was 410 nm. For ATP-dependent proton pumping measurements, an SLM 8000 fluorometer was used.

**ATP Synthesis and ATP-dependent Proton Pumping on Inner Membranes**—Inner membranes were washed twice with 25 mM Tris/HCl, 10% glycerol, 5 mM MgCl\(_2\), pH 7.5, diluted to 1 mg/ml, and reacted with 75 \( \mu \)M CuCl\(_2\) for 1 h at room temperature if necessary. 200 \( \mu \)l of Tris buffer were added to 50 \( \mu \)l inner membranes (50 \( \mu \)g) followed by 250 \( \mu \)l of reaction buffer (100 mM Tris/HCl, 10 mM ADP, 5 mM MgCl\(_2\), 10 mM K\(_2\)HPO\(_4\), pH 7.5). 100-\( \mu \)l samples were immediately quenched with 30 \( \mu \)l of 0.5 M trichloroacetic acid on ice to measure the amount of endogenous ATP. To the residual 460-\( \mu \)l sample, 50 \( \mu \)l NADH (4 \( \mu \), 0.5 mM stock) was added and incubated in a 37 °C water bath. The reaction was stopped after 5 min by adding 130 \( \mu \)l, 0.5 M trichloroacetic acid on ice. All samples including the background controls were diluted 25-fold with a buffer containing 0.1 mM Tris acetate, 2 mM EDTA, pH 7.7, and measured. The amount of ATP was determined for 100 \( \mu \)l of diluted sample, adding 50 \( \mu \)l of a 1:2 diluted luciferin/luciferase ATP assay mix (Sigma) and 150 \( \mu \)l of Tris acetate buffer. The emitted light was detected using a chemiluminometer after standardizing with preweighed ATP (also purchased from Sigma).

**Other Methods Used**—Inner membranes were isolated from the strain RA1 (11) according to the procedure of Foster and Fillingame (27). For the purification of ECF\(_{1F0}\), a method described by Foster and Fillingame (27) and modified by Aggeler et al. (28) was used.

**ATP hydrolysis** was measured using a regenerating system (29).

\[ \text{ECF}_{1F0} \text{ protein} + \text{ATP} \rightarrow \text{ECF}_{1F0} \text{ protein} + \text{ADP} + \text{P}_{\text{i}} \]

**TABLE I**

| Mutants  | Oligonucleotide | Restriction enzyme |
|----------|-----------------|--------------------|
| M49C     | C ATT AAG CCT GGT TGT | HinII (10214Δ) |
| A126C    | CTT GGC AAA GGC ATC TGT | BsiEI (10441Δ) |
| F61C     | G CAC GGT CAC GAA GAG | SfaI (10257+) |
| V130C    | TGC ATC TAT TGT GCC | SfaI (10465+) |
| A94C     | GAC GAA GCG CGA TGT | NcoI (10348A) |
| L128C    | GGC ATC GGC CGG CGT GCC | PvuII (10448Δ) |
| A101C    | GGC AAA CCT AAG TGC | Earl (10366A) |
| L121C    | GGC TCT GGC GAA TGT GCC | AaeI (10249A) |

**RESULTS**

Four different double mutants were used in this study, two with cysteine residues introduced at sites that are at the interface between the two domains (M49C/A126C or F61C/V130C, respectively) and two that place the two cysteines in close proximity at the interacting faces of the C-terminal \( \alpha \)-helical domain (A94C/L128C and A101C/L121C, respectively). Fig. 1 shows the position of these substitutions in the \( \epsilon \) subunit solution structure.

With ECF\(_{1F0}\) purified from each mutant, disulfide bond formation was readily obtained in essentially 100% yield when high enough concentrations of Cu\(^{2+}\) were used. The SDS-polyacrylamide gel in Fig. 2A shows the effect of internal cross-linking on the migration of \( \epsilon \) for the mutant eM49C/A126C. CuCl\(_2\) treatment led to a shift to apparent lower molecular weight but, at the same time, to a weaker overall staining of the \( \epsilon \) subunit band. This altered staining intensity made it difficult to quantitate cross-link yields. Therefore, the formation of the disulfide bond was also followed by fluorescence after labeling with E\(_{\text{M}}\). Fig.
2, B and C, shows the result of E5M labeling of eM49C/A126C using two different approaches. In one approach, cross-linking was induced by CuCl2, and the amount of e that had not become cross-linked was determined by modification of remaining exposed cysteines with E5M (Fig. 2B). As a complementary approach, cross-linked enzyme was reacted with NEM to block free cysteines, and then the disulfide bridge was broken by adding DTT to expose cysteine residues previously cross-linked. These were then modified with E5M (Fig. 2C). Both approaches gave the same results. High cross-linking yields with the mutant eM49C/A126C required 75 μM CuCl2. Fig. 2, D and E, shows cross-linking between the C-terminal helices in the mutant eA101C/L121C. In this case, uncross-linked e labeled with E5M gave a diffuse band (D, lane 3), an effect prevented by previous cross-link formation. As with the other mutants, cross-link yields were quantitated by E5M incorporation. Essentially, a 100% yield of cross-linking could be obtained at 10 μM CuCl2.

Functional Effects of Internal Cross-linking of the ε Subunit—The effects of cross-linking within the ε subunit on the function of the ATP synthase are summarized in Fig. 3A. Those mutants that formed a cross-link between N- and C-terminal domains had ATPase activities similar to wild-type enzyme. As shown for eM49C/A126C in Fig. 3B, ATPase activity increased in proportion to the cross-linking yield, giving a 2.5-fold higher turnover rate. Nevertheless, the cross-linked and activated enzyme retained similar levels of DCCD inhibition to untreated or wild-type enzyme. Moreover, this activation of ATPase was not accompanied by enhanced steady-state levels of ATP-dependent proton pumping when measured by ACMA quenching (Fig. 4) or of ATP synthesis (Fig. 3) when these activities were measured in inner membranes from E. coli. The ATPase activity of the mutant eA94C/L128C was significantly higher than wild-type even before the addition of Cu2+ and essentially the same as that of fully cross-linked enzyme from the mutant eM49C/A126C. Cu2+ treatment and the resulting cross-linking of eA94C/L128C in yields greater than 90% failed to increase activity much further. In contrast, the ATPase activity of the mutant eA101C/L121C was normal and not significantly activated by cross-linking. The DCCD sensitivity of both eA94C/L128C and eA101C/L121C was similar to wild-type (Fig. 4). For both of these mutants, disulfide bond formation between the two C-terminal α helices had no significant effect on proton pumping or ATP synthesis.

Nucleotide Dependence of Cross-linking—The nucleotide dependence of cross-linking of the mutant eM49C/A126C is shown in Fig. 5. Disulfide bond formation was most efficient with 5 mM Mg2+-AMP-PNP present. In the absence of Mg2+-ATP, which is rapidly hydrolyzed during the experiment, Mg2+-ADP, or Mg2+-ADP + P5 (5 mM respectively), the yields of cross-linking were 40–50%, compared with 90–100% in AMP-PNP. This can be seen clearly in the Coomassie-stained SDS-polyacrylamide gels (Fig. 5A). In Mg2+-AMP-PNP (lane 2), there is a nearly complete bandshift, whereas in ADP, ADP + P5, or ATP (lanes 4–6), two bands are clearly visible that have not been cross-linked and are not significantly inhibited by DCCD. Fig. 5B shows the Mg2+ dependence of ATP hydrolysis and ATP synthesis in reconstituted vesicles with or without CuCl2 treatment and E5M labeling. Lane 1, 5 mM DTT control; lane 2, zero control without E5M; lane 3, zero control with E5M; lane 4, 1 μM CuCl2 plus E5M; lane 5, 2 μM CuCl2 plus E5M; lane 6, 4 μM CuCl2 plus E5M; lane 7, 10 μM CuCl2 plus E5M; lane 8, 20 μM CuCl2 plus E5M. E5M fluorescence from noncross-linked cysteines of ECF1F0 (eA101C/L121C; lanes 1–6) show the same conditions as lanes 3–8 in D.

Figure 2. A, Coomassie Blue staining of ECF1F0 (eM49C/A126C) in reconstituted vesicles before and after CuCl2 treatment. Lane 1, zero control; lane 2, 100 μM CuCl2. B, fluorescence of noncross-linked cysteines from ECF1F0 (eM49C/A126C) in reconstituted vesicles before and after CuCl2 treatment. Lane 1, zero control; lane 2, 5 μM CuCl2; lane 3, 25 μM CuCl2; lane 4, 50 μM CuCl2; lane 5, 100 μM CuCl2. C, E5M fluorescence from cross-linked cysteines. Lane 1, zero control; lane 2, 5 μM CuCl2; lane 3, 25 μM CuCl2; lane 4, 50 μM CuCl2; lane 5, 100 μM CuCl2. For experimental details see “Materials and Methods.” D, Coomassie staining of ECF1F0 (eA101C/L121C) in reconstituted vesicles with or without CuCl2 treatment and E5M labeling. Lane 1, 5 mM DTT control; lane 2, zero control without E5M; lane 3, zero control with E5M; lane 4, 1 μM CuCl2 plus E5M; lane 5, 2 μM CuCl2 plus E5M; lane 6, 4 μM CuCl2 plus E5M; lane 7, 10 μM CuCl2 plus E5M; lane 8, 20 μM CuCl2 plus E5M. E, E5M fluorescence from noncross-linked cysteines of ECF1F0 (eA101C/L121C; lanes 1–6) show the same conditions as lanes 3–8 in D.

Figure 3. A, ATP hydrolysis and ATP synthesis activities of wild-type, eM49C/A126C, eA94C/L128C, and eA101C/L121C, respectively, along with the extent of DCCD inhibition caused by cross-linking. Data are presented for each ECF1F0 as the percentage of change of the particular activity due to cross-linking. The values above the bar graphs are the basal rates of ATP hydrolysis for each mutant before the addition of CuCl2. B, relationship between MgATPase activity and cross-link yield from the mutant eM49C/A126C as a function of CuCl2 concentration. Open circles, percentage cross-linked; open squares, MgATPase activity.
FIG. 4. ATP-dependent ACMA quenching of wild-type (A), eM49C/A126C (B), eA94C/L128C (C), and eA101C/L121C (D) in the presence of Mg2+ or 100 mM CuCl2 (dashed lines). All mutants had a yield of at least 90% internal cross-link based on E5M fluorescence.

FIG. 5. Nucleotide dependence of the interdomain cross-linking for the mutant eM49C/A126C examined by E5M fluorescence. Cross-linking of cysteines remaining after cross-linking A, Coomassie-stained polyacrylamide gel. Lane 1, zero control; lane 2, Mg2+; lane 3, Mg2+ + P; lane 4, Mg2+ + ADP; lane 5, Mg2+ + ATP. B, E5M fluorescence of the same samples.

Cross-linking of the mutant eF61C/V130C gave the same general patterns as for eM49C/A126C. The nucleotide dependence of cross-linking of mutants with both Cys in the C-terminal part was not examined in any detail.

Trypsin Cleavage of ECF1F0 after Cross-linking between the N- and C-terminal Domains—Earlier studies had shown a nucleotide dependence of the cleavage of e by trypsin in the wild-type ECF1F0 complex (34), and this proteolysis was found to activate the enzyme. Cleavage of the e subunit was followed in the mutant eM49C/A126C in the presence of Mg2+ + ADP and in Mg2+ + AMP-PNP. As with wild-type enzyme, cleavage of un-cross-linked enzyme was fast in the presence of AMP-PNP but slow in the presence of Mg2+ + ADP. If both the mutant was cross-linked and protease digestion was conducted in AMP-PNP, the rate of cleavage was fast. However, if cross-linking occurred in the presence of Mg2+ + ADP, and then the ADP was replaced by AMP-PNP before trypsin treatment, the cleavage was slow (Fig. 6). Clearly, cross-linking in ADP traps the e subunit in a conformation that is not subsequently modified by the addition of AMP-PNP. The inaccessibility of trypsin to cleavage sites could be a direct result of fixing the two domains of e together so that the C-terminal α helix cannot unfold. Alternatively, the cross-link could fix a conformation of the whole ECF1F0 where the cleavage sites in e are sterically shielded by other subunits.

DISCUSSION

The present study offers important insights into the arrangement and functioning of the e subunit in ECF1F0. Four mutants were generated, by reference to the structure of e from NMR studies of the isolated polypeptide (21). In each of these mutants, the positions of the cysteines are such that they should readily form disulfide bonds if the observed structure is retained in the enzyme complex. Internal cross-links were readily formed in essentially 100% yield between the introduced Cys in all four mutants when ECF1F0 was reacted with Cu2+. Two of the disulfide bridges are between one residue in the N-terminal β-barrel part and the very C-terminal α-helix of the C-terminal domain of the subunit. The other two disulfide bridges are between a residue in each of the C-terminal α-helices. This ready cross-linking would only be expected if the two domains and both C-terminal helices have the same position in ECF1F0 as in isolated e subunit. Therefore, it can be concluded that the structure of e in the intact complex is very similar to that already determined for purified e subunit.

The e subunit, along with γ, is now thought to be a part of the rotor that couples catalytic site events with proton pumping in the complex (11, 35). Nucleotide-dependent changes in interaction of the e subunit with other subunits in ECF1F0 have been resolved by cross-linking studies (36). These involve both domains of the subunit, and it has been suggested that the two domains move independently during functioning of the enzyme (35). The observation that the two domains can be tethered together in either of two different places without altering coupling argues against this possibility but does not rule out subtle
rotations of one domain relative to the other. The cross-linking studies here rule out that the C-terminal α-helices come apart during functioning (21). This is not to say that conformational changes are absent from ε. The nucleotide dependence of cross-linking in mutants forming S–S bridges between the two domains points to some structural changes within ε. This notion is also supported by the observation that rates of cleavage of ε by trypsin are affected by the nucleotide conditions in which cross-linking is conducted prior to the proteolysis step. When cross-linked in ADP the ε subunit was locked in a conformation characterized by very slow proteolysis, a conformation that was not altered by the addition of AMP-PNP before trypsin treatment. In the presence of AMP-PNP, proteolysis of the ε subunit is normally rapid.

The effect of cross-links within the ε subunit on functioning of ECF$_{F_0}$ adds to our understanding of the role(s) of the two domains of this subunit. Cross-linking to fix the N- and C-terminal domains increases ATP hydrolysis by the enzyme complex up to 2.5-fold. The introduction of Cys for Ala$^{394}$ plus Leu$^{128}$ also increases ATPase activity more than 2-fold, an activation that is not greatly enhanced by disulfide bond formation between the introduced Cys residues. The activation of ATP hydrolysis in these mutants is without effect on coupling because, in each case, the steady-state levels of proton pumping, as well as ATP synthesis rates, are unaltered. Therefore, our results support and extend previous studies that indicate that the ε subunit acts to regulate ATPase rates in the intact ATP synthase, i.e., that it is an inhibitor of this function in the intact complex (34, 38) (for the opposite view, see Refs. 39 and 40). This inhibitor function is probably provided by the N-terminal helical domain. Protease digestion of this domain by trypsin in ECF$_1$ (41) alters the inhibition by the ε subunit, while proteolysis and genetic deletion of part or all of this domain has no effect on coupling function (42–44). Our previous studies have shown that the C-terminal domain lies under the α$_\alpha$β$_\beta$ hexagon in the enzyme complex, where it associates with two α–β pairs (21, 36). This domain could regulate ATP hydrolysis by controlling catalytic site cooperativity. The N-terminal β barrel domain binds to the γ subunit (10) and the ε subunit ring (37), as would be expected of a key component of the coupling mechanism.

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