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Disulfide Bond Formation between the COOH-terminal Domain of the β Subunits and the γ and ε Subunits of the *Escherichia coli* F_1-ATPase

STRUCTURAL IMPLICATIONS AND FUNCTIONAL CONSEQUENCES*

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A set of mutants of the *Escherichia coli* F_1F_0-type ATPase has been generated by site-directed mutagenesis as follows: βE381C, βS383C, βE381C/εS108C, and βS383C/εS108C. Treatment of ECF_1 isolated from any of these mutants with $CuCl_2$ induces disulfide bond formation. For the single mutants, βE381C and βS383C, a disulfide bond is formed in essentially 100% yield between a β subunit and the γ subunit, probably at Cys^{87} based on the recent structure determination of F_1 (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) *Nature* 370, 621–628). In the double mutants, two disulfide bonds are formed, again in essentially full yield, one between β and γ, the other between a β and the ε subunit via Cys^{108}. The same two cross-links are produced with $CuCl_2$ treatment of ECF_1F_0 isolated from either of the double mutants. These results show that the parts of γ around residue 87 (a short α-helix) and the ε subunit interact with different β subunits.

The yield of covalent linkage of β to γ is nucleotide dependent and highest in ATP and much lower with ADP in catalytic sites. The yield of covalent linkage of β to ε is also nucleotide dependent but in this case is highest in ADP and much lower in ATP. Disulfide bond formation between either β and γ, or β and ε inhibits the ATPase activity of the enzyme in proportion to the yield of the cross-linked product. Chemical modification of the Cys at either position 381 or 383 of the β subunit inhibits ATPase activity in a manner that appears to be dependent on the size of the modifying reagent. These results are as expected if movements of the catalytic site-containing β subunits relative to the γ and ε subunits are an essential part of the cooperativity of the enzyme.

An H^+-ATPase is found in the plasma membrane of bacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts. This enzyme catalyzes both ATP synthesis coupled to an electrochemical gradient and ATP hydrolysis-driven proton translocation. The enzyme complex is made up of two parts: an F_1 part composed of five different subunits, α, β, γ, δ, and ε in the molar ratio 3:3:1:1:1 and an F_0 sector which, in bacterial enzymes such as that from *Escherichia coli* (ECF_1F_0),[1] contains subunits a, b, and c in the ratio 1:2:10–12 (reviewed in Cross, 1988; Senior, 1990; Boyer, 1993).

Low resolution cryoelectron microscopy studies show the F_1 part separated from the F_0 by a narrow stalk of around 45 Å in length (Gogol *et al.*, 1987; Lücken *et al.*, 1990). This stalk region includes parts of the γ, δ, and ε subunits of F_1 and b subunits of the F_0 part (Capaldi *et al.*, 1994; Wilkens *et al.*, 1994). Catalytic site events (ATP synthesis and ATP hydrolysis) appear to be linked to proton translocation via conformational changes in these stalk-forming subunits. Electron microscopy studies have also shown that the α and β subunits alternate in a hexagonal arrangement around a central cavity containing the γ subunit (Gogol *et al.*, 1989a, 1989b). The recent high resolution structure of MF_1 (mitochondrial F_1) (Abrahams *et al.*, 1994) adds detail to this picture. The α and β subunits have a very similar fold, each made up of three domains. At the top (farthest from the F_0 part) is an amino-terminal six-stranded β barrel. The more central domain contains the nucleotide-binding domain, which in the β subunits includes the (three) catalytic sites, while the α subunits contain the (three) non-catalytic sites. The third and bottom domain (closest to the F_0 moiety) consists of a carboxyl-terminal bundle of seven α helices in the α subunit and six α helices in the β subunit. In the COOH-terminal domain of the β subunits is the so-called "DELSEED" region which has been implicated in binding of the ε subunit (Dallmann *et al.*, 1992) and which is seen from the x-ray data to interact tightly with a segment of the γ subunit (Abrahams *et al.*, 1994).

We have been examining structure-function relationships in ECF_1F_0 by introducing Cys residues at various sites in the enzyme complex, which can be used to incorporate reporter groups of the environment of the site, as well as (conformational) changes that occur around the site during functioning of the enzyme. In recent work, we have introduced Cys residues into the γ and ε subunits, and by using fluorescent probes (Turina and Capaldi, 1994a, 1994b) as well as cross-linking reagents (*e.g.* Aggeler *et al.*, 1992, 1993), have provided evidence for large conformational changes in these subunits during coupling of ATP hydrolysis to proton translocation. Here we describe experiments in which we have introduced Cys residues into the DELSEED region of the β subunit in conjunction with mutating a Cys residue into the COOH-terminal region of the ε subunit (εS108C). With these mutants, we were able to reversibly generate disulfide bonds between β subunits and both the γ and ε subunits. The reactivity of the Cys residues in the DELSEED region with maleimides has been studied. In addition,

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[1] The abbreviations used are: ECF_1, soluble portion of the *E. coli* F_1F_0 ATP synthase; CM, *N*-[4-[7-(diethylamino)-4 methyl]coumarin-3-yl] maleimide; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; AMP·PNP, 5′-adenylyl-β,γ-imidodiphosphate; LDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; kb, kilobase(s).

the functional consequences of such modifications, and of disulfide bond formation between β and ε and between β and γ subunits both together and independently, have been examined.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Containing Mutations in the unc D and unc C genes—A 1.01-kb *Nco*I fragment encoding the COOH-terminal part of the β subunit was isolated from pRA100 (Aggeler *et al.*, 1992) and inserted into M13mp18 (New England Biolabs) in an *Nco*I site that was created by introduction of an *Nco*I linker (Boehringer Mannheim, GmbH.) into the *Sma*I site.

In the site-directed mutagenesis step (Kunkel *et al.*, 1987) the oligonucleotides CTTCAGACAGACAATCCATACCC and TTGTCTTCTTCACACAGTTCATCCAT were used for replacing a glutamate at position β381 and a serine at position β383 each with cysteine. The successful incorporation of the mutations was determined by sequencing (Sequenase from United States Biochemical Corp., Cleveland, OH).

The mutations were inserted in an unc operon containing plasmid in two steps: (i) the 5.8-kb *Xho*I/*Nsi*I fragment from pRA100 and pRA102, respectively, was inserted in pBluescript SK+ (Stratagene), which had been previously modified by placing an *Nsi*I linker (New England Biolabs) in the *Eag*I site. These plasmids, pRA13 and pRA14, contained the *unc* C coding for wild-type ε subunit and mutant εS108C, respectively; (ii) the *Nco*I fragments containing mutations βE381C and βS383C were then inserted in pRA13 and pRA14. Finally, the 5.8-kb *Xho*I/*Nsi*I fragments from these four constructs were ligated to the 6.8-kb *Xho*I/*Nsi*I portion of pRA100. The resulting plasmids were pRA133, pRA134, pRA135, and pRA136, containing the mutations βE381C, βE381C/εS108C, βS383C, and βS383C/εS108C, respectively.

$CuCl_2$ Induced Cross-linking of ECF_1 and ECF_1F_0—ECF_1-ATPase was precipitated for 1 h in 70% ammonium sulfate at 4 °C and collected by centrifugation at 10,000 × *g* for 15 min. The protein was dissolved in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol and passed through two consecutive centrifuge columns in Sephadex G-50 equilibrated in the same buffer at a concentration between 2–5 mg/ml, as described in Aggeler *et al.* (1992). Cross-linking was carried out in either of two ways: (i) the enzyme was passed through two centrifuge columns, equilibrated in 50 mM MOPS, pH 7.0, 10% glycerol, and 20 μM $CuCl_2$ (Bragg and Hou, 1986; Tozer and Dunn, 1986); (ii) the ATPase solution was diluted to 0.1 mM EDTA, supplemented with 2.5 mM $MgCl_2$ and 2 mM nucleotide, followed by incubation with 20 or 50 μM $CuCl_2$ for 3 h at room temperature.

ATP synthase was reconstituted into egg-lecithin vesicles by first adding 10% sodium deoxycholate to ECF_1F_0 from the sucrose gradient at 1 mg/ml to a final concentration of 0.5%. After 10 min on ice, 25 μl of 20 mg/ml egg-lecithin (in 1.5% sodium deoxycholate) was added per ml, followed by another 10-min incubation on ice. The mixture was then passed through a Sephadex G-50 column (medium, 1 × 45 cm) (Brunner *et al.*, 1978), equilibrated in 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, and 10% glycerol. Turbid fractions containing ATPase activity were pooled and vesicles pelleted by centrifugation for 1 h at 45,000 revolutions/min at 4 °C in a Beckman Ti60 rotor. The pellets were resuspended in the same buffer and kept frozen in liquid nitrogen. For cross-linking with $CuCl_2$, 1 mg of ECF_1F_0 was brought to a volume of 2 ml with 50 mM MOPS, pH 7.0, 2 mM $MgCl_2$, and 10% glycerol and centrifuged twice at 20 °C for 25 min at 70,000 revolutions/min in a Beckman TLA100.2 rotor to remove reducing agent. The pellet was resuspended in the same buffer at 0.7–1 mg/ml, samples were supplemented with 2 mM nucleotide, and cross-linked with 20 μM $CuCl_2$ for 3 h at room temperature. ATPase activity was measured with or without prior treatment with 10–20 mM DTT for 2 to 3 h at room temperature.

Maleimide Modifications of ECF_1—The rate of incorporation of [^{14}C]NEM into Cys^{381} of the β subunit of βE381C/εS108C was determined by reacting ATPase at a concentration of 0.4 mg/ml in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, 5 mM ATP, and 10% glycerol with 25 μM [^{14}C]NEM (specific activity 40 mCi/mmol) at room temperature. Aliquots were withdrawn at time intervals of 1, 10, 30, 60, and 150 min and quenched by the addition of 10 mM L-cysteine. For determination of stoichiometry of incorporation (mol [^{14}C]NEM/mol F_1), an additional aliquot denatured by 2% SDS was reacted with 200 μM [^{14}C]NEM for 150 min. The aliquots were then electrophoresed on a 10–18% SDS-polyacrylamide gel and the radioactivity in individual subunits measured as described in Aggeler *et al.* (1987). The change of ATPase activity, due to the incorporation of the maleimides NEM or CM into βCys^{381}, was determined in a parallel experiment under identical conditions. Aliquots of a reaction mixture containing 25 μM maleimide were quenched by the addition of 20 mM DTT at the time intervals 1, 10, 30, 60, and 150 min and ATPase activity determined.

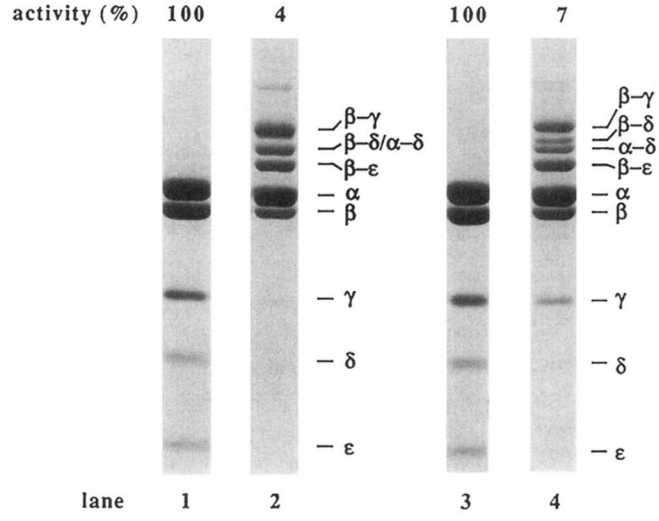

FIG. 1. **Cross-linking of ECF_1-ATPase from double mutants on $CuCl_2$ containing centrifuge columns.** 1 mg of ATPase, precipitated with ammonium sulfate, was dissolved in 0.2 ml of 50 mM MOPS, pH 7.0, 0.5 mM EDTA, and 10% glycerol and passed twice through a centrifuge column in the same buffer. Half of the sample was then applied on two consecutive columns containing 20 μM $CuCl_2$ and no EDTA. The other half was left untreated. After addition of 5 mM EDTA, ATPase activities were measured and expressed as relative values with the untreated sample as 100%. 50-μg samples were electrophoresed on an 10–18% polyacrylamide gel after addition of 20 mM NEM and dissociation buffer without reducing agent. βE381C/εS108C without (*lane 1*) and with $CuCl_2$ treatment (*lane 2*); βS383C/εS108C without (*lane 3*) and with $CuCl_2$ treatment (*lane 4*).

Other Methods—*E. coli* strains used for routine cloning procedures (Davis *et al.*, 1986; Maniatis *et al.*, 1982) and site-directed mutagenesis according to Kunkel *et al.* (1987) were *XL1*-Blue from Stratagene and *CJ236* from New England Biolabs. For expression of mutant ATPase and synthase, the plasmids pRA133-pRA136 were used to transform the *unc*$^-$ strain AN888 (Aggeler *et al.* 1992). ECF_1 and ECF_1F_0 were isolated as described by Wise *et al.* (1981), modified by Gogol *et al.* (1989a) and Aggeler *et al.* (1987). ε-Depleted ECF_1 was prepared by the use of the monoclonal antibody ε-4 as described by Dunn (1986). Protein concentrations were determined using the BCA protein assay from Pierce. ATPase activity was measured with a regenerating system described by Lötscher *et al.* (1984). For the analysis of cross-link products, SDS containing 10–18% polyacrylamide gels were run (Laemmli, 1970), and polypeptides were blotted on nitrocellulose membranes and identified with monoclonal antibodies (Aggeler *et al.*, 1990; Mendel-Hartvig and Capaldi, 1991a). Protein bands on gels were visualized by staining with Coomassie Brilliant Blue R according to Downer *et al.* (1976).

RESULTS

Mutants in the DELSEED region were prepared in which a cysteine was introduced in place of a glutamate at residue 381 (βE381C) or a cysteine replacing a serine at residue 383 (βS383C). In addition, double mutants were generated with the same mutations in the DELSEED region along with the mutation of a serine to a cysteine at position 108 of the ε subunit (βE381C/εS108C; βS383C/εS108C). The four different mutants grew well, and the activities of the isolated F_1 of each and F_1F_0 from the two double mutants were similar to wild-type enzyme, *i.e.* in the range 9–15 μmol ATP/min/mg for the F_1 and 25–35 μmol/min/mg for the F_1F_0 preparations. All four mutants showed normal LDAO activation, *i.e.* 8-fold for the F_1 and 3–4-fold for the F_1F_0.

The DELSEED Region Interacts with Both the γ and ε Subunits—$CuCl_2$ was used to induce disulfide bond formation using the isolated F_1 and F_1F_0 from the various mutants with or without gel filtration centrifugation. Fig. 1 shows data for the two double mutants. Passage of ECF_1 through a centrifuge

column in the presence of 20 μM of $CuCl_2$ generated four major cross-link products, as revealed by SDS-polyacrylamide gel electrophoresis of samples in the absence of reducing agents (Fig. 1), which Western blotting with monoclonal antibodies to the individual subunits of the enzyme showed to involve β+γ, β+ε, α+δ, and β+δ (result not shown). The effect of cross-linking on ATPase activity is listed above each lane. The residual activity of both mutants was less than 10% of that of the untreated enzyme. The only cross-linked products seen in wild-type ECF_1 treated similarly were between the α and δ subunits (results not shown, but see Mendel-Hartvig and Capaldi, 1991b). This cross-linking of α to δ had no effect on ATPase activity.

Cross-linking of β to ε via disulfide bond formation must involve the introduced Cys in the DELSEED region and the introduced Cys^{108} of the ε subunit. The yield of this product is close to 100% in both mutants, based on the disappearance of the ε subunit. The cross-linking between β and γ must involve the Cys introduced into the DELSEED region and either Cys^{87} or Cys^{112}, the two endogenous cysteines of the subunit. Based on the recently published x-ray structure of MF_1 (Abrahams *et al.*, 1994), it is more likely that Cys^{87} is involved. The yield of the β-γ cross-link is 90–95% in the mutant βE381C/εS108C and somewhat less in the mutant βS383C/εS108C. In wild-type ECF_1, the yield of the α+δ cross-linked product is essentially 100%. In the DELSEED mutants, the yield of α+δ is significantly less because of the presence of a β+δ cross-link product (discussed later). In the mutant βS383C/εS108C, the β+δ cross-linked product migrates differently from α+δ; this is not the case for the mutant βE381C/εS108C (Fig. 1).

Disulfide Bond Formation between β and ε Inhibits ATPase Activity and Is Nucleotide-dependent—When ECF_1 from either double mutant was reacted with $CuCl_2$ in the absence of the column centrifugation step, the same cross-link products were obtained. However, the yield in which the disulfide bonds were formed was now determined by the concentration of $CuCl_2$ used, and the length of time for which samples were incubated before the reaction was stopped by addition of EDTA to chelate the metal ion. Preliminary experiments showed that disulfide bond formation between β and ε was much greater than that between β+γ, α+δ, or β+δ under the conditions of cross-linking chosen. This made it possible to examine both the nucleotide dependence of this specific cross-link and its effect on ATPase activity (Fig. 2). As shown for the mutant βE381C/εS108C, the yield of the β+ε cross-link product was high in the absence of nucleotides, *i.e.* in Mg^{2+} alone (*lane 1*) and high in Mg^{2+}+ADP, either added directly (*lane 3*) or as generated by addition of ATP+Mg^{2+} followed by turnover of the enzyme (*lane 2*). However, the yield of this cross-link was very low (less than 10%) in the presence of AMP·PNP+Mg^{2+} (*lane 4*) or with ATP+Mg^{2+} when azide was added to prevent enzyme turnover (result not shown). The cross-linking yield obtained with the mutant S383C/εS108C was much lower (less than 10%). The inhibition of ATPase activity was in proportion to the yield of cross-linked products, *i.e.* in the range 70–75% in ADP for the mutant βE381C/εS108C, but below 10% in the presence of AMP·PNP. Fig. 2 shows also the important finding that the cross-linking could be reversed by adding DTT. This is true, whether the cross-linking was conducted by adding $CuCl_2$ with or without the gel filtration centrifugation step. The activity determination reported above each lane in Fig. 2, and in subsequent figures, is the rate of ATPase hydrolysis of the cross-linked enzyme as a percentage of the activity of the same sample after adding DTT to break the linkages.

Disulfide Bond Formation between β and γ Inhibits ATPase Activity and Has the Opposite Nucleotide Dependence of the

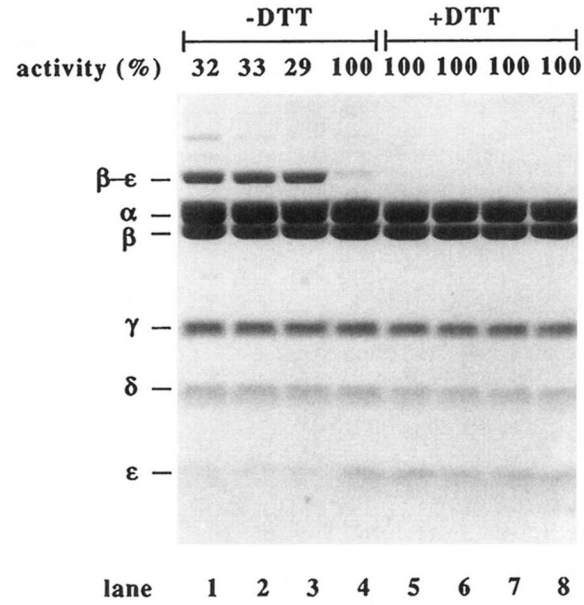

FIG. 2. **Cross-linking of ECF_1 from βE381C/εS108C without the use of centrifuge columns.** 2.5 mM $MgCl_2$ was added to ATPase in 50 mM MOPS, pH 7.0, 0.1 mM EDTA, and 10% glycerol at 0.3 mg/ml. 0.2 ml samples were supplemented with no nucleotides (*lanes 1* and *5*), 2 mM ATP (*lanes 2* and *6*), 2 mM ADP (*lanes 3* and *7*) and 2 mM AMP·PNP (*lanes 4* and *8*), respectively, incubated for 10 min at room temperature, and treated with 20 μM $CuCl_2$ for 3 h. 5 mM EDTA was added, and activities were measured after 2 h in the absence (*lanes 1–4*) or presence of 10 mM DTT (*lanes 5–8*). 30 μg of enzyme was applied per lane after addition of 40 mM NEM and DTT-free dissociation buffer. ATPase activities are expressed as percentage of DTT-treated samples. The ATPase activities of the control samples in *lanes 5–7* were 11 μmol of ATP hydrolyzed/min/mg. The activity of the sample reacted with AMP·PNP and then diluted in the assay buffer was 5 μmol of ATP hydrolyzed/min/mg.

β+ε Linkage—The nucleotide dependence and activity effects of the β+γ cross-link could be examined independently of the β+ε cross-link by using the single mutants in which an introduced cysteine was present in the DELSEED region, but the ε subunit was wild-type. Fig. 3*A* shows cross-linking of the single mutants βE381C and βS383C using the $CuCl_2$ gel filtration centrifugation procedure. The 90% yield of cross-linking of β to γ gave a 90% inhibition of ATPase activity. Fig. 3*B* shows cross-linking of β to γ in the mutant βE381C generated by addition of 50 μM $CuCl_2$ without a column step. The yield of β+γ was low in Mg^{2+}+ADP (*lane 1*), or in Mg^{2+}+ADP+P_i (*lane 2*). In contrast, the yield of this cross-linked product was high with Mg^{2+}+ATP added in the presence of azide to prevent enzyme turnover (*lane 3*) or with Mg^{2+}+AMP·PNP added (*lane 4*). Under all nucleotide conditions, the inhibition of activity mirrored the extent of β+γ cross-linking, being around 10% with ADP in catalytic sites (the experiment in Fig. 3), and 70% with ATP bound. The overall yield of cross-linked products obtained with the mutant βS383C was much lower than that for βE381C, when experiments such as those in Fig. 3*B* were conducted under the identical conditions of $CuCl_2$ reaction.

The Nucleotide Dependence of the β+γ Cross-linked Product Depends on the ε Subunit—Our previous work has shown that nucleotide-dependent conformational changes involving the γ subunit depend on the presence of the ε subunit (Aggeler and Capaldi, 1993; Turina and Capaldi, 1994a). To examine the effect of the ε subunit on β+γ cross-linking, ECF_1 from the mutant βE381C was depleted of the ε subunit by affinity chromatography using a monoclonal antibody against the ε subunit (Dunn, 1986). A comparison of the gel profiles of enzyme cross-linked under different nucleotide conditions by $CuCl_2$ (Fig. 4)

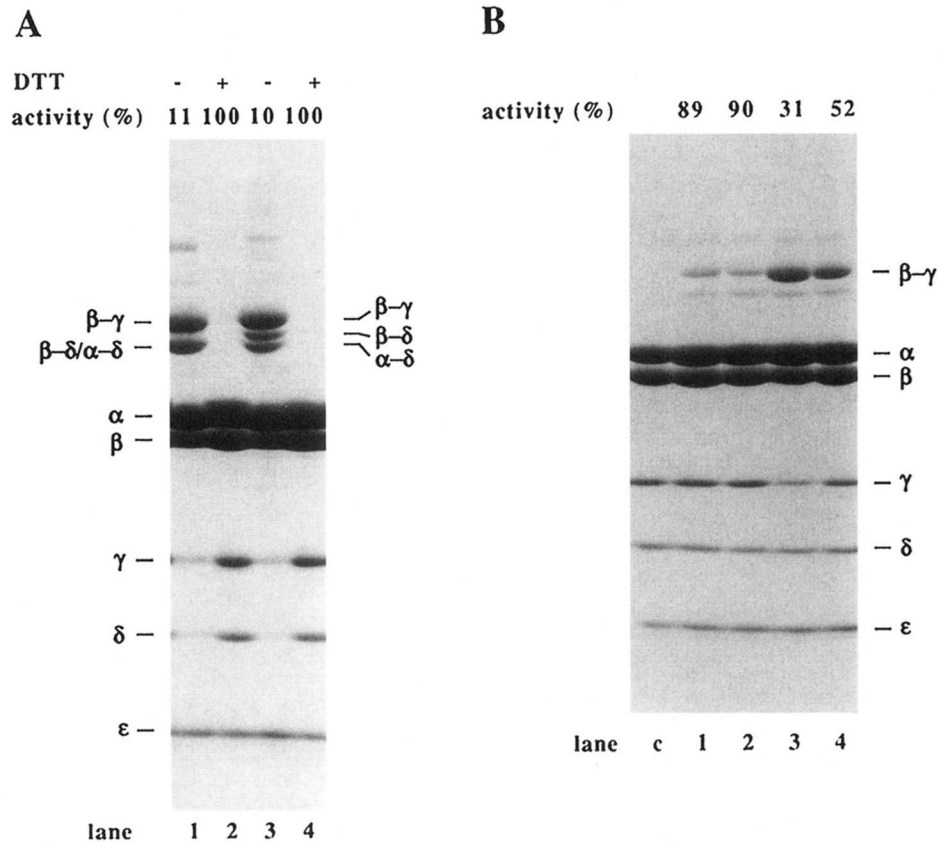

FIG. 3. **Cross-linking of ECF_1 from single mutants βE381C and βS383C.** *A*, 0.7 mg of ATPase from mutants βE381C (*lanes 1* and *2*) and βS383C (*lanes 3* and *4*) in 0.2 ml was passed, as described in Fig. 1, twice through EDTA containing columns, followed by two $CuCl_2$-containing columns. The cross-linked material (1.6 mg/ml) was either incubated in the absence (*lanes 1* and *3*) or presence of 20 mM DTT (*lanes 2* and *4*). 60 μg of ATPase was applied per lane. *B*, 2.5 mM $MgCl_2$ was added to 0.2-ml aliquots of 0.3 mg/ml ATPase from mutant βE381C in 50 mM MOPS, pH 7.0, 0.1 mM EDTA and 10% glycerol, followed by 2 mM ADP (*lane 1*), 2 mM ADP + 2 mM P_i (*lane 2*), 2 mM NaN_3 + 2 mM ATP (*lane 3*), and 2 mM AMP·PNP (*lane 4*). Samples were treated for 4 h with 50 μM $CuCl_2$ at room temperature and 30 μg of protein applied on a gel as described in Fig. 2. The ATPase activities of samples used in the experiments in *lanes 2* and *4* of *part A* and the controls for *lanes 1* and *2* of *part B* were in the range 7–9 μmol of ATP hydrolyzed/min/mg. The activity of the control for *lane 3B*, in which the sample with azide had been diluted in the assay buffer, was 4.3 and that for *lane 4B* where AMP·PNP had been added was 4.9 μmol of ATP hydrolyzed/min/mg.

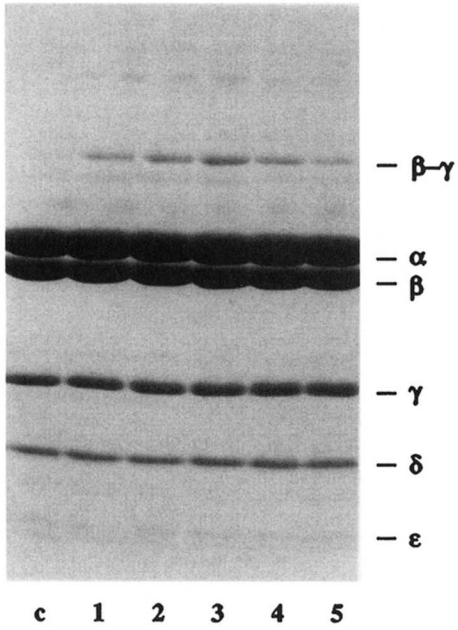

FIG. 4. **Cross-linking of ε-depleted ATPase from mutant βE381C.** 4 mg of ECF_1 at a concentration of 3 mg/ml was passed three times through an affinity column containing an ε-specific monoclonal antibody. ε-Free ATPase was treated at 0.5 mg/ml with 50 μM $CuCl_2$ as described for the intact enzyme in Fig. 3*B* in the presence of 2.5 mM $MgCl_2$ and 2 mM ATP (*lane 1*), 2 mM ADP (*lane 2*), 2 mM ADP + 2 mM P_i (*lane 3*), 2 mM NaN_3 + 2 mM ATP (*lane 4*), and 2 mM AMP·PNP (*lane 5*). *Lane c* is a control of untreated sample. 50 μg of protein was loaded per lane.

shows that there was essentially no nucleotide dependence of cross-linking of β to γ in ε-free ECF_1. This was confirmed by activity measurements which gave around 90% residual activity for all samples.

Chemical Modification of the DELSEED Region Inhibits ATPase Activity—To examine the relative exposure of the Cys residues introduced into the DELSEED region, ECF_1 from the mutants βE381C and βS383C was reacted with [^{14}C]NEM and with the larger maleimide, CM. [^{14}C]NEM was found to label the introduced Cys at either 381 or 383 in all three β subunits. Fig. 5 correlates ATPase activity and the incorporation of NEM into βE381C. Incorporation of close to 3 mol of the maleimide caused no inhibition of ATPase activity. Modification of the Cys at 381 with CM at the same concentration and for the same time intervals had a much more dramatic effect on activity. With this bulky maleimide, inhibition occurred rapidly in a time course that suggests that only 1 mol is necessary for maximal inhibition (95%).

Experiments similar to those shown in Fig. 5 were conducted using ε-free ECF_1 from the mutant βE381C, and identical results were obtained. Therefore, any observed effects of maleimide modification must be due to changes at the interface between β and γ, rather than the β and ε subunits. In one set of experiments, the effect of modifying the free β subunit, *i.e.* the one not associated with either the γ or ε subunit, was examined. ECF_1 from the mutant βE381C/εS108C was reacted with $CuCl_2$ to induce cross-linking of one β subunit to γ and a second β to ε in essentially 100% yield. The cross-linked sample was then reacted with CM to modify the fraction of Cys of the free β subunit that had not been cross-linked with the δ subunit (estimated at around 60%). Cross-links were then broken by DTT treatment, and the effect of incorporating the maleimide into the free β subunit was measured. The reaction with CM gave 60% inhibition of the ATPase activity.

Disulfide Bond Formation in ECF_1F_0—F_1F_0 was isolated from both double mutants and examined for disulfide bond formation upon $CuCl_2$ addition. Fig. 6*A* shows data for the mutant βS383C/εS108C on treatment with 20 μM $CuCl_2$ in the presence of ATP+Mg^{2+} and without the column centrifugation

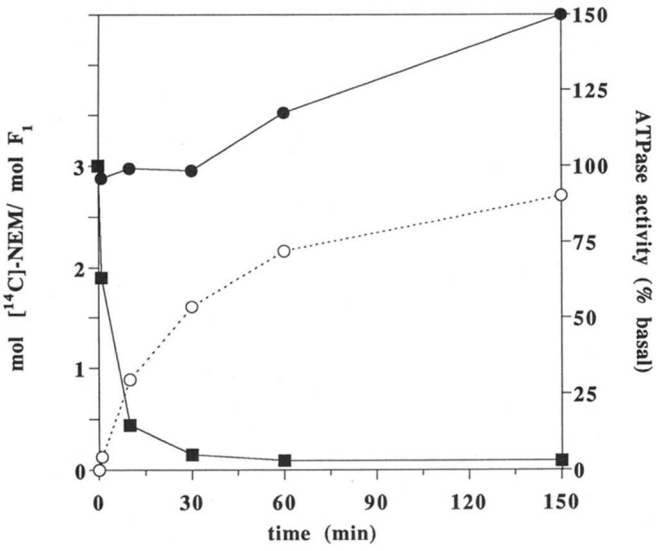

FIG. 5. **Correlation of ATPase activity of ECF_1 from βE381C/εS108C with incorporation of [^{14}C]NEM.** ATPase from the β/ε double mutant βE381C/εS108C was reacted at 0.4 mg/ml in 50 mM MOPS, pH 7.0, 0.5 mM EDTA, 5 mM ATP, and 10% glycerol with 25 mM [^{14}C]*N*-ethylmaleimide. The incorporation of [^{14}C]NEM into βCys^{381} was determined at 1, 10, 30, 60, and 150 min (mol [^{14}C]NEM/mol F_1 (*open circles*)). In a parallel experiment the change of ATPase activity due to the incorporation of 25 μM NEM (*circles*) or 25 μM CM (*squares*) into βCys^{381} of βE381C/εS108C was determined at 1, 10, 30, 60, and 150 min.

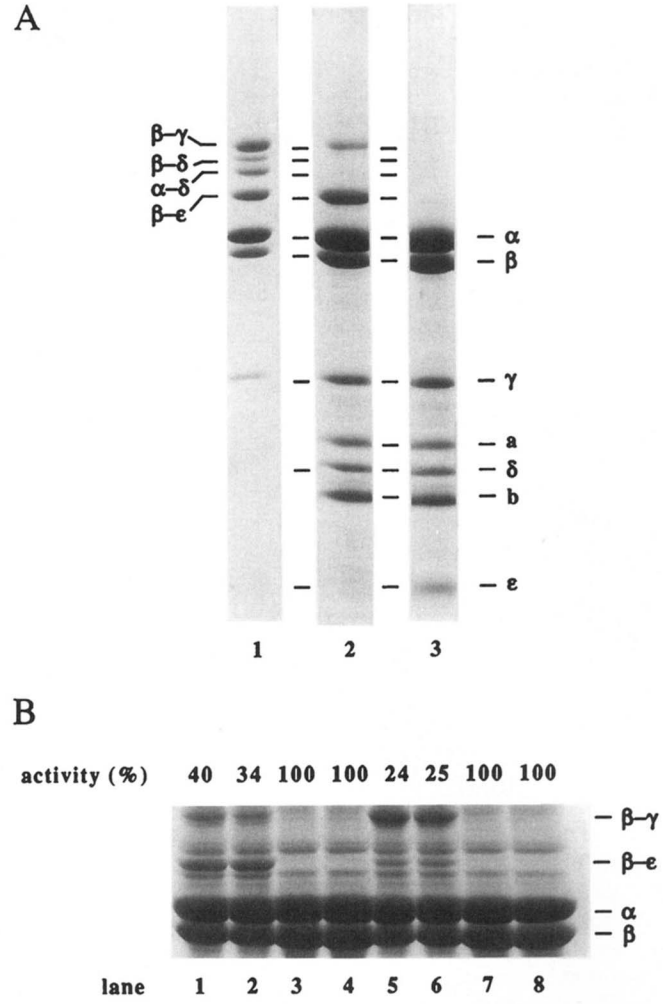

FIG. 6. **$CuCl_2$ induced cross-linking of ECF_1F_0 from the two β/ε double mutants.** *A*, 0.12 mg of reconstituted ECF_1F_0 from βS383C/εS108C in 0.2 ml of 50 mM MOPS, pH 7.0, 2 mM $MgCl_2$, 2 mM ATP, and 10% glycerol was incubated with 20 μM $CuCl_2$ for 3 h at room temperature. The sample was split in half and, after incubation for another 3 h in the absence (*lane 2*) or presence of 20 mM DTT (*lane 3*), 10 μl of 1 M NEM in Me_2SO was added to each 100-μl aliquot. Dissociation buffer without reducing agent was added, and 60 μg of protein was loaded on each lane of an 10–18% polyacrylamide gel. As a control, $CuCl_2$-treated ECF_1 (see Fig. 1) was applied (*lane 1*). *B*, 1.5 mg of reconstituted ECF_1F_0 from βE381C/εS108C was suspended in 1.5 ml of 50 mM MOPS, pH 7.0, 2 mM $MgCl_2$, and 10% glycerol. To 0.3-ml aliquots, 2 mM ATP (*lanes 1* and *3*), 2 mM ADP + 2 mM P_i (*lanes 2* and *4*), 2 mM NaN_3 + 2 mM ATP (*lanes 5* and *7*), and 2 mM AMP·PNP (*lanes 6* and *8*) were added, respectively. After incubation for 10 min and $CuCl_2$ treatment for 3 h, 100-μl samples were taken and not reduced (*lanes 1, 2, 5,* and *6*) or reduced with 20 mM DTT for 2 h (*lanes 3, 4, 7,* and *8*) and then quenched with 100 mM NEM. 80 μg of protein was loaded per lane. Only the top portion of the 10–18% gel is shown. The ATPase activity of the control lanes was 19 μmol of ATP hydrolyzed/min/mg for *lanes 3* and *4*, and 10 and 14 for *lanes 7* and *8* which had seen azide and AMP·PNP, respectively.

step (which is difficult to perform with the vesicular enzyme preparations). Both the β-ε and β-γ subunit cross-links were formed. However, no cross-linking between α or β plus δ was obtained in the intact ECF_1F_0. As with ECF_1, cross-linking between β and γ, and β and ε, caused inhibition of ATPase activity in proportion to the yield of the two cross-linked products. The cross-links were destroyed and activity restored to greater than 90% by addition of 20 mM DTT. Fig. 6*B* shows disulfide bond formation induced by 20 μM $CuCl_2$ in the mutant βE381C/εS108C under different nucleotide conditions. In the presence of ADP+Mg^{2+} (*lanes 1* and *2*), the yield of the β-ε cross-linked product was much greater than that of β-γ, while in the presence of ATP+Mg^{2+} (as ATP+Mg^{2+}+azide in *lane 5* and AMP·PNP+Mg^{2+} in *lane 6*), the yield of β-γ was much higher than that of β-ε subunits. Therefore, the same nucleotide dependence of cross-linked products is seen in ECF_1F_0 as in ECF_1 alone.

DISCUSSION

The studies presented here, using mutants in which Cys residues have been incorporated into the DELSEED region of the β subunit, provide new data on the structure of ECF_1 and help describe nucleotide-dependent conformational changes occurring in the complex. Using single mutants, in which Cys residues have been introduced into the DELSEED region, and double mutants with alterations of the DELSEED region along with introduction of a Cys into the ε subunit at position 108, it was possible to obtain disulfide bond formation between β and ε and/or β and γ. The cross-linking of β to γ is not surprising, based on the recent x-ray structure which shows the proximity of Cys^{87} of γ to the DELSEED region (Abrahams *et al.*, 1994). The cross-linking of ε to β from a Cys at 108 of ε to the DELSEED region was predicted from earlier work (Lötscher and Capaldi, 1984; Dallmann *et al.*, 1992). Dallmann *et al.* (1992) have shown that the cross-link of β to ε by the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide involves the DELSEED region and that part of the ε subunit COOH-terminal to residue 96. The cross-link, which was shown to be an ester linkage, was not seen in the mutant εS108C, implying that 1 of the 3 serines, either at 106, 107, or most likely at 108, was involved. The data presented here confirm that it is the region of ε near residue 108 that binds to the β subunit.

Importantly, the fact that both β+γ and β+ε cross-links can be obtained in near 100% yield in the same ECF_1 molecule when the double mutants βE381C/εS108C or βS383C/εS108C were used, establishes that the small α-helix of the γ subunit (around residue 87) and the COOH-terminal part of the ε subunit, interact with different β subunits. Cross-linking of

these mutants in the high yields obtained here means that it is now possible to experimentally distinguish the three β subunits in any F_1 molecule, based on the interaction of these catalytic subunits with the small subunits. This will be very useful in future studies to examine the affinity of the three different β subunits for ADP, ATP, etc.

When cross-linking on ECF_1 was conducted by addition of $CuCl_2$ without the column procedure, the rate and/or yield of the cross-linking of both β-γ and β-ε was much less in βS383C/εS108C than in βE381C/εS108C. It appears, therefore, that the Glu at 381 is better positioned for interaction with both the endogenous Cys of γ (Cys^{87}) and Cys^{108} of the ε subunit than is the Ser at 383 of the β subunit.

The recently published 2.8-Å resolution structure of MF_1 (Abrahams *et al.*, 1994) has provided details of the arrangement of the α, β, and γ subunits and new insights into catalysis by the enzyme. Briefly, this structure confirmed that the three β subunits are in different conformations and are structurally different, with the asymmetry related both to nucleotide occupancy of catalytic sites and interactions of the γ subunit. Thus, in one β subunit the catalytic site is closed around an Mg^{2+}·ADP (β_{DP}) and the β subunit is closest to the NH_2-terminal α-helical region of the γ subunit. A second β subunit has a partly open catalytic site containing AMP·PNP+Mg^{2+} (β_{TP}), and it is this β subunit which interacts with the short α-helix of the γ subunit containing Cys^{87}. The third β subunit is empty of nucleotide (β_E), and this β has interactions with the long COOH-terminal α-helix of the γ subunit. It is interesting then that there is nucleotide dependence of (the rate of) cross-linking between the DELSEED region and the γ and ε subunits. The disulfide link between the DELSEED region of that β subunit which interacts with the short central α-helix of the γ subunit is generated in higher yield with uncleaved ATP (either ATP+azide, or AMP·PNP) in catalytic sites. This suggests that the interaction between the region of γ and β is more stable with ATP than ADP in the catalytic site. In contrast, the interaction between the ε subunit and another β subunit is more stable in ADP than ATP, based on the data presented here, and on our previous studies of the interaction of the ε with the core ECF_1 complex (Mendel-Hartvig and Capaldi, 1991a; Aggeler *et al.*, 1992).

The important role of the ε subunit in determining the properties of the F_1 is now well established. It has been shown that ε-free ECF_1 has both altered rates of unisite ATP hydrolysis, due to an increased off rate of product, P_i (Dunn *et al.*, 1987), and a greatly enhanced (10-fold increased) rate of multisite ATP hydrolysis (Dunn *et al.*, 1987; Aggeler *et al.*, 1990; Mendel-Hartvig and Capaldi, 1991a). The functional effect of the ε subunit could result from the direct interaction of the ε with a β subunit, or with the γ subunit, or both. We have previously found that the nucleotide-dependent conformational changes in the γ subunit, as measured by fluorescence probes bound to a cysteine introduced at position 8 of the γ subunit, are lost on removal of the ε subunit (Turina and Capaldi, 1994a), as is the nucleotide dependence of cross-linking from this site to the β subunit by the tetrafluorophenylazide maleimide series of bifunctional reagents (Aggeler and Capaldi, 1993). Here we show that the nucleotide dependence of disulfide bond formation between the short central α-helix of γ and β_{TP} is lost on removal of the ε subunit. Taken together, these data suggest that the ε subunit may regulate the functioning of the F_1, at least in part, by controlling the conformation of the γ subunit.

The key observation related to function is that covalent linkage of a β subunit to the ε subunit through a single disulfide bond, from either the Cys at position 381 or position 383 in β to a Cys at position 108 of ε, blocks ATPase activity. Similarly, the linkage of the Cys at 381 or 383 in β to a Cys at position 87 in the γ subunit completely inhibits enzyme activity. When the disulfide bonds are broken, full activity is restored.

The DELSEED region of the β subunit is arranged as a loop linking two α-helices and is in a domain distinct from that containing the catalytic site. As with much of the β subunit, the DELSEED region appears unaltered (and the structures can be superimposed) whether ATP or ADP are bound in catalytic sites (Abrahams *et al.*, 1994). It is unlikely, therefore, that disulfide bond formation between γ and ε and the DELSEED region causes conformational changes within the β subunit which alter nucleotide binding in the catalytic sites. Rather, the most straightforward interpretation of the inhibitory effect of covalent bond formation between β-ε or β-γ is that these linkages block movements of the (α+) β subunits relative to γ and ε that are necessary for catalytic cooperativity. Based on our previous studies (*e.g.* Gogol *et al.*, 1990; Wilkens and Capaldi, 1994) and on the features of the structure of the F_1 moiety (Abrahams *et al.*, 1994), we believe that there are translocations of the γ and ε subunits between different β subunits driven by ATP hydrolysis (and ATP synthesis in the reverse direction of enzyme functioning) which, as first suggested by Boyer and Kohlbrenner (1981), cause alternation of catalytic sites between the three nucleotide-binding states (*i.e.* between β_{TP}, β_{DP}, and β_E). The chemical modification studies described here are similarly best interpreted in terms of an effect on movement of subunits relative to one another. Incorporation of a small molecule (NEM) into the DELSEED region has no significant effect on the functioning of the enzyme even when close to 3 mol of reagent are bound and cannot therefore be inducing major conformational changes in the β subunit. In contrast, when the more bulky maleimide CM was bound into the β subunit at position 381 at 1 mol equivalent (*i.e.* 1 mol of CM/mol of ECF_1), activity was dramatically reduced. Inhibition of activity was observed when free β subunit (*i.e.* that β not in contact with γ or ε) was modified. Such a result would be expected if the incorporation of CM prevents the free β subunit from "switching" to become associated with the γ or ε subunits during catalytic site alternation.

In addition to the cross-linked products between β and γ, and β and ε, discussed above, $CuCl_2$ treatment of the double mutant led to disulfide bond formation between α and δ, and β and δ. The cross-linking of α to δ by disulfide bond formation has been observed before and obtained in essentially 100% yield (Bragg and Hou, 1986; Tozer and Dunn, 1986; Mendel-Hartvig and Capaldi, 1991b). It involves Cys^{140} of the δ subunit (Mendel-Hartvig and Capaldi, 1991b). In the DELSEED mutants, there is competition between the β and α subunits for the δ subunit. In contrast to the linkage of β to ε, or β to γ, there is no disulfide bond formation between α and δ, or β and δ in ECF_1F_0, implying that the association of the δ subunit in ECF_1 is not physiological.

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