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Arrangement of the ϵ subunit in the *Escherichia coli* ATP synthase from the reactivity of cysteine residues introduced at different positions in this subunit

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Abstract

ECF_1F_0 has been purified from three mutants in which a Cys has been incorporated by site-directed mutagenesis in the ϵ subunit: these mutants are ϵS10C, ϵH38C and ϵS108C, respectively. ECF_1F_0 from the mutant ϵS10C had a 2-fold higher activity than wild-type enzyme, due to altered association of the ϵ subunit with the rest of the complex, and yet showed normal proton pumping function. The other two mutants had ATPase activities similar to wild-type enzyme. The introduced Cys was exposed for reaction with maleimides in ϵS10C and ϵS108C. In ϵH38C, the introduced Cys reacted readily with *N*-ethylmaleimide in isolated ECF_1, but was unavailable for reaction with this or other maleimides in ECF_1F_0. When this Cys at position 38 in the ϵ subunit was reacted with various maleimides in isolated ECF_1 and then the ECF_1 bound back to F_0, the interaction between the two parts was perturbed. While ECF_1F_0 reconstituted with unmodified ECF_1 functioned normally, enzyme with maleimide-reacted Cys-38 showed much reduced proton pumping, had only around 50% of the DCCD inhibition of unmodified or wild-type enzyme, and had a much higher LDAO activation (as much as 8.3-fold, c.f. 4-fold for wild type). Nucleotide-dependent conformational changes have been observed previously, in studies of ECF_1 from the mutants ϵS10C and ϵS108C. Identical nucleotide-dependent structural changes were observed in cross-linking experiments with tetrafluorophenylazide maleimides when the intact ECF_1F_0 from these mutants was examined. Taken together, the Cys reactivity data and cross-linking results provide the orientation of the ϵ subunit in the enzyme complex.

Keywords: ATP synthase; ϵ subunit; Topology; Cross-linking; Coupling

1. Introduction

The H^+ ATPase of *Escherichia coli* (ECF_1F_0) catalyzes both ATP synthesis coupled to an electron chemical gradient and ATP hydrolysis-driven proton translocation. The enzyme 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 containing subunits, a, b and c in the ratio 1:2:10-12 [1,2]. The F_1 part contains a total of six nucleotide binding sites, three catalytic sites located mainly on β subunits, and three so-called non-catalytic sites located mainly on α subunits. The proton channel is located in the F_0 sector and is constituted by the c subunits and possibly the a subunit [2,3].

Low resolution cryoelectronmicroscopy studies show the F_1 part separated from the F_0 by a narrow stalk of around 45 Å in length [4,5]. This stalk region appears to include parts of the γ, δ, and ϵ subunits of the F_1 part and the b subunits of the F_0 part [6,7]. It is now generally accepted that events in the catalytic sites of the F_1 part are coupled to proton translocation in the F_0 part via long-distance conformational changes transmitted through the stalk region [2,8]. We are studying structure–function relation-

Abbreviations: MBB, maleimidobutyrylbiocytin; CM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; RM, tetramethylrhodamine-5-(and -6)-maleimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, dicyclohexylcarbodiimide; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; DTE, dithioerythritol; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; TFPAM, tetrafluorophenylazide maleimide; AMP-PNP, adenyl-5′-yl-β,γ-imidodiphosphate.

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ships by introducing Cys residues into the stalk-forming subunits by site-directed mutagenesis. These sites can then be used for accessibility studies using maleimides and, if exposed, for attachment of cross-linkers, gold maleimides [9] and fluorophores [10] as reporters of the structural changes occurring.

We have previously described two mutants in which a Cys has been engineered genetically into the ϵ subunit, ϵS10C and ϵS108C respectively [11]. Both Cys residues were reactive to NEM. The Cys at position 10 was found to be close to the γ subunit by cross-linking with the TFPAM series of cross-linkers, while the Cys at position 108 was close to an α subunit [11]. The C-terminal region of ϵ around S108 is also close to the β subunit based on EDC cross-linking of ECF_1 [12,13]. A third Cys-containing mutant of the ϵ subunit (ϵH38C) has been described recently by Skakoon and Dunn [14]. Cross-linking studies showed the Cys at 38 to be close to the γ subunit.

In the present study, we have examined the NEM reactivity of the Cys residues at positions 10, 38 and 108 of the ϵ subunit in the intact ATP synthase, i.e., ECF_1F_0. Both Cys-10 and Cys-108 were found to react readily with this, and other maleimides, in ECF_1F_0. However, the Cys at position 38 was not modified by NEM, or other maleimides. This observation was followed up by reconstitution studies in which ECF_1 from the mutant ϵH38C was modified by NEM or other maleimides and then rebound to F_0. Functional studies of the reconstituted ECF_1F_0 indicate the importance of the region of ϵ around residue 38 for coupling of ATP hydrolysis to proton translocation by the complex.

2. Experimental procedures

Bacterial strains, plasmids. The *E. coli* strains used in this study were XL1-Blue from Stratagene for cloning, AN1460 [15] for isolation of the wild-type ATP-synthase and the unc$^-$ strain AN888 as the host for the plasmids containing the unc operon with ϵ subunit mutations. The plasmids pRA101 (ϵS10C) and pRA102 (ϵS108C) were described in Aggeler et al. [11].

An unc operon containing plasmid with the ϵH38C mutation was constructed by inserting the *Aat*II/*Pst*I fragment of pES4 [14], a generous gift of Dr. Stanley D. Dunn (University of Western Ontario), into pBluescript containing the 2.8 kb *Nde*I/*Eag*I fragment followed by one containing the 6.1 kb *Nde*I/*Xho*I fragment, and finally the 5.8 kb *Nsi*I/*Xho*I fragment into pRA100, as described in Aggeler et al. [11] to obtain pRA122. Standard subcloning procedures were carried out as described in Maniatis et al. [16] and Davis et al. [17].

Labeling and Cross-linking of E. coli F_1 ATPase and F_1F_0 ATP-synthase - ECF_1-ATPase from the ϵH38C mutant was modified with TFPAM-6 in 50 mM Mops (pH 7.0), 0.5 mM EDTA and 10% glycerol followed by removal of excessive label and photo cross-linking as described in Aggeler et al. [11].

ECF_1F_0 was reconstituted in egg-lecithin vesicles by dialysis as described before [18]. After centrifugation at 50000 rpm at 4° C for 1 h in a Beckman Ti60 rotor, the liposomes were resuspended in 50 mM Mops (pH 7.0), 5 mM $MgCl_2$, 20 μM DTT and 10% glycerol. At a concentration of 0.5 to 1.0 mg/ml of enzyme, 200 μM TFPAM-6 was added. After incubation at room temperature for 2 h, excess label was removed by two consecutive spins for 40 min at 70000 rpm in a Beckman TLA100.2 rotor. Photolysis was carried out for 3 h at room temperature under constant stirring with a 6 W 366 nm UV lamp (UVP, Model UVL-56, Blak-Ray lamp) at a distance of 2.5 cm.

[^{14}C]NEM modification of ECF_1F_0 was carried out by reacting the enzyme for 1 h with 300 μM [^{14}C]NEM, followed by the addition of 5 mM cysteine for 15 min. Samples were electrophoresed on $NaDodSO_4$-polyacrylamide gels, and radioactivity in the subunits was quantified as described in Aggeler et al. [18].

Rebinding of ECF_1 to ECF_0. ATPase and ATP-synthase were isolated as described by Gogol et al. [19] and Aggeler et al. [18], respectively. ECF_0 containing vesicles were obtained by subjecting the ECF_1F_0 vesicles, either liposomes described above or inverted inner membranes, to low salt conditions according to Lötscher et al. [20], or by the extraction procedure with 1 M KSCN as described in Perlin et al. [21] and resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM DTE and 10% glycerol. Free ECF_1 of the various mutants, with or without prior modification with 200 μM maleimide for 1 h at room temperature followed by addition of 2 mM cysteine, was rebound to stoichiometric amounts of ECF_0 in the presence of 5 mM ATP by incubation for 8 h at room temperature. The liposomes were pelleted twice by centrifugation for 40 min at 70000 rpm in a Beckman TLA100.2 rotor to remove unbound F_1 ATPase.

Other Methods. Protein concentrations were determined with the BCA protein assay from Pierce Chemical Co. after addition of 1% $NaDodSO_4$ to the samples containing egg-lecithin vesicles. The cross-link products were analyzed after electrophoresis on $NaDodSO_4$ containing 10–18% polyacrylamide gels [22], followed by blotting onto nitrocellulose membranes with monoclonal antibodies [23]. The effect of DCCD on the ATPase activity was assayed after incubation of ECF_1F_0 with 20 μM DCCD for 2 h at room temperature with a regenerating system [20] in the absence and presence of 0.5% LDAO. The quenching of ACMA fluorescence was measured on inner membranes with an SLM 8000 fluorometer at 480 nm with an excitation wavelength of 410 nm essentially according to Lötscher et al. [20]. To a suspension of membranes at a protein concentration of 0.2 mg/ml in 10 mM Hepes (pH 7.5), 100 mM KCl and 5 mM $MgCl_2$ were added sequentially 3.6 μM valinomycin, 1 μM ACMA, 0.5 mM NADH, 2 mM KCN, 2 mM ATP and 3.6 μM nigericin.

3. Results

Mutants in which a Cys residue has been introduced into the ϵ subunit are ϵS10C, ϵS108C [11] and ϵH38C [14]. For the studies presented here, the ϵH38C mutation was incorporated into the unc operon as described in the Experimental procedures section and ECF_1 and ECF_1F_0 prepared from this and the other mutants listed above.

ATPase activities of ECF_1F_0 for the mutants ϵH38C and ϵS108C were similar to that of wild-type enzyme, i.e., in the range 15–25 μmol ATP hydrolyzed per min per mg protein, the variability depending mostly on the purity of different preparations. In contrast, ECF_1F_0 isolated from the mutant ϵS10C, reproducibly gave an ATPase activity of 40–43 μmol ATP hydrolyzed per min per mg protein. This value is close to that obtained by trypsin treatment of wild-type ECF_1F_0 [23] and higher than the activity of ECF_1 isolated from the mutant ϵS10C, i.e., 30–32 μmol ATP per min per mg [11].

3.1. Characterization of the ϵH38C mutant

The ATPase activity of ECF_1 isolated from the mutant ϵH38C of 18 μmol ATP hydrolyzed per min per mg F_1 was increased 8-fold by addition of 0.5% LDAO, indicating that the mutant ϵ subunit binds and efficiently inhibits ATPase activity, as reported by Skakoon and Dunn [14]. These workers also described cross-linking of ϵ through the Cys at position 38 to the γ subunit using *p*-azidophenacyl bromide and *N*-[4-(*p*-azidosalicylamido)butyl]-3′-(2′-pyridyldithio)propionamide as cross-linkers, but these reagents gave very low yields of product, precluding activity studies.

Fig. 1 shows cross-linking of ECF_1 isolated from the mutant ϵH38C with TFPAM-6, a recently developed cross linker [11], which has generated cross-linked products in high yields in our previous studies [11,24]. Fig. 1, part A shows the $NaDodSO_4$ polyacrylamide gel of samples cross-linked in both ADP + Mg^{2+} and ATP + EDTA containing buffers, after staining with Coomassie brilliant blue; part B shows a Western blotting analysis of the sample reacted with TFPAM in ATP + EDTA. A cross-linked product between the γ and ϵ subunits was obtained under both nucleotide conditions that migrates under the β subunit (resolved by antibody blotting). The yield of this cross-linked product in ADP + Mg, ATP + EDTA, and AMP.PNP + Mg (not shown) was similar and 20–30% based on the disappearance of the γ and ϵ subunits from the gel profile. At these yields of cross-linked product, there was no significant effect on ATPase activity, i.e., 21–25 μmol ATP hydrolyzed per min per mg enzyme before photolysis compared with 21–23 μmol ATP hydrolyzed per min per mg enzyme after photolysis. In this respect, the data for the ϵH38C mutation are the same as with the ϵS10C mutation, where cross-linking of ϵ to the γ subunit had very little effect on ATPase activity [11]. In contrast, the cross-linking of ϵ to the α subunit via a Cys at ϵ108 inhibited activity [11].

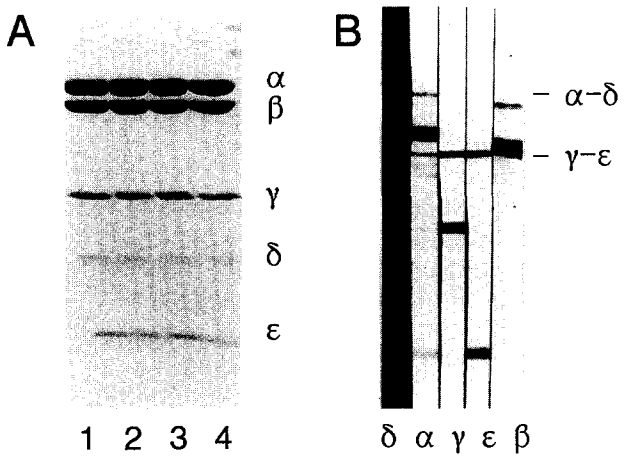

Fig. 1. Cross-linking of ϵH38C mutant F_1 ATPase. (A) 540 μg of ECF_1 in 100 μl of buffer (50 mM Mops (pH 7.5), 0.5 mM EDTA and 10% glycerol) was labeled with 200 μM TFPAM-6 for 1 h at room temperature. Excess label was removed by two consecutive passages through centrifuge columns in Sephadex G50 in the same buffer. 5 mM ATP (lanes 1 and 2) and 5 mM ATP + 5.5 mM $MgCl_2$ (lanes 3 and 4), respectively were added to this buffer, and samples either kept in the dark (lanes 1 and 3) or photolyzed at 366 nm for 2 h at room temperature (lanes 2 and 4). 50 μg protein was applied on each lane and electrophoresis carried out on a $NaDodSO_4$ containing 10–18% gradient polyacrylamide gel, which was stained with Coomassie brilliant blue. (B) Immunoblot of 30 μg ECF_1, which was cross-linked in the presence of 5 mM ATP + 0.5 mM EDTA (as lane 2 in (A)), using monoclonal antibodies against the indicated subunits.

3.2. Accessibility of introduced Cys residues in the various ϵ subunit mutants

Previous studies have shown that the Cys introduced at positions 10, 38, and 108 of the ϵ subunit is, in all cases, reactive to maleimides in isolated ECF_1 [11,14]. To examine the reactivity of the Cys at these sites in intact ECF_1F_0, this complex was purified from each of the mutants and the binding of [^{14}C]NEM measured. The data are summarized in Table 1. [^{14}C]NEM was readily incorporated into the Cys at positions 10 or 108. However, ϵ subunit in ECF_1F_0 from the mutant H38C was unreactive to [^{14}C]NEM, the small amount of radioactivity running at the position of the ϵ subunit being about the same as that for enzyme preparations not containing a Cys in the ϵ subunit (Table 1).

3.3. Reconstitution studies with ECF_1 from the ϵ subunit mutants

Shielding of the Cys at position 38 of the ϵ subunit from modification in ECF_1F_0, but not in isolated ECF_1, suggests that this site is in the interface between the F_1 and F_0 parts, a possibility that was examined further in reconstitution experiments. ECF_1 isolated from the three ϵ sub-

Table 1
[^{14}C]NEM incorporation into ECF_1F_0 isolated from the ϵ subunit mutants

Mutant	NEM incorporation (mol ^{14}C per mol subunit)		
	γ subunit [a]	δ subunit [b]	ϵ subunit [c]
Control (γS8C) [d]	0.78	0.62	0.08
ϵS10C	0.07	0.72	0.80
ϵH38C	0.11	0.87	**0.10**
ϵS108C	0.15	1.02	0.70

[a] There are two intrinsic Cys residues in the wild-type γ subunit but these are unreactive as indicated by the incorporation of [^{14}C]NEM into this subunit in the ϵ subunit mutants.
[b] There is one reactive intrinsic Cys residue in the δ subunit.
[c] There are no intrinsic Cys residues in the wild-type ϵ subunit and hence the small amount of labeling by [^{14}C]NEM in the gel in the region of this subunit in the γ subunit mutants represents background labeling.
[d] γS8C is described in Aggeler and Capaldi [24].

unit mutants, with or without subsequent modification of the introduced Cys residue, was reacted with vesicles containing F_0 (obtained as described in the Experimental procedures section), and bound ECF_1 separated from free enzyme by two centrifugation steps. Reconstituted ECF_1F_0 preparations were then examined for proton pumping activity, DCCD sensitivity and for LDAO-activated ATPase activity. Modification of the Cys at positions 10 (Fig. 2) or 108 (result not shown) by NEM had no effect on proton translocation as measured by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) (Fig. 2). There was no effect on DCCD sensitivity of modification of the Cys at positions 10 or 108 by any of several maleimides of different sizes and hydrophobicity. In both mutants, the DCCD inhibition of ATPase activity both before and after Cys modification was 56–64%, which is the same as for wild-type ECF_1F_0 under identical conditions (Table 2). Finally, the LDAO-induced activation of the ATPase activity was unaltered by the various modifications of Cys-10 or 108 (Table 3). LDAO disrupts the interaction between the F_1 and F_0 parts, relieving inhibition of ATPase activity caused by this association [25]. Therefore, the difference in activity with and without LDAO present in the assay is a measure of the integrity of the F_1F_0 interaction.

In contrast to the results for Cys-10 and -108, modification of the Cys at position 38 drastically altered the F_1F_0 interaction. As shown in Fig. 2, unmodified ECF_1 from this mutant showed good proton pumping function in the reconstitution system while, with NEM-modified enzyme, proton translocation was drastically impaired. Also, with maleimide modification of the Cys at position 38, DCCD inhibition was significantly altered (Table 2) as was LDAO activation of the ATPase activity. Thus after NEM modification, DCCD inhibition was only 30%, half of that obtained with the unmodified enzyme. The LDAO activation was 6.2 as opposed to 4.3 before treatment. Modification of the Cys at position 38 with more bulky maleimides

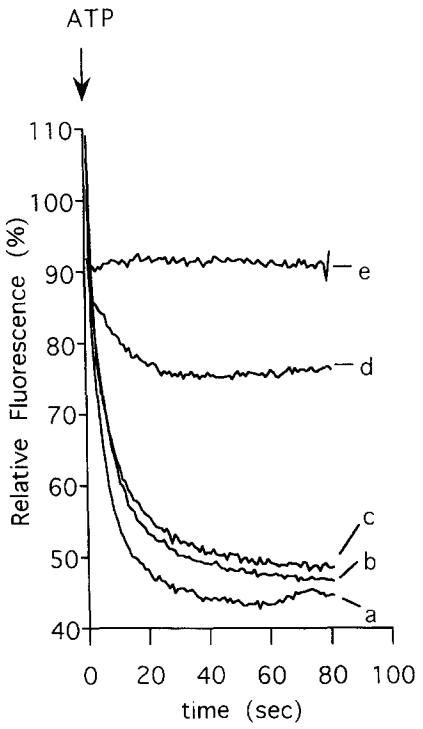

Fig. 2. ACMA quenching of fluorescence in ϵH38C membranes. Membranes of ϵH38C were stripped of ECF_1 with 50 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 M KSCN, washed twice with 5 mM Tes (pH 7.0), 0.5 mM DTT, 0.5 mM EDTA, 40 mM 6-aminohexanoic acid and 15% glycerol, and twice with 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM DTE and 10% glycerol. To 1 mg of ECF_1-depleted membranes in 0.25 ml of the same buffer with 5 mM ATP were added 150 μg of ECF_1 and the mixture was incubated for 5 h at room temperature and 16 h at 4° C. To a membrane suspension at 0.2 mg/ml in 10 mM Hepes (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$ were added 3.6 μM valinomycin (as a K^+ ionophore), followed by 1 μM ACMA (quenchable fluorescent dye to follow pH changes), 0.5 mM NADH (electron transfer substrate), 2 mM KCN (to block the terminal oxidase of the respiratory chain), 2 mM ATP (as substrate) and 3.6 μM nigericin (to dissipate the ion gradients) in that order. ECF_1 from ϵS10C, unmodified (scan a) and NEM-modified (scan b); ECF_1 from ϵH38C, unmodified (scan c) and NEM-modified (scan d); no ECF_1 added (scan e).

Table 2
DCCD inhibition of ECF_1F_0 from the various ϵ subunit mutants

Modifying reagent	ϵ subunit mutant		
	S10C	S108C	H38C
–	64	56 ± 6(4)	64 ± 7(8)
NEM	60	n.d.	30(2)
TFPAM-6	60	59(2)	28 ± 2(4)
CM	51	n.d.	26(2)
RM	59	55	25
MBB	60	55(2)	28 ± 7(3)

Numbers in parentheses show the number of independent measurements. Others are the results of a single experiment in duplicate. ECF_1 (76 μg) was added to ECF_0 (28 μg) reconstituted in egg lecithin vesicles suspended in 0.5 ml 50 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 5 mM ATP, 1 mM DTE and 10% glycerol, and the mixture incubated at room temperature for 8 h and then at 4° C for 16 h. After removal of free ECF_1, DCCD inhibition was determined. n.d. = not determined. Values represent the inhibition of ATP hydrolysis of ECF_1F_0 by DCCD expressed as a percentage of this activity of the uninhibited enzyme.

resulted in somewhat smaller levels of DCCD inhibition and higher LDAO activity, e.g., with CM, LDAO activation was 8.3. These reconstitution studies, therefore, establish that modification of the Cys at position 38 – but not that at positions 10 or 108 – of the ϵ subunit affect the interaction between F_1 and F_0.

3.4. Cross-linking studies with ϵ mutants in ECF_1F_0

Cross-linking studies with ECF_1 from the various mutants have provided information on near-neighbor interactions in the complex, and in the cases of the mutants ϵS10C and ϵ108C, showed nucleotide dependent conformation changes in the protein [11]. Similar cross-linking studies were conducted using ECF_1F_0 isolated from the three mutants. Fig. 3 shows the results for the mutant ϵS10C. Two cross-linked products were obtained on TFPAM-6 modification and subsequent UV photolysis: one involving subunits α and δ, the second involving γ and ϵ subunits; both of these products were obtained in experiments using isolated ECF_1 [11]. As evident in Fig. 3, the yield of cross-linking from the Cys at position 10 of ϵ to the γ subunit was nucleotide-dependent, being significantly higher in Mg^{2+} AMP.PNP or ATP + EDTA than in ADP + Mg^{2+} (generated in catalytic sites by addition of ATP + Mg^{2+} followed by enzyme turnover). This is the same result obtained in isolated ECF_1, and indicates that the interaction of F_1 with F_0 does not hinder the nucleotide dependent conformational change around position 10 of the ϵ subunit. The α-δ subunit cross-linked product was present in the different nucleotide conditions, as observed with isolated ECF_1. Note that a β-δ product seen in low yield in ECF_1, was not observed in ECF_1F_0 (in any of the mutants studied).

Studies with the mutant ϵS108C using TFPAM-6 showed cross linking of ϵ to an α subunit; the yield of which was lower in AMP · PNP + Mg than in ADP + Mg (result not shown) as reported already for isolated ECF_1. Cross-linking was obtained between the ϵ and γ subunits in ECF_1F_0 from the mutant ϵH38C after first reacting the F_1 part with the maleimide group of TFPAM-6 (in the dark) and then rebinding to F_0 before photolysis (result not shown). There was no obvious nucleotide dependence of cross-linking from the Cys at position 38 in ECF_1 or in the reconstituted ECF_1F_0.

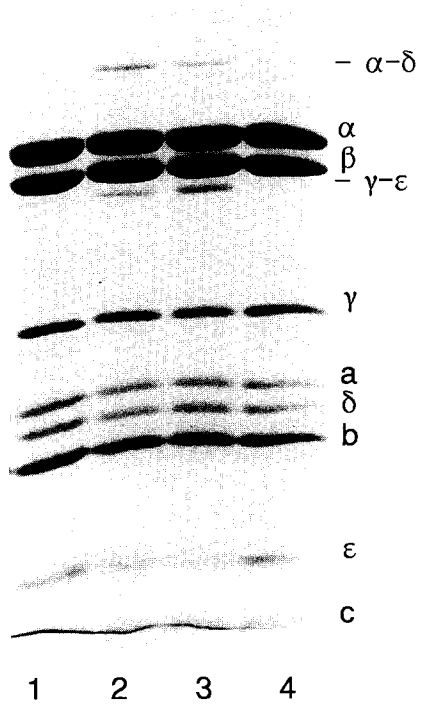

Fig. 3. Cross-linking of ECF_1F_0 of ϵS10C mutant. ECF_1F_0 was reconstituted in egg-lecithin vesicles at lipid:protein = 1:1 (w/w), by dialysis and collected by centrifugation at 4° C for 1 h at 45000 rpm in a Beckman Ti60 rotor. The vesicles were resuspended in 50 mM Mops (pH 7.0), 2 mM $MgCl_2$, 20 μM DTT and 10% glycerol. 400 μg ATP-synthase at a concentration of 1 mg/ml was labeled with 200 μM TFPAM-6. Excess of label was removed by centrifugation at 100000× g for 40 min. The pellet was resuspended in 0.8 ml of the same buffer and split in half. One sample was supplemented with 2 mM ATP (lanes 1 and 2), the other with 2 mM AMP-PNP (lanes 3 and 4). The ATP-synthase was then kept at room temperature under stirring for 3 h either in the dark (lanes 1 and 4) or photolyzed (lanes 2 and 3). 150 μl aliquots containing 70 μg enzyme were applied on a 10–18% gradient polyacrylamide gel, which was stained with Coomassie brilliant blue.

Table 3
LDAO activation of vesicles reconstituted with ECF_1F_0 from the different ϵ subunit mutants

Modifying reagent	ϵ subunit mutant		
	S10C	S108C	H38C
–	3.6	4.3 ± 0.3(4)	4.3 ± 0.7(8)
NEM	3.7	n.d.	6.2(2)
TFPAM-6	3.9	4.8(2)	7.2 ± 0.6(5)
CM	3.8	n.d.	8.3 ± 0.3(3)
MBB	3.4	3.3(2)	6.0 ± 0.8(3)

Numbers in parentheses are the number of independent measurements as in Table 2. n.d., not determined. Values are the ATPase activity of ECF_1F_0 in the presence of LDAO divided by this activity in the absence of the detergent.

4. Discussion

The arrangement of the ϵ subunit in the ECF_1F_0 complex and the functions that this subunit plays remains to be fully elucidated. Unfortunately, in the recently-studied crystal form of the mitochondrial F_1 (MF_1) [26], the δ subunit, which is the homologue of the ϵ subunit of ECF_1, was disordered and no high-resolution structure of this subunit could be obtained. Sequence prediction algorithms suggest that the N-terminal two-thirds of the ϵ subunit is predominantly a β-sheet structure, while the C-terminal third is arranged as an α-helical structure. Our ongoing NMR determination of the purified ϵ subunit supports this

structural arrangement (Wilkens, S. and Capaldi, R.A., unpublished data). Moreover, we find that a truncated form of the ϵ subunit, containing residues 1–95, is mostly β-sheet with no observable α-helical structure. Such data indicate a two-domain structure of the ϵ subunit, as first suggested by the functional studies of Jounouchi et al. [27] and Kuki et al. [28]. These workers have shown that a mutant in which the ϵ subunit has been truncated to remove the C-terminal one third (residues 79–138 are missing) can still grow by oxidative phosphorylation.

Evidence that the ϵ subunit is a part of the stalk has been obtained by cryoelectron microscopy studies of ECF_1 [9] as well as from low resolution X-ray structural studies of MF_1 (which show the δ subunit of this complex is in a 40 Å extension) [29]. The results presented here are a clear indication that the ϵ subunit must be in the stalk with the N-terminal domain around residue 38 in the interface with one of the F_0 subunits. Thus, a Cys at position 38 was found to be shielded from reaction with NEM in intact ECF_1F_0, although this Cys was reacted readily with maleimides as large as a 27 Å gold-containing maleimide in ECF_1 [9]. Modification of ECF_1 from the mutant ϵH38C with NEM did not prevent the F_1 part from rebinding to F_0, but the interaction between the two parts was altered, as judged by a reduced DCCD sensitivity of the ATPase activity and an increased LDAO activation. Most importantly, NEM modification of the Cys at position 38 uncoupled ATP hydrolysis from proton pumping in the reconstituted F_1F_0.

Additional evidence of the close functional relationship between the N-terminal part of the ϵ subunit and the F_0, specifically the c subunit, comes from studies reported recently by Zhang et al. [30]. These workers showed that an uncoupled mutant of the c subunit in which Gln-42 was converted to Glu is suppressed by a change of a Glu at position 31 of the ϵ subunit to a Gly, Val or Lys. Such data are suggestive, but not conclusive evidence, of the interaction of the N-terminal domain with the F_0. Suppressor effects can occur at a distance as indicated by the recent studies of Futai and colleagues on the β and γ subunits [31–33]. For example, these workers have shown that the mutation of Gly-149 to a Cys inhibits the ATPase activity of ECF_1, but that this deleterious mutation is suppressed by a change of Ser-174 to a Phe, Val-198 to an Ala, or Glu-192 to a Val, all residues significantly distant from Gly149 [31].

It is important to note that the region of the ϵ subunit around His-38 (Val-20–Ala-44) is the only region that has strong homology with the ϵ subunit of chloroplasts and the δ subunit of mitochondrial F_1. LaRoe and Vik [34] have shown that some mutations at positions 31 and 38 affect the coupling of ATP hydrolysis with proton pumping.

As reported by Aggeler et al. [11] for ECF_1, and as shown here for ECF_1F_0, the ϵ subunit can be cross-linked to an α subunit via Cys-108 using the TFPAM series of cross-linkers. The ϵ subunit can also be cross-linked to a β subunit in ECF_1 and in ECF_1F_0 by EDC [12,23,35]. Dunn and colleagues have shown that this zero length crosslink generated in high yield by EDC is between the so-called DELSEED region of the β subunit (residues 380–386) and a region of ϵ close to Ser-108 [13]. We have now confirmed the close proximity of the ϵ subunit at Ser-108 to the DELSEED region, by disulfide bond formation in the double mutant βE381C;ϵS108C (Aggeler, R. and Capaldi, R.A., unpublished data).

An important and not yet fully answered question is: what is the role of the C-terminal part of the ϵ subunit? As described already, the mutational studies of Kuki et al. [28] indicate that the C-terminal 60 residues are dispensable for oxidative phosphorylation by ECF_1F_0. One suggestion is that the ϵ subunit is an inhibitor of free ECF_1 in the cell, thereby preventing depletion of ATP during F_1F_0 assembly [36]. Removal of the ϵ subunit causes as much as a 10-fold increase in the rate of ATP hydrolysis of isolated ECF_1, and this inhibitory function has been localized to the C-terminal part of the ϵ subunit by proteinase digestion [35] and to the region 80–93 in mutagenesis experiments [28]. Cleavage of the C-terminal region of the ϵ subunit in ECF_1F_0 increases ATPase activity without affecting ATP-driven proton translocation [23].

We have previously found that ECF_1 from the mutant ϵS10C has a much higher basal activity than the wild-type enzyme, i.e., 3-fold higher [11]. It is shown here that ECF_1F_0 for this mutant also has a higher activity than the wild-type, in this case a 2-fold activation, supporting a role of the ϵ subunit as at least a partial inhibitor of the ATPase activity in ECF_1F_0.

Based on cross-linking data in ECF_1 [11], and now in ECF_1F_0, the Cys at position 10 of the ϵ is in close proximity to the γ subunit. Moreover, there is a nucleotide dependence of the yield of the γ-ϵ crosslink product in both preparations. There is also cross-linking between the Cys at position 38 of ϵ and the γ subunit. This is significant because, with the data presented here, it places the γ subunit close to subunits of F_0. We are now studying mutants containing cysteines introduced in the polar loop of subunit c, and have been able to obtain cross-links from c to the γ subunit (Watts, S. and Capaldi, R.A., unpublished results). If the γ subunit contacts the proton channel containing c subunits (and possibly the a subunit), transmission of conformational changes between catalytic sites and the proton channel could be entirely within the γ subunit, with the ϵ subunit acting only as a regulator of conformational coupling. More studies are needed to distinguish whether the ϵ subunit acts directly in the coupling process or indirectly in this postulated regulatory role.

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