Introduction of Reactive Cysteine Residues in the ε Subunit of *Escherichia coli* F_1 ATPase, Modification of These Sites with Tetrafluorophenyl Azide-Maleimides, and Examination of Changes in the Binding of the ε Subunit When Different Nucleotides Are in Catalytic Sites†

Robert Aggeler,‡ Kathy Chicas-Cruz,‡ Sui-Xiong Cai,§ John F. W. Keana,§ and Roderick A. Capaldi*,‡

Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403

Received October 14, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: Cysteine residues have been exchanged for serine residues at positions 10 and 108 in the ε subunit of the *Escherichia coli* F_1 ATPase by site-directed mutagenesis to create two mutants, ε-S10C and ε-S108C. These two mutants and wild-type enzyme were reacted with [^{14}C]*N*-ethylmaleimide (NEM) to examine the solvent accessibility of Cys residues and with novel photoactivated cross-linkers, tetrafluorophenyl azide-maleimides (TFPAM's), to examine near-neighbor relationships of subunits. In native wild-type F_1 ATPase, NEM reacted with α subunits at a maximal level of 1 mol/mol of enzyme (1 mol/3 α subunits) and with the δ subunit at 1 mol/mol of enzyme; other subunits were not labeled by the reagent. In the mutants ε-S10C and ε-S108C, Cys_{10} and Cys_{108}, respectively, were also labeled by NEM, indicating that these are surface residues. Reaction of wild-type enzyme with TFPAM's gave cross-linking of the δ subunit to both α and β subunits. Reaction of the mutants with TFPAM's also cross-linked δ to α and β and in addition formed covalent links between Cys_{10} of the ε subunit and the γ subunit and between Cys_{108} of the ε subunit and the α subunit. The yield of cross-linking between sites on ε and other subunits depended on the nucleotide conditions used; this was not the case for δ-α or δ-β cross-linked products. In the presence of ATP + EDTA the yield of cross-linking between ε-Cys_{10} and γ was high (close to 50%) while the yield of ε-Cys_{108} and α was low (around 10%). In the presence of ATP + Mg^{2+} the yield of ε-Cys_{10}-γ was lower than in ATP + EDTA (only 22%) and the yield of cross-linking between ε-Cys_{108} and α was higher (now around 30%). These changes in cross-linking of ε to near-neighbor subunits support previous work [Mendel-Hartvig, J., & Capaldi, R. A. (1991) *Biochemistry 30*, 1278-1284] in showing that there are ligand-dependent conformational changes and/or binding changes of the ε subunit. Cross-linking of the ε subunit to γ had very little effect on ATPase activity, while cross-linking of the ε subunit to an α subunit inhibited ATPase activity dramatically.

An F_1F_O-type ATP synthase is found in the bacterial plasma membrane, the mitochondrial inner membrane, and the chloroplast thylakoid membrane, catalyzing ATP synthesis in response to a transmembrane proton gradient. This enzyme can also generate a proton gradient by using the energy released from hydrolysis of ATP.

The *Escherichia coli* ATP synthase, ECF_1F_O, is made up of eight different subunits, five of which, α, β, γ, δ, and ε, form the ECF_1[1] part (Senior, 1988; Futai et al., 1989). There are three copies each of the α and β subunits and three catalytic sites per F_1 moiety (e.g., Cross & Nalen, 1982), now thought to be located at the interface between α-β subunit pairs (Vogel & Cross, 1991; Rao et al., 1988; Ida et al., 1991). The three other subunits in ECF_1, γ, δ, and ε, are present in one copy each.

We have focused our recent studies on the γ, δ, and ε subunits. These three subunits are in the interface between the F_1 and F_O parts (Sternweis, 1978; Sternweis & Smith, 1977) and are, therefore, likely to be involved in the coupling between catalytic site events and proton channeling through the F_O part of the complex. Evidence has been presented that the ε subunit changes conformation and/or changes binding sites during ATP hydrolysis and ATP synthesis (Mendel-Hartvig & Capaldi, 1991a,b; Bragg & Hou, 1987; Richter & McCarty, 1987). During ATP hydrolysis these changes or shifts are related to P_i binding in the catalytic sites containing ADP + Mg^{2+} and are blocked by DCCD modification of F_O (Mendel-Hartvig & Capaldi, 1991b).

A clear definition of the role of the ε subunit (as well as γ and δ subunits) in energy transduction within the ATP synthase will require kinetic analyses, in which structural and/or binding changes are correlated with individual steps in catalysis and proton translocation. To this end we are introducing reporter groups into the γ, δ, and ε subunits that are able to monitor structural changes. Our approach is to introduce Cys residues at selected sites in the various subunits and then modify these sites with probes such as photoactivatable cross-linkers and fluorescence reagents that can be used for time-resolved analysis of conformational changes. Here we describe mutagenesis of the ε subunit to introduce Cys residues in the N- or C-terminal parts of the polypeptide. These sites

† This work was supported by NIH Grants HL24526 to R.A.C. and GM27137 to J.F.W.K.

‡ Institute of Molecular Biology.

§ Department of Chemistry.

[1] Abbreviations: F_1, membrane-extrinsic portion of the proton-translocating ATPase complex; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; DCCD, dicyclohexylcarbodiimide; DTT, DL-dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; P_i, inorganic phosphate; TFPAM, tetrafluorophenyl azide-maleimide.

0006-2960/92/0431-2956$03.00/0

were modified by a new class of photoactivated cross-linking reagents, and the interaction of the ϵ subunit with near-neighbor subunits was then monitored as a function of nucleotide binding in the catalytic sites.

Experimental Procedures

Materials. The *E. coli* strains used for cloning and site-directed mutagenesis were XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB*, *lacI*^{q}ZΔM15, Tn10 (Tet^r)]) (Bullock et al., 1987) from Stratagene and CJ236 (*dut ung1 thi-1 relA1*/pCJ105 (Cm^r) (see Kunkel et al., 1987) from New England Biolabs. An888 (*uncB*$^+$Mu::416 *argH pyrE entA nalA*, *recA*) (Lightowlers, 1987) and AN1460 (pAN45/*unc413*::Mu *argH pyrE entA nalA recA*) (Downie et al., 1980) were gifts from Graeme B. Cox (The Australian National University).

The plasmid pBR322 and M13mp18 were obtained from New England Biolabs, and pBluescript II SK$^+$ and KS$^+$ were from Stratagene. pAN45, containing the *unc* operon in the plasmid pACYC184 [*uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$ (Cm^r)] (Downie et al., 1980) was obtained from the *E. coli* strain AN1460.

N-Ethyl[2,3-^{14}C]maleimide ([^{14}C]NEM, 7 mCi/mmol) was from Amersham Corp. Enzymes and reagents for routine subcloning and sequencing procedures were obtained from Boehringer Mannheim, Bethesda Research Laboratories, New England Biolabs, and U.S. Biochemicals.

Synthesis of Cysteine-Specific Photo-Cross-Linkers: (*A*) *N-(4-Azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide* (**4**, *TFPAM-3*). A solution of 23.1 mg (0.0868 mmol) of *N*-succinimidyl 3-maleimidopropionate (Sigma), 25.2 mg (0.0982 mmol) of 4-azido-2,3,5,6-tetrafluorobenzylamine hydrochloride [mp 185–186 °C with decomposition, prepared from compound **14** in Keana and Cai (1990)] and 18.1 mg (0.179 mmol) of triethylamine in $CHCl_3$ (2 mL) was stirred at 25 °C for 16 h. The solution was applied to a preparative TLC plate (20 × 20 cm) and developed (1:1 THF–hexane) to give 32.1 mg of **4** (R_f = 0.45) as a white solid. The solid was dissolved in acetone (1 mL) and the solution was added dropwise into hexane (10 mL) to give a precipitate, which was centrifuged and dried to leave 26 mg (80%) of **4** as a white solid, mp 130–131 °C. ^{1}H NMR: 2.536 (t, J = 6.9 Hz, 2 H), 3.823 (t, J = 6.9 Hz, 2 H), 4.504 (d, J = 5.7 Hz, 2 H), 5.946 (m, 1 H), 6.693 (s, 2 H). IR: 2122, 1713, 1684, 1602, 1496, and 1237 cm^{-1}. MS: 343 (30, $M^+ - N_2$), 219 (68), 152 (38), 110 (100). High-resolution MS calculated for $C_{14}H_9F_4N_5O_3 - N_2$, 343.0579; found, 343.0567.

(*B*) *N-(4-Azido-2,3,5,6-tetrafluorobenzyl)-6-maleimidohexanamide* (*5*, *TFPAM-6*). Amide **5** was prepared in a manner similar to **4**. From 30.7 mg (0.100 mmol) of *N*-succinimidyl 6-maleimidohexanoate (Sigma) and 22.2 mg (0.100 mmol) of 4-azido-2,3,5,6-tetrafluorobenzylamine **1** (free base) was obtained 24 mg (58%) of **5** as a white solid, mp 119–120 °C. ^{1}H NMR: 1.251 (m, 2 H), 1.618 (m, 4 H), 2.164 (t, J = 7.4 Hz, 2 H), 3.495 (t, J = 7.1 Hz, 2 H), 4.517 (d, J = 5.8 Hz, 2 H), 5.815 (br s, 1 H), 6.681 (s, 2 H). IR: 2122, 1708, 1653, 1494, 1380, and 1238 cm^{-1}. MS: 385 (100, $M^+ - N_2$), 262 (15), 219 (20), 162 (4), 110 (40). High-resolution MS calculated for $C_{17}H_{15}F_4N_5O_3 - N_2$, 385.1049; found, 385.1058.

Construction of Plasmids Containing Mutated uncC Genes. The *uncC*-containing 0.73-kb *Pst*I/*Dra*I fragment of pAN45 was inserted in the *Pst*I and *Sma*I sites of the bacteriophage ds-M13mp18, and site-directed mutagenesis was carried out according to Kunkel et al. (1987) in CJ236 and XL1-Blue by the use of oligonucleotides GACGTCGTCTGCGCAGAG for S10C and AGCAGCTGTCACGGCGAC for S108C, respectively. The successful introduction of the mutations in the *uncC* gene was shown by restriction enzyme cleavage since the new restriction sites *Fsp*I and *Pvu*II were obtained in the case of S10C and S108C, respectively.

The mutations were then introduced in a modified pAN45 vector in three steps: (i) The 2.8-kb *Nde*I/*Eag*I fragment of pAN45 was placed into pBluescript II KS$^+$ after introducing an *Nde*I site in that plasmid by inserting the 0.6-kb *Hin*dIII/*Cla*I fragment of M13mp18. The 589-bp *Aat*II/*Pst*I fragment from M13mp18, containing the *uncC* mutations, was inserted in this construct. (ii) The *Eag*I/*Nde*I fragment was then inserted in a pBluescript II SK$^+$ that had been modified with the 0.6-kb *Nde*I-site containing *Cla*I/*Sal*I fragment of M13mp18 and contained the 6.1-kb *Nde*I/*Xho*I fragment of pAN45. (iii) The 5.8-kb *Nsi*I/*Xho*I fragment of (ii) was inserted in the 6.8-kb fragment obtained from pAN45 by *Nsi*I and *Xho*I cleavage. The resulting plasmids were 12.6 kb long and contained all genes encoding the ECF_1F_0 ATPase.

These plasmids, pRA100, pRA101, and pRA102, containing no mutation, ϵ-S10C, and ϵ-S108C mutations, respectively, were used to transform AN888, which is deficient in all but the a-subunit of ATPase, producing pRA100/AN888 (isogenic wild type) and pRA101/AN888 and pRA102/AN888 (ϵ-S10C and ϵ-S108C mutants).

Labeling and Cross-Linking of ECF_1 ATPase. *E. coli* F_1 ATPase was isolated from AN1460 and from pRA100/AN888, pRA101/AN888, and pRA102/AN888 by a modification of the method of Wise et al. (1981) described in Gogol et al. (1989a). For labeling experiments with [^{14}C]NEM and cross-linking experiments with TFPAM's, the ATPase was passed through two consecutive centrifuge columns (Penefsky, 1977) (Sephadex G50, fine, 0.5 × 5.5 cm) equilibrated with 50 mM MOPS buffer, pH 7.0, containing 0.5 mM EDTA and 10% glycerol to remove thiol-containing and primary amino group containing buffer components and to establish a defined nucleotide condition of the ATPase (Mendel-Hartvig & Capaldi, 1991a).

The ATPase was labeled at a concentration of 0.6–1.0 mg/mL with 400 μM [^{14}C]NEM (8 mM stock solution in pentane) and 200 μM TFPAM (20 mM stock solution in DMSO), respectively, for 1 h at room temperature. Excessive label was quenched with 15 mM cysteine or removed by ammonium sulfate precipitation (70%) followed by one passage through a centrifuge column. In the case of the [^{14}C]*N*-ethylmaleimide, the ATPase was electrophoresed on a NaDodSO$_4$–polyacrylamide gel, and the radioactivity in the individual subunits was determined as described in Aggeler et al. (1987). In the case of the cross-linkers, photolysis was carried out after removal of excess reagent and addition of 5 mM ATP either with or without 5.5 mM $MgCl_2$ for 2 h at room temperature in the tip of an Eppendorf tube (1.5 mL) at a distance of 2 cm from a 6-W 366-nm UV lamp (UVP, Inc., Model UVL-56, Blak-Ray lamp). The ATPase activity was measured with an ATP regenerating system, according to Lotscher et al. (1984). The cross-linked products were analyzed with the help of monoclonal antibodies (Aggeler et al., 1990) after the polypeptides were blotted from NaDodSO$_4$–polyacrylamide gels onto nitrocellulose membranes (Mendel-Hartvig & Capaldi, 1991a).

Other Methods. The isolation of plasmid DNA from *E. coli*, ligation, transformation of *E. coli*, restriction enzyme digests and other routine procedures in subcloning were carried out according to standard protocols (Davis et al., 1986; Maniatis et al., 1982). Protein concentrations were determined ac-

Table I: Effect of Modification of Cys Residues on ATPase Activity[a]

	no treatments	maleimide reaction, no photolysis[b]
AN1460	12.4 ± 1.2 (8)	12.0 ± 2.0 (12)
ϵ-S10C	31.8 ± 2.9 (9)	53.7 ± 6.1 (11)
ϵ-S108C	17.9 ± 1.4 (6)	20.6 ± 3.5 (14)

[a] ATPase activities of ECF_1 were measured with an ATP-regenerating system and are expressed as micromoles of ATP hydrolyzed per minute per milligram of enzyme. [b] The effect of the cysteine modification by maleimides was measured after 1 h of incubation at room temperature with 200 μM TFPAM-3 or TFPAM-6.

cording to Lowry et al. (1951) as modified by Markwell et al. (1978).

Results

ATPase Activity of ϵ Mutants. Mutants in the ϵ subunit were constructed by site-directed mutagenesis of the *uncC* gene in M13mp18, which was then introduced into a modified pAN45 plasmid for subsequent transformation of the *E. coli* strain AN888. Two sites were chosen for mutation, Ser_{10} in the N-terminal part and Ser_{108} close to the C-terminus of the polypeptide.

The modified pAN45 plasmid contained the entire *unc* operon (Downie et al., 1980) minus the *uncI* gene, along with the entire pACYC184 sequence. The use of *NsiI* in the last cloning step leads to loss of a 7-kb DNA fragment 3′ of the *unc* operon, and the resulting vector is therefore 12.5 kb long instead of the 19.5 kb of the original pAN45.

Three plasmids were constructed, pRA100, pRA101, and pRA102, and these were used to transform AN888 to give the isogenic strain and the mutants ϵ-S10C and ϵ-S108C, respectively. All three strains grew on the nonfermentable carbon source succinate at the same rates as AN1460. Thus, neither the introduction of the mutations nor removal of DNA 3′ of the *unc* operon altered bacterial respiratory function.

The AN888 derivatives are overproducing strains and ECF_1 could be isolated in yields of around 30 mg from 10-L cultures of each of the transformants.

ECF_1 obtained from the isogenic strain had identical ATPase activity to enzyme isolated from AN1460. The exchange of a Cys for a Ser at position 108 of the ϵ subunit had a minimal effect on activity (Table I). However, conversion of Ser_{10} to Cys caused an increase in basal activity from 12 to 32 μmol of ATP hydrolyzed min^{-1} mg^{-1}.

Sulfhydryl Labeling by NEM. Preparations were reacted with [^{14}C]NEM to determine both the exposure to solvent of the Cys residues introduced into the ϵ subunit and the stoichiometry of mutated ϵ in the enzyme complex. The α, β, γ, and δ subunits contain 4, 1, 2, and 2 Cys residues, respectively, on the basis of sequence data (Walker et al., 1984; Stan-Lotter et al., 1986). The ϵ subunit of wild-type enzyme contains no Cys residues. Figure 1A shows the labeling of different preparations with [^{14}C]NEM under native conditions. As we have shown previously, ECF_1 from the wild-type strain AN1460 is labeled by [^{14}C]NEM in the α and δ subunits with incorporation of reagent in the δ subunit approaching 1 mol/mol of subunit and reactivity of the α subunit ranging from 0.5 to 1.0 mol/mol of enzyme (Mendel-Hartvig & Capaldi, 1991c). The site of labeling of the δ subunit was identified tentatively as Cys_{140} from peptide mapping experiments (Mendel-Hartvig & Capaldi, 1991c). More recently we have conducted site-directed mutagenesis experiments in which Cys_{140} was replaced by Ser and in this mutant there was no reaction of the δ subunit with maleimides (R. Aggeler and R. A. Capaldi, unpublished results), indicating that the second Cys in this subunit, Cys_{64}, is buried in the native structure.

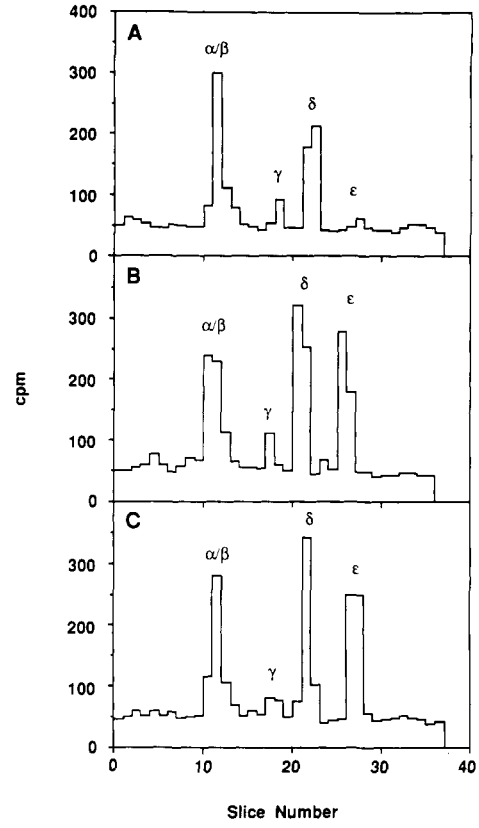

FIGURE 1: *N*-Ethylmaleimide labeling of mutant F_1 ATPases. ECF_1 was precipitated with 70% ammonium sulfate, dissolved in 50 mM MOPS, pH 6.8, 10% glycerol, 2 mM $MgCl_2$, and 20 μM DTT (0.6 mg/mL), and labeled with 400 μM [^{14}C]NEM at room temperature for 1 h. The reaction was quenched with 15 mM cysteine, and samples were electrophoresed on a $NaDodSO_4$-containing 10–18% polyacrylamide gel. After staining of the gel with Coomassie brilliant blue, the radioactivity in 3-mm slices was determined in a liquid scintillation counter. (A) 55 pmol of pRA100/AN888 ATPase (isogenic wild type); (B) 84 pmol of pRA101/AN888 ATPase (ϵ-S10C mutant); (C) 71 pmol of pRA102/AN888 ATPase (ϵ-S108C mutant).

The labeling of the α subunit by [^{14}C]NEM could represent labeling of a Cys on one of the three α subunits, on the basis of the reported asymmetry of the α subunits in the F_1 complex (Nalin et al., 1985), or it could occur by reaction of the maleimide with a small amount of denatured enzyme, leading to modification of many or all of the 4 Cys/α subunit of 12 Cys on this subunit/mol of ECF_1.

Panels B and C of Figure 1 show the labeling of the ϵ-S10C and ϵ-S108C mutants of ECF_1, respectively. The labeling of α and δ subunits in these mutants was similar to that of the wild-type strain. In both mutants there was labeling of the ϵ subunit through the introduced Cys residues at stoichiometries of 0.8 for ϵ-S10C and 1.0 for ϵ-S108C of the labeling of the exposed Cys_{140} in the δ subunit. These results show that the introduced Cys residues are in both cases on the surface of the enzyme. Moreover, it appears that there is very little loss of mutant ϵ subunit from the complex during isolation of the enzyme.

Cross-Linking Studies. Cys residues introduced into selected locations in the complex allow site-specific cross-linking to be conducted when a bifunctional reagent containing a maleimide or other sulfhydryl-reacting group is used. In prototype experiments the two ϵ mutants were reacted with the reagents shown in Figure 2, which contain a maleimide and a tetrafluorophenyl azide as reactive groups, separated by different lengths of linkers. In a typical experiment, enzyme

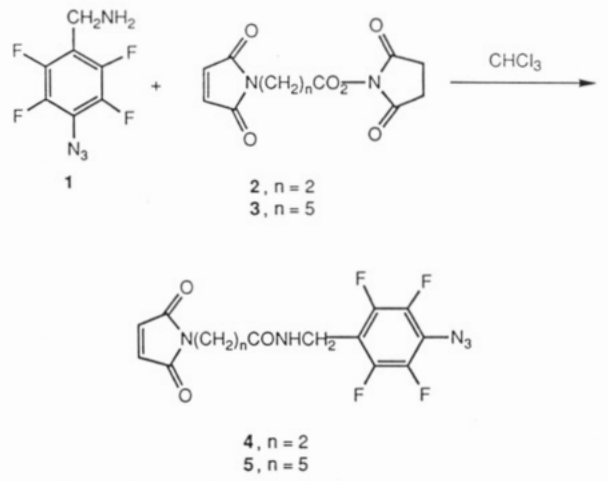

FIGURE 2: Synthesis and structures of the heterobifunctional photo-cross-linkers: $n = 2$, TFPAM-3; $n = 5$, TFPAM-6.

was reacted with one of these reagents in the dark under conditions favoring reaction of Cys residues with the maleimide, excess reagent was removed, and the samples were irradiated with UV light to activate the phenyl azide to a nitrene for covalent insertion into a nearby group. Samples at various stages in the reaction procedure were examined for activity and for cross-linked products using $NaDodSO_4$–polyacrylamide gel electrophoresis. Table I lists the effect of maleimide modification on the activity of wild-type and mutant enzyme.

Modification of the wild type and ϵ-S108C mutant with NEM or with cross-linker via the maleimide group did not alter activity appreciably. Reaction of the ϵ-S10C mutant with the reagent led to an activation, but the activity obtained (52 μmol of ATP hydrolyzed min^{-1} mg^{-1}) is considerably less than expected if the ϵ subunit had been released from the enzyme [cf. 93 units/mg for ϵ-free enzyme; see Aggeler et al. (1990)].

Photolysis of the labeled ECF_1 led to cross-linked products in all samples, which were not evident in the unphotolyzed samples. Figure 3 shows an experiment in which TFPAM-3 was used. The use of TFPAM-6 led to equivalent results (not shown). Two new bands, running above the α subunit, were resolved in wild-type enzyme that were also seen in the mutants. These products were found to be covalent adducts of α–δ and β–δ, respectively, by Western analysis, using monoclonal antibodies to each of the subunits (Figure 4). They occur presumably by reaction of the maleimide with Cys_{140} in δ and then nitrene insertion into one or the other of the two large subunits. The yield of these cross-linked products was low and independent of the nucleotide conditions during the photolysis step.

Figure 3 also shows cross-linking of both the ϵ-S10C and ϵ-S108C mutants in ATP + EDTA and ATP + Mg^{2+}, respectively. There is one cross-linked product visible in the ϵ-S10C mutant not found in wild type, which Western analysis established involved γ and ϵ (Figure 4). One new band was also seen in the ϵ-S108C mutant, identified as a cross-linked product between α and ϵ.

Attempts were made to quantitate the yield of cross-linked products using densitometry based on the disappearance of free subunits from Coomassie brilliant blue stained gels. By this approach the yield of γ–ϵ was estimated at 48% (55% loss of ϵ; 42% loss of γ) in ATP + EDTA and 22% in ATP + Mg^{2+} (21% loss of ϵ; 23% loss of γ) in the experiment in Figure 3. The yield of α–ϵ could only be estimated from the disappearance of ϵ, with values of 31% calculated for cross-linking in ATP + Mg^{2+} and 10% in ATP + EDTA in the experiment

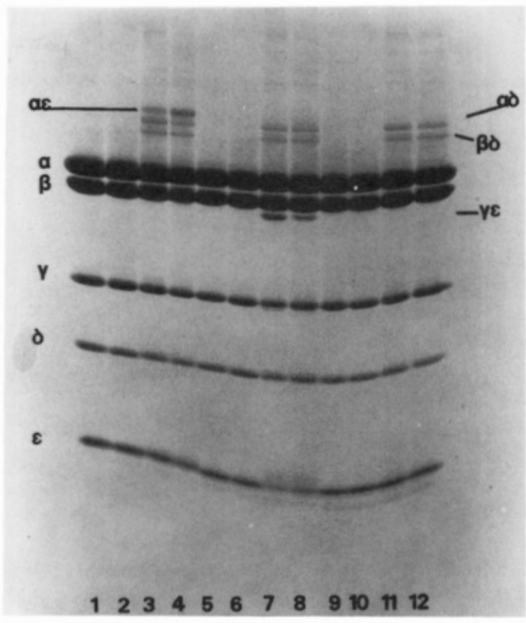

FIGURE 3: Nucleotide dependence of photoactivated cross-linking of mutant F_1 ATPases. ECF_1 (500 μg) was labeled in 500 μL of buffer (50 mM MOPS, pH 7.0, 10% glycerol, and 0.5 mM EDTA) by addition of 5 μL of 20 mM TFPAM-3 (in DMSO) and incubation for 1 h at room temperature in the dark. The enzyme was precipitated with 70% ammonium sulfate, dissolved in 100 μL of buffer, and passed through a centrifuge column. After addition of 5 mM ATP (lanes 1, 3, 5, 7, 9, and 11) or 5 mM ATP and 5.5 mM $MgCl_2$ (lanes 2, 4, 6, 8, 10, and 12), samples were either kept in the dark (lanes 1, 2, 5, 6, 9, and 10) or photolyzed for 2 h at 366 nm (lanes 3, 4, 7, 8, 11, and 12). Fifty-microgram quantities of ϵ-S108C (lanes 1–4), ϵ-S10C (lanes 5–8), and AN1460 (lanes 9–12) ATPases were electrophoresed on an $NaDodSO_4$-containing 10–18% polyacrylamide gel that was then stained with Coomassie brilliant blue.

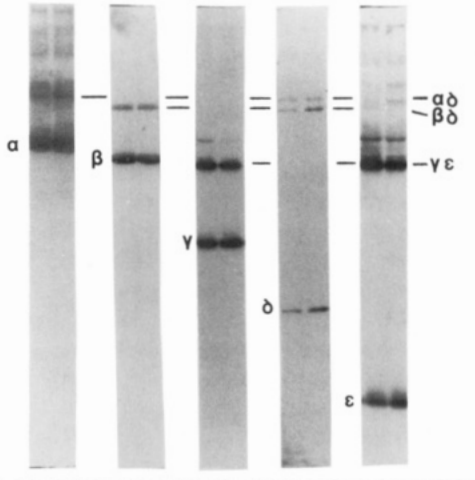

FIGURE 4: Immunoblot analysis of cross-linked products of the ϵ-S10C mutant. Samples containing 25 μg of F_1 ATPase from pRA101/AN888 (ϵ-S10C), cross-linked as described for Figure 3, were electrophoresed on an $NaDodSO_4$-containing 10–18% polyacrylamide gel. The polypeptides were transferred onto a nitrocellulose membrane and characterized by the use of monoclonal antibodies against α (A), β (B), γ (C), δ (D), and ϵ (E). The two lanes show duplicate experiments.

shown in Figure 3. The general features of cross-linking, with a high yield of γ–ϵ and low yield of α–ϵ in ATP + EDTA but a low yield of γ–ϵ and high yield of α–ϵ in ATP + Mg^{2+}, were reproducible in many different experiments, using either the short or the long cross-linker.

The effect of cross-linking on the activity of the enzyme was measured in various experiments. Photolysis of AN1460 under conditions used for cross-linking had no effect on activity if the enzyme had not been modified with the cross-linker.

Photolysis of wild-type enzyme that had been reacted with TFPAM-3, with resulting cross-linking of δ to α and β subunits, gave an activity of 11.3 ± 1.0 (five determinations); cf. 12.0 ± 2.0 for nonphotolyzed enzyme. Covalent cross-linking of ϵ to γ in the ϵ-S10C mutant in the presence of ATP + EDTA, when the yield of cross-linked product was highest (i.e., around 50%), caused at most 16% inhibition of ATPase activity. Only with the ϵ-S108C mutant was there a quantitative relationship between the extent of inhibition of activity and yield of cross-linked product. Thus, when the photolysis reaction was carried out in ATP + Mg^{2+} in the experiment of Figure 3, the 31% yield of cross-linked product was associated with a 30% loss of ATPase activity. In two other experiments conducted in ATP + Mg^{2+}, the ratios of loss of activity to yield of cross-linked product were 0.7 and 0.9, respectively.

Discussion

The present work begins a series of experiments in which the ATP synthase of *E. coli* is being mutated at selected sites on various subunits by converting Ser into Cys residues, an exchange of an S for O in the side chain. These introduced Cys residues are then being used as sites for enzyme modification with cross-linking reagents, heavy-atom derivatives (for cryoelectron microscopy), and fluorescence reagents (for time-resolved analysis of conformational changes).

In the present study we have focused on the ϵ subunit and introduced Cys at Ser_{10} and Ser_{108} near the N- and C-termini of the polypeptide, respectively. Mutants were engineered in an overproducing strain from which large amounts of ECF_1 could be isolated for study.

There are a total of 19 Cys in ECF_1: four per α subunit, one per β, two per γ, and two per δ. Of these, no more than one Cys of the 12 in α subunits is reactive with maleimides along with one Cys on δ. Novel bifunctional reagents containing maleimide bridged by different length linkers to a photoactivatable tetrafluorophenyl azide moiety were used for cross-linking. Perfluorophenyl azides have been introduced recently as an advance over nitrophenyl azides for photolabeling (Keana & Cai, 1990). The presence of fluorine atoms instead of protons in the benzene ring prevents rearrangement reactions and thereby increases the effectiveness of insertions by the photogenerated nitrene into C–H as well as into N–H bonds (Keana & Cai, 1990). The TFPAM's, described here for the first time, offer an opportunity of probing near-neighbor subunits to defined Cys residues and, in conjunction with peptide mapping and sequencing, can give precise data on topology of protein complexes.

The ϵ subunit of ECF_1 is well characterized as an inhibitor of ATPase activity (Sternweis & Smith, 1980). The inhibitory effect of the subunit has been found to result from a decreased off rate of product P_i (Dunn et al., 1987). An increased residency of P_i in the catalytic site(s) when the ϵ subunit is bound in the core ECF_1 complex is also indicated by ^{18}O exchange experiments (Wood et al., 1987). Our recent studies indicate that the ϵ subunit is sensitive to ligand binding in the catalytic site. We have found that the ϵ subunit is rapidly cleaved by trypsin in ATP + EDTA but only slowly cleaved by the protease in ATP + Mg^{2+} (Mendel-Hartvig & Capaldi, 1991a). Similar results have been presented by Bragg and Hou (1987). Additional experiments showed that the conformational change and/or change in environment of the ϵ subunit was a function of P_i binding (Mendel-Hartvig & Capaldi, 1991a), indicating a reciprocal relationship between the structure of the ϵ subunit and P_i binding.

We further showed that cross-linking of the ϵ subunit to a β subunit induced by EDC is sensitive to ligands in the catalytic site. Conditions which cause slow proteolysis of ϵ, e.g., ATP + Mg^{2+}, gave cross-linking in high yield between β and ϵ, while conditions in which cleavage of the ϵ subunit is fast (ATP + EDTA) gave a low yield of this cross-linked product (Mendel-Hartvig & Capaldi, 1991a).

The conformation or accessibility of the ϵ subunit not only is sensitive to ligand binding in catalytic sites but also is modulated by DCCD binding in the F_O, on the basis of experiments in *E. coli* (Mendel-Hartvig & Capaldi, 1991b), and by light-driven proton translocation, on the basis of experiments with CF_1F_O (Richter & McCarty, 1987). Taken together, the above data lead us to believe that conformational changes in the ϵ subunit are an integral part of coupling catalytic-site events with proton translocation in the ATP synthase.

Both the ϵ-S10C and ϵ-S108C mutant ϵ subunits remain bound to the core ECF_1 complex throughout isolation. ϵ subunit in which Ser_{108} was converted to Cys inhibited ATPase activity to nearly the same extent as wild-type ϵ subunit. ϵ subunit in which Ser_{10} was converted to a Cys also inhibited ATPase activity but to a lesser extent than unmodified ϵ subunit, indicating some disruption of the binding of the subunit to the core complex. Reaction with the bulky cross-linking reagent further reduced the inhibition, indicating that the effect of the modification is a steric one and Ser_{10} is in or very near the binding domain.

Photolysis of the wild-type ECF_1 after reaction with the cross-linking reagent generated two intersubunit cross-linked products, identified as δ–α and δ–β. A close association of δ and α in ECF_1 has been suggested before, on the basis of disulfide bond formation between these two subunits under oxidizing conditions such as in the presence of $CuCl_2$ (Bragg & Hou, 1986b; Tozer & Dunn, 1986), and from studies in which the N-terminus of the α subunit was cleaved with the concomitant loss of binding of the δ subunit (Dunn et al., 1980). Interaction of the δ with a β subunit and, by inference, a location at an α–β interface has not been reported in previous cross-linking. However, a δ-subunit-specific mAb was seen to superimpose on a β subunit in projection images of the hexagonal view of ECF_1 obtained in cryoelectron microscopy studies (Gogol et al., 1989b).

The α–δ and β–δ cross-linked products were observed in experiments with wild-type and ϵ-mutant enzyme. The yield of these products was independent of the nucleotide conditions used during the photolysis reaction. ϵ subunit containing a Cys at residue 10 was found to cross-link selectively to the γ subunit, while ϵ containing a Cys at 108 cross-linked specifically to the α subunit. Interaction between the ϵ and γ has been reported before on the basis of cross-linking (Aris & Simoni, 1983; Bragg & Hou, 1986), as well as direct binding between the purified subunits (Dunn, 1982).

A linkage between α and ϵ has not been documented previously. As described already, the ϵ subunit can be cross-linked to a β subunit in high yield by the water-soluble reagent EDC (Lotscher et al., 1984). Linkage of ϵ to both α and β indicates that the small subunit must bind at the interface between the two large subunits. Linkage of ϵ to α, β, and γ can only occur at one end of the ECF_1 structure and is consistent with a model in which the large subunits are interdigitated in their hexagonal arrangement (Gogol et al., 1989a) but is difficult to reconcile in a model in which the α and β subunits are in two tiers (Amzel et al., 1982; Tiedge et al., 1983; Tsuprun et al., 1984).

An intriguing aspect of the cross-linking data is the ligand dependence of the yields of γ–ϵ and α–ϵ in the Cys_{10} and Cys_{108} mutants. In ATP + Mg^{2+}, the yield of α–ϵ is high but that of γ–ϵ is low. In ATP + EDTA, the yield of α–ϵ is low and

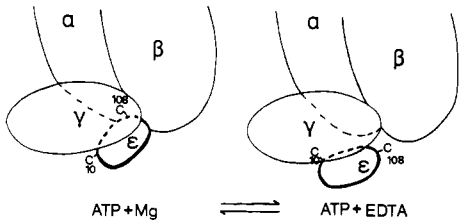

FIGURE 5: Model of the nucleotide-dependent interaction of ε with the γ and the α subunit.

that of γ–ε is high. Thus the cross-linking results with the tetrafluorophenyl azide–maleimides support and extend both proteolysis and EDC cross-linking studies (Mendel-Hartvig & Capaldi, 1991a,b) in establishing that the ε subunit undergoes ligand-dependent conformational and/or binding changes. The cross-linking studies presented here indicate that the ε subunit is altered in its binding, so that it interacts more tightly with α (as well as β from EDC experiments) when ATP + Mg^{2+}, or more specifically ADP + Mg^{2+} + P_i, are present in catalytic sites. This interaction appears to be weakened in ATP + EDTA, with the ε subunit shifting more toward the γ subunit as shown schematically in Figure 5. We suggest that such ligand-dependent changes in the conformation and/or binding of ε not only control the rate of ATP hydrolysis but also are important in linking catalytic events to the proton channel in F_O. The binding of ε to α and β subunits could alternate with each α, β subunit pair being involved sequentially (Boyer, 1987; Gogol et al., 1990). If such a rotation of the ε + γ subunits were a part of the enzyme mechanism, covalent linkage of ε to α (or β) should inhibit activity, as is the case in the study reported here.

References

Aggeler, R., Zhang, Y.-Z., & Capaldi, R. A. (1987) *Biochemistry 26*, 7107–7113.

Aggeler, R., Mendel-Hartvig, J., & Capaldi, R. A. (1990) *Biochemistry 29*, 10387–10393.

Amzel, L. M., McKinney, M., Narayanan, P., & Pederson, P. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5852–5856.

Aris, J. P., & Simoni, R. D. (1983) *J. Biol. Chem. 258*, 14599–14609.

Boyer, P. D. (1987) *Biochemistry 26*, 8503–8507.

Bragg, P. D., & Hou, C. (1986a) *Arch. Biochem. Biophys. 244*, 361–372.

Bragg, P. D., & Hou, C. (1986b) *Biochim. Biophys. Acta 851*, 385–394.

Bragg, P. D., & Hou, C. (1987) *Biochim. Biophys. Acta 894*, 127–137.

Bullock, W. O., Fernandez, J. M., & Short, J. M. (1987) *BioTechniques 5*, 376–379.

Cross, R. L., & Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874–2881.

Davis, L. G., Dibner, M. D., & Battey, J. F. (1986) *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., Inc., New York.

Downie, J. A., Langman, L., Cox, G. B., Yanofsky, C., & Gibson, F. (1980) *J. Bacteriol. 143*, 8–17.

Dunn, S. D. (1982) *J. Biol. Chem. 257*, 7354–7359.

Dunn, S. D., Heppel, L. A., & Fullmer, C. S. (1980) *J. Biol. Chem. 255*, 6891–6896.

Dunn, S. D., Zadorozny, V. D., Tozer, R. G., & Orr, L. E. (1987) *Biochemistry 26*, 4488–4493.

Futai, M., Noumi, T., & Maeda, M. (1989) *Annu. Rev. Biochem. 58*, 111–136.

Gogol, E. P., Lucken, U., Bork. T., & Capaldi, R. A. (1989a) *Biochemistry 28*, 4709–4716.

Gogol, E. P., Aggeler, R., Sagermann, M., & Capaldi, R. A. (1989b) *Biochemistry 28*, 4717–4724.

Gogol, E. P., Johnston, E., Aggeler, R., & Capaldi, R. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9585–9589.

Ida, K., Noumi, T., Maeda, M., Fukui, T., & Futai, M. (1991) *J. Biol. Chem. 266*, 5424–5429.

Keana, J. F. W., & Cai, S. X. (1990) *J. Org. Chem. 55*, 3640–3647.

Kironde, F. A. S., & Cross, R. L. (1987) *J. Biol. Chem. 262*, 3488–3495.

Kunkel, T. A., Roberts, J. D., & Zakour, M. A. (1987) *Methods Enzymol. 154*, 367–382.

Lightowlers, R. (1987) Ph.D. Thesis, Australian National University.

Lotscher, H. R., deJong, C., & Capaldi, R. A. (1984) *Biochemistry 23*, 4134–4140.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206–210.

Mendel-Hartvig, J., & Capaldi, R. A. (1991a) *Biochemistry 30*, 1278–1284.

Mendel-Hartvig, J., & Capaldi, R. A. (1991b) *Biochemistry 30*, 10987–10991.

Mendel-Hartvig, J., & Capaldi, R. A. (1991c) *Biochim. Biophys. Acta 1060*, 115–124.

Nalin, C. M., Snyder, B., & McCarty, R. E. (1985) *Biochemistry 24*, 2318–2324.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Rao, R., Cunningham, D., Cross, R. L., & Senior, A. E. (1982) *J. Biol. Chem. 263*, 5640–5645.

Richter, M. L., & McCarty, R. E. (1987) *J. Biol. Chem. 262*, 15037–15040.

Senior, A. E. (1988) *Physiol. Rev. 68*, 177–231.

Stan-Lotter, H., Clarke, D. M., & Bragg, P. D. (1986) *FEBS Lett. 197*, 121–124.

Sternweis, P. C. (1978) *J. Biol. Chem. 253*, 3123–3128.

Sternweis, P. C., & Smith, J. B. (1977) *Biochemistry 16*, 4020–4025.

Sternweis, P. C., & Smith, J. B. (1980) *Biochemistry 19*, 526–531.

Tiedge, H., Schafer, G., & Mayer, F. (1983) *Eur. J. Biochem. 132*, 37–45.

Tozer, R. G., & Dunn, S. D. (1986) *Eur. J. Biochem. 161*, 513–518.

Tsuprun, V. L., Mesyanzhinova, I. V., Kozlov, I. A., & Orlova, E. V. (1984) *FEBS Lett. 167*, 285–290.

Vogel, P. D., & Cross, R. L. (1991) *J. Biol. Chem. 266*, 6101–6105.

Walker, J. E., Saraste, M., & Gay, N. J. (1984) *Biochim. Biophys. Acta 768*, 164–200.

Wise, J. G., Latchney, L. R., & Senior, A. E. (1981) *J. Biol. Chem. 256*, 10383–10389.

Wood, J. M., Wise, J. G., Senior, A. E., Futai, M., & Boyer, P. D. (1987) *J. Biol. Chem. 262*, 2180–2186.