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Inhibition of the Adenosinetriphosphatase Activity of *Escherichia coli* F_1 by the Water-Soluble Carbodiimide 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide Is Due to Modification of Several Carboxyls in the β Subunit†

Hans-Ruedi Lötscher, Catherina deJong, and Roderick A. Capaldi*

ABSTRACT: Reaction of the ATPase of *Escherichia coli* (ECF_1) with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) resulted in a time- and concentration-dependent inhibition of ATPase activity. The inactivation was greatly reduced by Mg^{2+} ions. Close to 13 mol of EDC per mol of ECF_1 was incorporated into the enzyme at 95% inhibition of ATPase activity. Two-thirds of the label was found to be associated with subunit β with a stoichiometry of about 3 mol of EDC per mol of β. Cleavage of EDC-modified subunit β with cyanogen bromide and fractionation of the peptides by high-pressure liquid chromatography revealed a short segment of 33 amino acids (CB8, residues 162–194) containing 3 mol of EDC per mol of peptide. In tryptic peptide maps, two EDC-labeled fragments could be identified (T18, residues 166–183, and T20, residues 186–202). The analyses were complicated by significant internal cross-linking within the β subunit induced by EDC. The results show that EDC modifies multiple sites in a short segment of subunit β which includes the glutamic acids modified by dicyclohexylcarbodiimide in F_1 from both *E. coli* and PS3. In addition to covalent modification, EDC also promoted the formation of intersubunit cross-links. The predominant cross-linked product was identified as a β–ϵ complex by antibody binding experiments.

The ATP synthase of plants, animals, and bacteria is similarly a multisubunit enzyme catalyzing the reversible reaction ATP $\rightleftarrows$ ADP + P_i. In all cases, the enzyme is composed of a part extrinsic to the bilayer (F_1) made up of 5 different subunits and an intrinsic transmembrane section (F_0) of 3–13 different polypeptides depending on the source of the enzyme [reviewed recently by Fillingame (1980), Futai & Kanazawa (1983), and Senior & Wise (1983)].

The F_1 portion is readily purified in a water-soluble form which catalyzes ATPase but not ATP synthase activity. The presence of the F_0 portion is needed for coupled function, probably by the F_0 acting as a reversible proton channel through the membrane (Fillingame, 1980; Futai & Kanazawa, 1983). The sequences of the five subunits of ECF_1[1] have been obtained by sequencing the unc operon [e.g., see Gay & Walker (1981a,b) and Kanazawa et al. (1981, 1982)]. There is a high degree of homology in the sequences of the α and β subunits from ECF_1, beef heart, and maize chloroplast F_1 (Walker et al., 1982a,b; Futai & Kanazawa, 1983). The δ subunit of ECF_1 appears to be homologous with the so-called "oligomycin-sensitivity" conferring protein of beef heart F_1 with no equivalent to the δ subunit of beef heart being present in ECF_1 (Walker et al., 1982a,b). Another apparent difference between animal and bacterial F_1 is the presence of a small inhibitor protein in the former (Pullman & Monroy, 1963). It has been suggested that the ϵ subunit of ECF_1 is equivalent to this inhibitor protein (Sternweis & Smith, 1980).

Several recent studies have established the importance of carboxyl groups in F_1 ATPase activity. It has been shown that DCCD, a hydrophobic carboxyl-modifying reagent, inhibits the ATPase activity of F_1 from *Escherichia coli* (Satre et al., (1979), PS3 (Yoshida et al., 1981), beef heart (Pougeois et al., 1979), and chloroplast (Shoshan & Selman, 1980), predominantly be modification of one carboxyl per β subunit. The site has been identified in ECF_1 (Yoshida et al., 1982) and in beef heart (Esch et al., 1981) as a highly conserved residue, Glu-193. In PS3, it is another conserved carboxyl, Glu-182 (Yoshida et al., 1981) (numbered for *E. coli* sequences).

More recently it has been found that water-soluble carbodiimides including EDC and *N*-(ethoxycarbonyl)-2-ethoxy-

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[1] Abbreviations: ECF_1–F_0, complete H^+-translocating ATPase complex of *E. coli*; ECF_1, water-soluble, extrinsic ATPase sector of the F_1–F_0 complex of *E. coli*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; [^{14}C]ETC, 1-ethyl-3-[3-[^{14}C](trimethylammonio)propyl]carbodiimide; DCCD, dicyclohexylcarbodiimide; HPLC, high-pressure liquid chromatography; Mops, 3-(*N*-morpholino)propanesulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TPCK, tosylphenylalanine chloromethyl ketone.

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1,2-dihydroquinoline inhibit F_1 ATPase activity by reaction in at least two different sites (Pougeois et al., 1978; Pougeois, 1983). On the basis of competition experiments, it has been claimed that these sites of reaction of water-soluble carbodiimides are different from the residues altered by DCCD.

The inhibitory effect of DCCD has been found to be less in the presence of the nucleotides ADP and ATP and dramatically reduced by having Mg^{2+} but not Ca^{2+} in the medium (Satre et al., 1979; Pougeois et al., 1979). This has led to the suggestion that the carboxyl group modified by this reagent is involved in Mg^{2+} binding in the catalytic site (Satre et al., 1979).

We have examined the effect of the water-soluble carbodiimide EDC on the isolated ECF_1 as well as on isolated ECF_1–F_0 and the membrane-bound ATP synthase. Results presented here confirm that EDC inhibits ATPase activity of ECF_1 and show that Mg^{2+} protects the F_1 from this inhibition. Studies have been conducted to locate the sites of modification of EDC. We show that the reaction of water-soluble carbodiimide is much more complex than DCCD and involves modification of several carboxyls as well as both intra- and intersubunit cross-linking.

Experimental Procedures

Materials. [^{14}C]ETC was synthesized according to the procedure of Sheenan et al. (1961) by reacting the free base EDC with $^{14}CH_3I$ (53 mCi/mmol; Research Products International Corp.). The product was diluted with unlabeled ETC to a specific activity of 10 mCi/mmol. Unlabeled ETC was synthesized by the same method. [^{14}C]DCCD (54 mCi/mmol) was obtained from Research Products International. Subunit-specific antibodies were raised in rabbits. Anti-ϵ antibody was kindly provided by Dr. L. A. Heppel, Cornell University, Ithaca, NY.

Source of Cells and Preparation of ECF_1. *E. coli* strain KY 7485 containing a λ-transducing phage which carries all eight ATPase genes of the unc operon was kindly provided by Dr. R. H. Fillingame, University of Wisconsin, Madison, WI. The F_1 complex was purified from this strain after induction of the λ phage (Foster et al., 1980) according to the procedure described by Foster & Fillingame (1979). Protein was determined according to the procedure described by Lowry et al. (1951).

Removal of Subunit δ and Subunit ϵ from ECF_1 by Limited Trypsin Proteolysis. ECF_1 (1.6 mg/mL) was dialyzed overnight against 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 10% sucrose, 10% glycerol, 2 mM EDTA, and 1 mM ATP. TPCK-treated trypsin was added at a weight ratio of 1:1000 (protease/ECF_1) as described by Dunn et al. (1980). After incubation at 25 °C for 2 h, the proteolysis was stopped by addition of phenylmethanesulfonyl fluoride (13 375:1 phenylmethanesulfonyl fluoride:trypsin molar ratio). The sample was then passed over a Sephadex G50 column in 25 mM Mops, pH 7.0, and 100 mM KCl. The specific activity of ECF_1 increased from 30 units/mg to about 95 units/mg during the incubation with trypsin.

Reaction of ECF_1 with EDC and DCCD and Purification of Subunit β. ECF_1 (0.5–2 mg/mL) in 20 mM Mops, pH 7.0, was incubated at room temperature with 4 mM EDC or 50 μM DCCD in the presence or absence of 10 mM $MgCl_2$. At the required times, aliquots were withdrawn and immediately assayed for ATPase activity. Incubations with [^{14}C]ETC and [^{14}C]DCCD were carried out in the same way.

In large-scale labeling experiments, ECF_1 (50 mg, 5–10 mg/mL) was reacted with 2 mM [^{14}C]ETC (3000 cpm/nmol) until 80–90% inactivation was reached. The reaction was then stopped by addition of 0.5 M sodium acetate, and ECF_1 was precipitated by 65% ammonium sulfate. The pellet was dissolved in 10 mM tris(hydroxymethyl)aminomethane, pH 7.2, 100 mM NaCl, 8 M urea, 1 mM dithiothreitol, and 0.02% NaN_3. Subunit β was purified by gel filtration and ion-exchange chromatography as described by Runswick & Walker (1983).

ATPase Activity Assay. The ATPase activity was assayed in 1 mL of a solution containing 25 mM Tris, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 5 mM KCN, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.5 mM NADH, 30 units of L-lactic acid dehydrogenase, and 30 units of pyruvate kinase at 37 °C. The absorbance change at 340 nm was followed in a Beckman DU7 spectrophotometer. The specific activity is expressed as units per milligram; 1 unit corresponds to 1 μmol of ATP hydrolyzed/min.

NaDodSO$_4$ Gel Electrophoresis. ECF_1 was dissolved in 6% $NaDodSO_4$, 10% mercaptoethanol, 20% glycerol, and 0.125 M Tris, pH 6.2, after precipitation with 5 volumes of acetone. One-millimeter-thick slab gels were run as described by Laemmli (1970) with a 5% polyacrylamide stacking gel and a 12–18% polyacrylamide separating gel (linear gradient), both containing 0.2% $NaDodSO_4$. Staining with Coomassie brilliant blue R and destaining were carried out according to Downer et al. (1976). $NaDodSO_4$ gels containing radiolabeled protein were fractionated into 0.8-mm slices on a Mickel gel slicer. The slices were then dissolved in 0.5 mL of 15% H_2O_2 at 60 °C for 10 h, mixed with 3 mL of Omnifluor (New England Nuclear; 2.66 g/L in 2:1 toluene:Triton X-100), and counted in a Beckman LS 7000 scintillation counter.

Fluorescent Antibody Staining. ECF_1 subunits were separated by $NaDodSO_4$ gel electrophoresis and transferred electrophoretically to nitrocellulose paper by using a Bio-Rad transblot apparatus and buffer containing 0.19 M glycine, 0.02 M Tris, 0.1% $NaDodSO_4$, and 20% methanol (Towbin et al., 1979). Electrophoresis was carried out at 20 V for 2 h. The nitrocellulose paper was soaked in 2% poly(vinylpyrrolidine) and 2% Ficoll for 30 min to block nonspecific binding and washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The paper was then incubated with antibody in 5% bovine serum albumin for 3 h at room temperature, washed with phosphate-buffered saline, and incubated for 2 h with fluorescein isothiocyanate labeled goat anti-rabbit antibody. The fluorescent antibody labeled bands were visualized under UV light.

Fractionation of Cyanogen Bromide Fragments and Tryptic Fragments of [^{14}C]ETC-Labeled Subunit β by HPLC. Purified [^{14}C]ETC-labeled subunit β (5–10 mg) was extensively dialyzed against 1 mM sodium phoshate, pH 6.8, and lyophilized. The sample was dissolved in 80% formic acid, and solid cyanogen bromide was added to give a 10-fold excess by weight over protein. After incubation for 16 h at room temperature, the sample was dried under a stream of nitrogen, dissolved in 5 mM sodium phosphate, pH 7.0 (buffer A), and injected into an Altex 334 HPLC with a Brownlee RP-300 column and an Altex 155–10 detector set at 210 nm. The peptides were eluted with 80% 2-propanol (buffer B) as indicated in the figures. The flow rate was 0.8 mL/min. Five percent of each fraction was analyzed for ^{14}C. The amino acid composition of purified peptides was determined after the samples were hydrolyzed in 6 M HCl at 100 °C for 24 in vacuo. In the trypsin cleavage experiments, [^{14}C]ETC-labeled subunit β was adjusted to 100 mM $NaHCO_3$, pH 8.0, 0.1% $NaDodSO_4$, and 1% octyl glucoside. TPCK-treated trypsin (Worthington, Inc.) was then added (20:1 w/w) and the

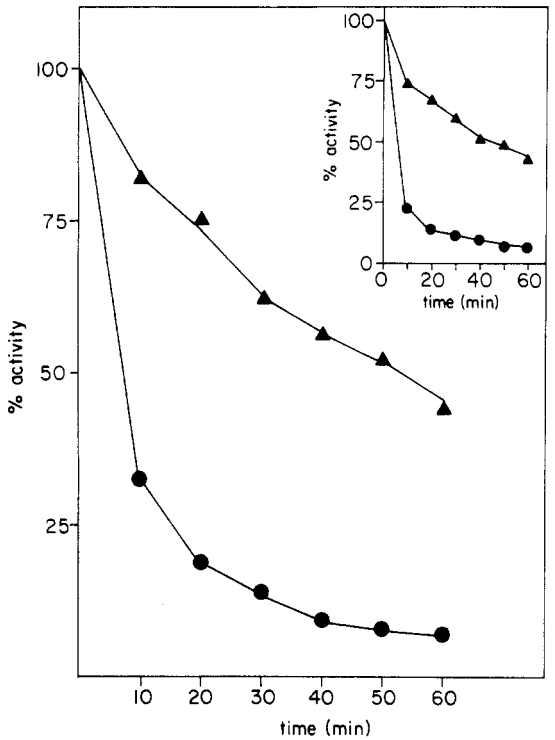

FIGURE 1: Inactivation of ECF_1 by EDC. ECF_1 (0.8 mg/mL) was incubated with 4 mM EDC in the absence (circles) or presence (triangles) of 10 mM $MgCl_2$. At the indicated times, 5-μL aliquots were withdrawn and assayed for ATPase activity. 100% activity corresponds to 25 units/mg. No loss of activity was observed in the absence of EDC. Insert: Inactivation profile obtained with 50 μM DCCD.

mixture incubated overnight at 37 °C. The sample was dried under a stream of nitrogen and dissolved in buffer A. The peptides were separated on HPLC as described above.

Results

EDC Inhibits ECF_1 ATPase Activity in a Mg^{2+} Concentration Dependent Manner. As reported by Satre et al. (1979), EDC inhibits ECF_1 in a time-dependent and concentration-dependent manner. ETC had qualitatively and quantitatively the same effect, and the two very similar carbodiimides are here used interchangeably, ETC being the ^{14}C-labeled compound. Figure 1 shows the effect of 4 mM EDC on the ATPase activity of ECF_1 in the presence and absence of Mg^{2+} (10 mM) in the reaction mixture. The reaction time for half-maximal inhibition in the presence of Mg^{2+} was 50 min in the experiment in Figure 1, compared with 8 min in the absence of Mg^{2+}. This protection from EDC modification afforded by Mg^{2+} was the same as that seen for DCCD modification (insert to Figure 1).

Incorporation of EDC into Different Subunits of ECF_1. EDC and carbodiimides, in general, react predominantly with carboxyl groups and to a lesser extent with tyrosines and sulfhydryls. Modification of carboxyls occurs via an unstable *O*-acylisourea intermediate which can react in three ways (Hoare & Koshland, 1967): it may be hydrolyzed off to regenerate the carboxyl; it may rearrange to a stable *N*-acylisourea (thereby incorporating radioactivity in the protein when [^{14}C]ETC is used, as here); or it may be subjected to nucleophilic attack by a nearby group, e.g., a primary amino group, to generate a cross-link within the protein with release of the radioactivity. ECF_1 that had been reacted to give 95% inhibition of ATPase activity incorporated close to 13 mol of ETC per mol of ECF_1. The distribution of reagent among the different subunits was analyzed by separating polypeptides by

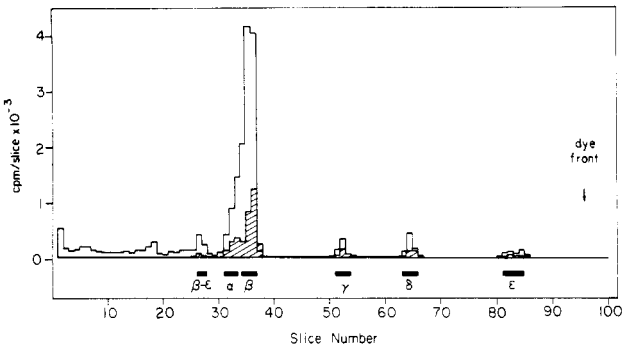

FIGURE 2: Modification of ECF_1 by [^{14}C]ETC. ECF_1 (2 mg/mL) was incubated with 4 mM [^{14}C]ETC (2×10^4 cpm/nmol) for 60 min in the absence and presence of 10 mM $MgCl_2$. The samples were precipitated with acetone, dissolved in $NaDodSO_4$ denaturing buffer, and subjected to $NaDodSO_4$ gel electrophoresis. The distribution of radioactivity was determined after the gel was stained and destained. The bars indicate the positions of Coomassie blue stained bands. The hatched area represents the distribution of radioactivity of ECF_1 labeled in the presence of 10 mM $MgCl_2$. 20 μg of ECF_1 was applied.

Table I: Modification of F_1 by [^{14}C]ETC in the Presence and Absence of Mg^{2+} [a]

	[^{14}C]ETC incorporated (mol/mol)	
	$-Mg^{2+}$	$+Mg^{2+}$
F_1	12.8 (100)[b]	3.7 (100)
α	0.9 (21)	0.3 (23)
β	2.8 (66)	0.8 (65)
γ	0.4 (3)	0.2 (5)
δ	0.6 (5)	0.2 (5)
ϵ	0.3 (2)	0.1 (3)

[a] The amount of [^{14}C]ETC incorporated into ECF_1 subunits was calculated from the distribution of radioactivity on $NaDodSO_4$ gels as shown in Figure 2. The calculations are based on a $\alpha_3\beta_3\gamma\delta\epsilon$ stoichiometry of the F_1 complex with a molecular weight of 380 000. [b] Values in parentheses represent the percent of [^{14}C]ETC incorporated.

$NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). Two-thirds of a radioactivity was bound to the β subunit and most of the rest to the α subunit (Table I). Figure 2 and the data in Table I show a comparison of the labeling of two aliquots of an ECF_1 preparation reacted with [^{14}C]ETC under identical conditions except for the presence of 10 mM Mg^{2+} in one sample and no Mg^{2+} in the other. The protective effect of Mg^{2+} was found to be the same for incorporation of [^{14}C]ETC into each subunit.

Intersubunit Cross-Linking of ECF_1 by EDC. In addition to being incorporated into ECF_1, EDC caused both intersubunit (Figure 3) and intrasubunit cross-linking (see later). The major cross-linked product resolved in $NaDodSO_4$–polyacrylamide gel electrophoresis had an apparent molecular weight of 67 000 (Figure 3A, lane 2). Identification of the subunits involved in this cross-linked product was made by the immunoblotting technique of Towbin et al. (1979), using antibodies raised against individual subunits. Figure 3B shows the cross-reactivity of the 67 000-dalton band to both the β and ϵ antibodies.

It has been claimed that ϵ is an inhibitor of ECF_1 but is released at low concentrations of enzyme used in standard assays (Laget & Smith, 1979). Inhibition of ECF_1 could therefore, result, at least in part, from retention of ϵ on ECF_1 due to being cross-linked to the β subunit. To test this possibility, ECF_1 was treated with trypsin to remove the ϵ (and δ) subunits before reaction with EDC. Figure 3 (lanes 3 and 4) confirms that trypsin cleavage removes δ and ϵ and shows that without ϵ present, no intersubunit cross-linked products

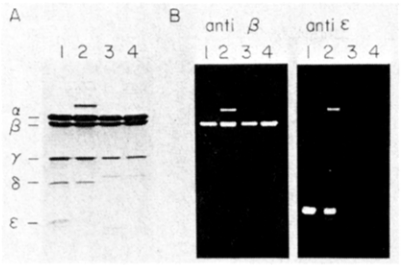

FIGURE 3: Formation of cross-links by EDC. ECF_1 (0.9 mg/mL) and trypsin-treated ECF_1 (1.4 mg/mL) were incubated with 4 mM EDC for 60 min in the absence of Mg^{2+}. The samples were then dissolved in $NaDodSO_4$ denaturing buffer, subjected to $NaDodSO_4$ gel electrophoresis, electrophoretically transferred to nitrocellulose paper, and incubated with rabbit antibodies specific for subunit β and subunit ϵ. Fluorescein-labeled goat anti-rabbit antibodies were used to visualize the rabbit antibodies. The fluorescent bands were photographed under UV light. (A) Coomassie blue stained gel; (B) fluorescent antibody staining: Lane 1; ECF_1, control; lane 2, ECF_1, EDC treated; lane 3, trypsin-treated ECF_1, control; lane 4, trypsin-treated ECF_1, EDC treated.

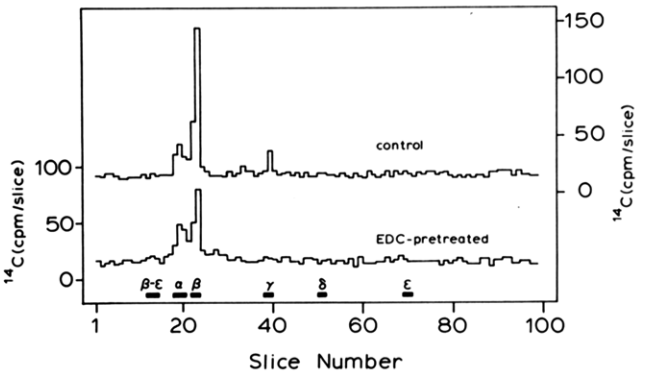

FIGURE 4: Labeling of ECF_1 with [^{14}C]DCCD after incubation with EDC. ECF_1 was treated with 4 mM cold EDC for 90 min to give more than 95% inactivation. Then 160 μM [^{14}C]DCCD (4.5×10^4 cpm/nmol) was added. After 3 h, the samples were dissolved in $NaDodSO_4$ and subjected to $NaDodSO_4$ gel electrophoresis. The control sample was treated in the same way but without EDC. The distribution of radioactivity was determined after the gel was stained and destained. The bars indicate the positions of Coomassie blue stained bands. 16 μg of ECF_1 was applied.

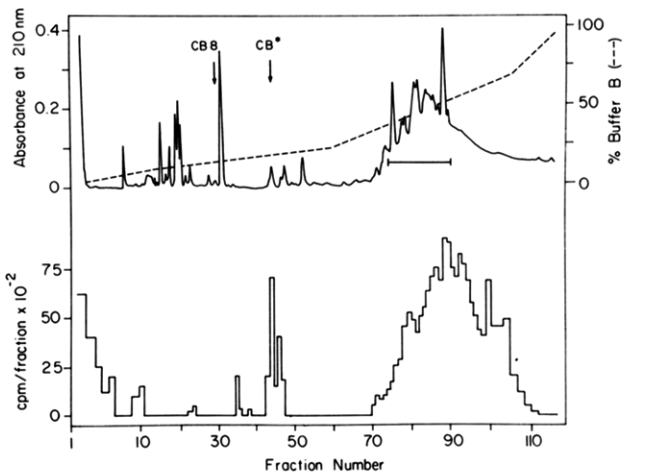

FIGURE 5: Separation of cyanogen bromide fragments of [^{14}C]-ETC-labeled β on HPLC. [^{14}C]ETC-labeled β was digested with cyanogen bromide and subjected to HPLC as described under Experimental Procedures. Two-(fractions 1–10) and one-minute fractions (fractions 10–115) were collected, and 5% of each fraction was analyzed for radioactivity. Fractions 74–91 indicated by the bar were pooled, concentrated under a stream of nitrogen, and adjusted to 100 mM $NaHCO_3$, pH 8.0, for the trypsin cleavage experiment (see text).

are formed. Comparison of the time-dependent inactivation of intact ECF_1 and trypsin-treated ECF_1 by EDC revealed no significant difference, indicating that the inhibition does not result from cross-linking of ϵ to β or any other modification of the δ or ϵ subunits (not shown).

In one set of experiments, the reaction of ECF_1 with EDC was done in the presence of a large excess of glycine ethyl ester. This reagent has been found to inhibit the rearrangement of the *O*-acylisourea intermediate in the reaction of carboxyls with carbodiimides by providing a competing external nucleophile (Hoare & Koshland, 1967). It does not compete for reaction of carbodiimides with other residues such as tyrosines in a protein. The presence of glycine ethyl ester in the medium did not prevent the inhibition of ATPase activity but did reduce significantly the labeling of ECF_1 with [^{14}C]ETC. It did not alter the generation of the β–ϵ cross-link, indicating that this is a specific reaction between closely spaced groups in the molecule.

Location of the Sites of EDC Modification in the β Subunit. Several lines of evidence indicate that the β subunit of F_1 ATPases, including ECF_1, contains the nucleotide binding site that is involved in enzyme catalysis. DCCD has been shown to inhibit ATPase activity by specific modification of Glu-193 of the β subunit. The possibility that EDC reacts at the same site was explored first by competition experiments. Figure 4 shows the labeling profiles of two aliquots of ECF_1, one of which had been modified with unlabeled EDC to 95% activity prior to the reaction with [^{14}C]DCCD. There is clearly much less incorporation of DCCD into the β subunit of the sample modified with EDC, implying that they react at the same site.

A more detailed localization of the sites of modification of EDC was begun by analysis of the labeling of CNBr fragments of the β subunit. Figure 5 shows a peptide map of the CNBr fragments as resolved by HPLC on a reverse-phase column. This fragmentation pattern, reproduced almost exactly in two different experiments, differed from that of control β subunit (not treated with ETC) in two important respects. A major peak in the control of unreacted β subunit, identified by amino acid analysis as CB8, was missing from the profile of ETC-treated subunit. In turn, the broad peak of protein of the ETC-treated sample eluting at relatively high concentrations of buffer B was missing from the control sample.

There were three major peaks of radioactivity eluting from the HPLC column after labeling with [^{14}C]ETC. The radioactivity at the beginning of the chromatogram was not associated with protein and probably represents free 1-ethyl-3-[3-[^{14}C](trimethylamino)propyl]urea. The doublet labeled CB* in Figure 5 contained approximately 12% of the [^{14}C]ETC incorporated into the entire β subunit. Both peaks of the doublet had identical amino acid compositions, identifying both as modified forms of CB8, altered in migration from unmodified CB8 by incorporation of ETC (Table II). Both contained 3 mol of ETC per mol of peptide. The difference in the two fractions could result from internal cross-linking or from another modification such as deamidation of an amido-containing residue (discussed later).

Most of the radioactivity eluting from the chromatogram shown in Figure 5 was associated with the broad peak at about 50% buffer B. $NaDodSO_4$ gel electrophoresis showed that this material contained a peptide of $M_r \sim 15\,000$, a molecular weight larger than that of any single CNBr fragment and thus either a concatamer or a cross-linked product. Amino acid analyses were done on several aliquots across this peak. All had similar amino acid compositions which were best fitted by assuming a 1:1 mixture of CB8 + CB13.

Table II: Amino Acid Composition of Radioactive Peptides Isolated by HPLC[a]

fragment	CB* (CB8)	T* (T18 + T20′)	D (T20)	D1	D2	F (T18 + T20)	F1
Asp	2.8 (3)	3.3 (3)	5.0 (5)	2.8 (3)	2.5 (2)	4.0 (6)	5.7 (5)
Thr	0.6 (1)	0.9 (0)	0.7 (1)	0.5 (1)	0 (0)	1.5 (1)	0.4 (1)
Ser	2.0 (2)	2.5 (2)	0.7 (1)	0.3 (0)	1.0 (1)	3.8 (3)	1.9 (3)
Glu	3.9 (5)	3.7 (4)	2.2 (2)	2.4 (2)	0.3 (0)	3.6 (4)	2.9 (3)
Gly	4.2 (4)	4.0 (4)	1.2 (1)	1.4 (1)	0.3 (0)	4.5 (4)	4.3 (4)
Ala	2.1 (2)	2.2 (2)	0.3 (0)	0.2 (0)	0.1 (0)	2.4 (2)	1.0 (1)
Val	1.7 (2)	1.6 (2)	0.5 (1)	0 (0)	0.5 (1)	3.5 (3)	2.6 (3)
Met	0 (0)	0 (0)	0.4 (1)	0.4 (1)	0 (0)	0.8 (1)	0.4 (1)
Ile	1.0 (3)	0.6 (2)	0.3 (1)	0 (0)	0.5 (1)	1.0 (3)	0.8 (1)
Leu	1.1 (1)	0.2 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.2 (0)
Tyr	1.7 (2)	1.6 (2)	1.1 (1)	0.8 (1)	0.2 (0)	1.5 (2)	1.5 (2)
Phe	2.0 (2)	1.6 (2)	1.0 (1)	0.9 (1)	0 (0)	1.7 (2)	1.6 (2)
His	1.9 (2)	1.7 (2)	0.7 (1)	1.2 (1)	0 (0)	1.6 (2)	2.3 (2)
Lys	0 (0)	0.7 (0)	0.7 (1)	0 (0)	1.1 (1)	1.1 (1)	0.7 (1)
Arg	2.7 (3)	1.4 (1)	0.2 (0)	0 (0)	0 (0)	2.1 (1)	1.3 (1)
position in sequence	162–194	166–183 186–194	186–202	186–196	197–202	166–183 186–202	171–183 186–202

[a] The amino acid composition of radioactive peptides obtained by cyanogen bromide and/or proteolytic cleavage of [^{14}C]ETC-labeled subunit β is shown. The fragments are labeled as CB*, T*, D, D1, D2, F, and F1 as indicated in Figures 6 and 7. The numbers in parentheses show the amino acid composition derived from the sequence.

The composition of the major radioactive peak was further analyzed by pooling the fractions (tubes 74–91 in Figure 5), lyophilizing the sample, and then subjecting the material to tryptic digestion. Trypsin released many peptides, one of which contained approximately 50% of the radioactivity. This peak (T*; Table II) was identified by amino acid analysis as a part of CB8 containing T18 and a part of T20 (184–194). It could be a concatamer (T18, T19, and T20) or could be a cross-linked product of T18 and T20. It contained 2.5 mol of ETC per mol of peptide(s).

The peptide chromatogram of the ETC-modified β subunit subjected to trypsin cleavage without prior CNBr cleavage is shown in Figure 6. There are many peaks of radioactivity, the major areas of which are labeled A–F. The broad peak of radioactivity eluting last in the chromatogram represents cross-linked material as in the chromatogram of CNBr fragments. This peak was largely absent in the chromatogram of tryptic fragments of unmodified β subunit. The complicated chromatogram in Figure 6 was reproduced almost exactly in four different experiments. Three of the six radioactive peaks could be identified unambiguously. Peak D is T20 (Table II) with 1 mol of ETC incorporated per mol of peptide. Identification of this peptide was confirmed by *Staphylococcus aureus* digestion of peak D (insert I to Figure 6) which generated two peptides, D1 and D2, with the amino acid compositions of residues 186–196 and 197–202, respectively (Table II). Most of the radioactivity was lost during digestion with *S. aureus* protease, presumably being hydrolyzed off in the slightly alkaline buffer conditions used, and it was not possible to decide unambiguously which of these segments of T20 were labeled.

Peak F had the amino acid composition of a 1:1 complex of T18 and T20 (Table II) and contained 2 mol of ETC per mol of peptide. This peak eluted in the chromatogram well shifted from where T18 or T20 run as pure peptides. Cleavage of this material with *S. aureus* protease generated only one major peak (insert II in Figure 6). This fragment had an amino acid composition similar to but not identical with that of the undigested material. It appears to be T18 + T20 from which a small portion has been removed by cleavage at Glu-170. The combined data indicate that peak F is a cross-linked product of T18 and T20. It is unlikely that all but one of the carboxyls in these two peptides are either involved in cross-links or have ETC incorporated in them, and resistance to cleavage by *S. aureus* protease is probably a conformational effect due

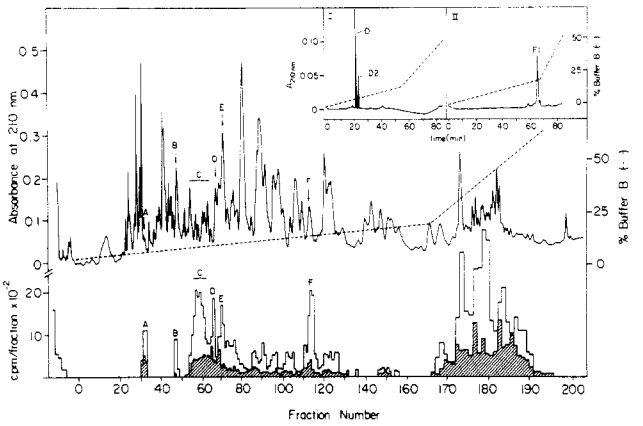

FIGURE 6: Fractionation of a tryptic digest of [^{14}C]ETC-labeled β by HPLC. [^{14}C]ETC-labeled β was digested with trypsin and subjected to HPLC as described under Experimental Procedures. Two-(fractions 1–20 and 160–200) and one-minute fractions (fractions 20–160) were collected. Fragments containing a major amount of radioactivity are indicated with letters (A–F). The broad peak of radioactivity eluting at around 40% buffer B was not further analyzed. The hatched area in the histogram represents the extent of modification when ECF_1 was reacted with [^{14}C]ETC in the presence of 10 mM Mg^{2+}. Inserts I and II show *S. aureus* V8 proteolytic digests of peaks D and F, respectively, fractionated by HPLC.

to one or two internal cross-links.

The region of the chromatogram labeled C contained six closely spaced peaks of labeled peptides. Amino acid compositions of each of these peaks were similar to one another and contained a unitary ratio of tyrosine, phenylalanine, and lysine along with the high ratio of aspartic acid to glutamic acid that is characteristic of T20. The reason that one peptide (T20) should run as so many different peaks remains unclear, and several factors may be involved. It is probable that there is cross-linking within this fragment via the lysine and even via peptide amide groups. A similarly complicated chromatogram was seen in our studies of EDC modification of cytochrome *c* oxidase, where a small fragment of subunit II was modified both by inclusion of EDC and by internal cross-linking (Millett et al., 1983). Also, Yoshida et al. (1982) observed multiple forms of T20 in their study of the DCCD binding site in the β subunit of ECF_1. They attributed this to a deamidation reaction or an $\alpha \rightarrow \beta$ inversion of a peptide bond at an aspartate residue. There are two asparagines and three aspartates in this sequence.

FIGURE 7: Amino acid sequence around the DCCD and EDC binding sites in subunit β. The sequence from Glu-162 to Lys-202 is shown. Cyanogen bromide fragment 8 (CB8), trypsin fragments 18–20 (T18–T20), and *S. aureus* V8 fragments of T20 (SA_1 and SA_2) are indicated. The boxed glutamic acid residues represent DCCD binding sites [Glu-182 in the thermophilic bacterium PS3 (Yoshida et al., 1981) and Glu-193 in *E. coli* (Yoshida et al., 1982)].

Peaks A, B, and E could not be identified unambiguously from their amino acid composition. Peaks A and B had an amino acid composition suggestive of the presence of T20 and peak E a composition more like T18 than any other fragment of mixture of fragments, but in all cases the amino acid analyses suggested the presence of other peptides in the peaks. Further digestion of each fraction with *S. aureus* protease supported the interpretation that they contained either T18 or T20 but failed to resolve the identity of the associated peptides.

Figure 6 also compares the distribution of the label in trypsin fragments after [^{14}C]ETC labeling in the presence and absence of Mg^{2+} ions. The tryptic peptide chromatograms for the samples labeled with and without Mg^{2+} present were identical, with the same amount of cross-linked material present whether the divalent metal ion was in the reaction mixture or not. Only the labeling profile is shown in Figure 6 (hatched areas). It is clear that all of the sites labeled by ETC in the absence of Mg^{2+} were labeled when this divalent metal ion was present, but to a lower extent. Most significantly, the reduction in labeling due to the presence of Mg^{2+} was about the same for all peaks, i.e., about 30% of the counts that were incorporated with Mg^{2+} present.

Discussion

There has been considerable interest recently in the effect of the hydrophobic carbodiimide DCCD on the ATPase activity of F_1 from different sources. DCCD has been found to inhibit F_1 ATPase activity by the specific reaction of a single glutamic acid residue in the β subunit. The residue involved in beef heart and ECF_1 is the same one, Glu-193 of the *E. coli* sequence (Esch et al., 1981; Yoshida et al., 1982). In F_1 from PS3, a different residue is involved. This is a conserved glutamic acid, Glu-182 (numbering in *E. coli* sequence) (Yoshida et al., 1981). The effect of water-soluble carbodiimides on the ATPase activity from beef heart and ECF_1 has been surveyed by Satre et al. (1979) and Kiel & Hatefi (1980). In both studies, it was established that these reagents inhibit ATPase activity. On the basis of competition studies, Satre et al. (1979) have concluded that inhibition by water-soluble carbodiimides is at least in part due to modification of a site other than that reacted by DCCD.

In our study, we have examined reaction of the water-soluble carbodiimide EDC with ECF_1 in detail. Several interesting conclusions can be drawn from the results. The modification of ECF_1 is considerably more complex than with DCCD and involves inter- and intrasubunit cross-linking as well as incorporation of EDC into the protein. The major intersubunit cross-link is between β and ϵ, indicating that these two subunits are near neighbors in the protein. Cross-links between β and ϵ were obtained by using bifunctional reagents in the studies of Bragg & Hou (1975) but never in the yield obtained here. Dunn (1982) has shown the ϵ subunit binds to the γ subunit by gel filtration experiments and fluorescent quenching studies using purified, native subunits. Therefore, the ϵ subunit must be at the interface between β and γ subunits in the ECF_1 complex.

Reaction of ECF_1 with EDC at levels which result in 95% inactivation led to incorporation of around 1 mol of EDC into the α subunit and 3 mol of reagent into the β subunit. Analyses of the sites of labeling in β were complicated by significant internal cross-linking within this subunit induced by the carbodiimide. However, it is clear that there are multiple modifications of a short segment of the 41-residue part of the β subunit from residues 162–202 which includes the glutamic acids modified by DCCD in F_1 from both *E. coli* and PS3 (Figure 7). At least three of the eight carboxyls in this region of the sequence must be modified by incorporation of EDC, and this segment must undergo internal cross-linking as judged by the separation of a cross-linked product of T18 + T20 in tryptic peptide maps. Moreover, this segment appears to cross-link to other portions of the sequence.

No evidence was obtained for incorporation of ETC in other parts of the sequence of the β subunit although this possibility has not been completely ruled out. Most if not all of the modification of ECF_1 by ETC is clearly in the same part of the sequence of the β subunit that is modified by DCCD, supporting the idea that this segment is important for activity. It has been found that prior reaction of DCCD with F_1 from chloroplasts prevents the binding of nucleotide into the catalytic site, suggesting to Shoshan & Selman (1980) that the DCCD-modified residue is in the catalytic site. F_1 from *E. coli*, beef heart, PS3, and chloroplast is protected from modification by DCCD when Mg^{2+} ions are present, but not significantly with Ca^{2+} in the reaction mixture (Satre et al., 1979; Pougeois et al., 1979; Shoshan & Selman, 1980). This has been taken to indicate that the carboxyl group(s) modified by DCCD act(s) to bind Mg^{2+} in the active site. In this study, we have examined the effect of Mg^{2+} more broadly. The presence of this ion in the medium protects all subunits from labeling by ETC to about the same extent and affects the labeling of different sites on the β subunit about equally. It does not affect either intersubunit cross-linking or intrasubunit cross-linking within the β subunit. These results are taken to indicate either that the effect of Mg^{2+} is a direct shielding effect or that Mg^{2+} ions "tighten" the structure of ECF_1 without altering the conformation of individual subunits. There is evidence not only for a Mg^{2+} binding site on the β subunit but also for the presence of a tight binding site for this ion on the α subunit (Senior et al., 1980; Senior, 1981; A. E. Senior, personal communication). In addition, binding of F_1 to F_0, which involves the δ and ϵ subunits at least in the case of the enzyme from *E. coli* (Futai et al., 1974), requires Mg^{2+} and is disrupted by the presence of EDTA. Any or all of these metal binding sites may be important for the structure of ECF_1.

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Registry No. EDC, 91158-61-1; ATPase, 9000-83-3.

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Interconversion of High and Low Adenosinetriphosphatase Activity Forms of *Escherichia coli* F_1 by the Detergent Lauryldimethylamine Oxide†

Hans-Ruedi Lötscher, Catharina deJong, and Roderick A. Capaldi*

ABSTRACT: The amphipathic detergent lauryldimethylamine oxide (LDAO) stimulated ATP hydrolytic activity of *Escherichia coli* membranes and isolated ECF_1 and ECF_1-F_0 ATPase complexes in a concentration-dependent manner. The enzyme was maximally activated 3-fold in membranes and 5-6-fold for isolated ECF_1 or the ECF_1-F_0 complex. The maximal specific activity of activated ECF_1 was 140-160 μmol of ATP hydrolyzed min^{-1} mg^{-1}. The activation by LDAO was reversible. LDAO specifically released subunit δ from ECF_1, generating a four subunit enzyme (α, β, γ, and ε subunits). The removal of subunit δ was not responsible for the stimulation of ATPase activity as evidenced by the full activation of the four subunit enzyme by LDAO. Treatment of ECF_1 with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide generated a β-ε cross-link in high yield [Lötscher, H. R., DeJong, C., & Capaldi, R. A. (1984) *Biochemistry* (accompanying paper in this issue)]. The formation of this cross-link was greatly reduced in the presence of LDAO, indicating that the detergent perturbated the interaction between ε and β subunits although ε was not removed from the ECF_1 complex. The results suggest that the interconversion of ECF_1 from a low to a high ATPase activity form by LDAO is in major part due to a release of the inhibitory action of subunit ε on subunit β.

The oligomeric proton translocating ATPases[1] from bacteria, mitochondria, and chloroplasts can exist in forms with low ATPase activity and forms with high ATP hydrolytic activity [for recent reviews, see Futai & Kanazawa (1983), Senior & Wise (1983), and Hammes (1983)]. In mitochondria and chloroplasts, the modulation of ATPase activity is due in large part to an endogenous inhibitor protein (Pullman & Monroy, 1963; Pederson et al., 1981). Binding of this small polypeptide converts the F_1 from a high activity to a low activity ATPase,

† From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received February 23, 1984.* This work was supported by NIH Grant HL 24526 to R.A.C. H.-R.L. is a recipient of a fellowship from the Schweizerische Stiftung für medizinisch-biologische Stipendien.

[1] Abbreviations: LDAO, lauryldimethylamine oxide; ECF_1-F_0, complete H^+-translocating ATPase complex of *E. coli*; ECF_1, water-soluble, extrinsic ATPase sector of the F_1-F_0 complex of *E. coli*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; TPCK, tosylphenylalanine chloromethyl ketone.

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