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Modification of the F_0 Portion of the H^+-Translocating Adenosinetriphosphatase Complex of *Escherichia coli* by the Water-Soluble Carbodiimide 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide and Effect on the Proton Channeling Function†

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ABSTRACT: 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), a water-soluble carbodiimide, inhibited ECF_1-F_0 ATPase activity and proton translocation through F_0 when reacted with *Escherichia coli* membrane vesicles. The site of modification was found to be in subunit c of the F_0 portion of the enzyme but did not involve Asp-61, the site labeled by the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD). EDC was not covalently incorporated into subunit c in contrast to DCCD. Instead, EDC promoted a cross-link between the C-terminal carboxyl group (Ala-79) and a near-neighbor phosphatidylethanolamine as evidenced by fragmentation of subunit c with cyanogen bromide followed by high-pressure liquid chromatography and thin-layer chromatography.

The ATP synthase of animals, bacteria, and plants is a multisubunit enzyme often called F_1F_0, which is able both to catalyze the synthesis of ATP in response to a proton gradient and to generate a transmembrane proton gradient driven by ATP hydrolysis (Pederson et al., 1981; Fillingame, 1980; Senior & Wise, 1983; Futai & Kanazawa, 1983).

The F_1F_0 complex can be divided into a water-soluble part, F_1, which is extrinsic to the lipid bilayer and made up of five different subunits, generally called α, β, γ, δ, and ϵ. These subunits are most likely present in a stoichiometry of 3:3:1:1:1, respectively. It is the F_1 portion which contains the catalytic sites of ATP hydrolysis and ATP synthesis. The F_0 portion is intrinsic to the lipid bilayer and provides the transmembrane part, being composed of three different subunits in *Escherichia coli* called a, b, and c that are present in a stoichiometry of 1:2:(6-10), respectively (Foster & Fillingame, 1982).

Studies with the hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD)[1] have established that the functionality of F_1 is critically dependent on its binding to F_0. At low concentrations, this reagent binds specifically to a carboxyl in subunit c and inhibits both ATP synthesis and ATP hydrolysis by the intact complex [for a review, see Sebald & Hoppe (1981)].

The F_0 portion of the ATP synthase has been shown to act as a proton channel. DCCD blocks proton movement through this channel (Sebald & Hoppe, 1981).

In the ATP synthase of *E. coli*, called here ECF_1-F_0, the effect of DCCD is due to modification of Asp-61 of subunit c (Wachter et al., 1980a). Recently, there has appeared evidence that carboxyls of subunit c in addition to Asp-61 are important for proton channeling through ECF_1-F_0. Altendorf and colleagues have claimed that water-soluble carbodiimides react with a carboxyl near the N terminus of subunit c to inhibit proton translocation through F_0 (Wachter et al., 1980b; Altendorf et al., 1980). Patel & Kaback (1976) have also

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ECF_1-F_0, H^+-translocating ATPase 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; FCCP, carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone; HPLC, high-pressure liquid chromatography; LDAO, lauryldimethylamine oxide; Mops, 3-(*N*-morpholino)propanesulfonic acid; PE, phosphatidylethanolamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate.

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reported that water-soluble carbodiimides block proton translocation by modification of subunit c at a site different from that reacted by DCCD. Here we confirm the results of Altendorf and colleagues and of Patel and Kaback that water-soluble carbodiimides, in our case EDC, inhibit proton translocation through ECF_1–F_0. However, we find that the effect of EDC is due to a very different type of modification of subunit c than claimed by these workers.

Experimental Procedures

Materials. [^{14}C]ETC was synthesized according to the procedure of Sheehan et al. (1961) by reacting the free base EDC with [^{14}C]CH_3I (53 mCi/mmol; Research Products International Corp.). The product was diluted with unlabeled ETC to a specific activity of 10 mCi/mol. L-α-Phosphatidyl[2-^{14}C]ethan-1-olamine and dioleoyl[^{14}C]PE (49 mCi/mmol) were obtained from Amersham. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was kindly provided by Dr. R. Simoni, Stanford University.

Source of Cells and Preparation of Membranes (ECF_1–F_0 and 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. Membranes, ECF_1–F_0, and ECF_1 were each prepared from this strain after induction of the λ phage as described by Foster et al. (1980) and Foster & Fillingame (1979). ECF_1-stripped membranes were prepared by incubating membranes of 5 mg of protein/mL in 1 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 1 mM dithiothreitol, 0.5 mM EDTA, and 5% glycerol for 1 h at 25 °C. The membranes were collected by centrifugation at 100000*g* for 90 min, resuspended in the same buffer, and incubated for an additional hour. After centrifugation, the stripped membranes were resuspended in 20 mM Mops, pH 7.0, and 1 mM $MgCl_2$ at 10–15 mg of protein/mL. Protein was determined according to the procedure described by Lowry et al. (1951).

ATPase Activity Assay. The ATPase activity of membranes, ECF_1–F_0, and ECF_1 was assayed in 1 mL of a medium 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. To assay the ATPase activity of F_1 alone, i.e., separated from the F_0 portion, the assay medium was supplemented with 0.5% lauryldimethylamine oxide (LDAO; Ammonyx LO, Onyx Chemical Co.). The absorbance change at 340 nm was followed in a Beckman DU7 spectrophotometer. The specific activity is expressed as units per milligram. One unit corresponds to 1 μmol of ATP hydrolyzed per min.

ACMA Fluorescence Assay. The fluorescence of ACMA was measured with an SLM 8000 spectrofluorometer (excitation 410 nm, emission 490 nm) in a medium containing 50 mM Tris, pH 7.8, 2.5 mM $MgCl_2$, 1 μM ACMA, and membranes (0.5 mg/mL) at 25 °C. The following additions were made: 0.5 mM NADH, 0.5 mM KCN, 1 mM ATP, and 3 μM FCCP.

Reconstitution of the F_0 Sector and the H^+-Translocation Assay. The purified ECF_1–F_0 was reconstituted with asolectin (lipid to protein ratio 50:1, w/w) by the dialysis technique as described by Foster & Fillingame (1979). The liposomes were collected by centrifugation at 100000*g* for 3 h and resuspended in 1 mM Tris, pH 8.0, 1 mM dithiothreitol, 0.5 mM EDTA, and 5% glycerol to remove the F_1 sector. After incubation for 1 h at room temperature, the vesicles were collected by centrifugation, and the extraction procedure was repeated. The final pellet was suspended in 20 mM potassium phosphate, pH 7.5, 200 mM K_2SO_4, and 1 mM DTE and subjected to mild sonication as described by Sone et al. (1981). The H^+ conductivity of these K^+-loaded vesicles was measured at 25 °C in a medium containing 0.2 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, pH 7.5, 200 mM Na_2SO_4, and 5 mM $MgSO_4$ after an artificial K^+ diffusion potential was imposed by valinomycin (inside negative). The uptake of protons was followed with a pH meter (Beckman Model SS-2 with a 39505 combination electrode).

Isolation of Subunit c and Analysis of Cyanogen Bromide Fragments on HPLC. Subunit c was extracted from membranes with chloroform/methanol (2:1), precipitated by ether, and purified on DEAE-cellulose as described by Fillingame (1976). For the cyanogen bromide cleavage experiments, isolated subunit c was dissolved in 80% formic acid and incubated with cyanogen bromide (10-fold excess by weight) overnight at 25 °C. The sample was then dried under a stream of nitrogen and dissolved in 5 mM sodium phosphate, pH 7.0, or 0.2% trifluoroacetic acid (buffer A). After removal of insoluble material by centrifugation or filtration, the peptide mixture was 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 a gradient of 80% 2-propanol (buffer B) as indicated in Figure 4. The amino acid composition of each purified peptide was determined after the sample was hydrolyzed in 6 M HCl at 110 °C for 24 h in vacuo.

Thin-Layer Chromatography of Cyanogen Bromide Fragments. Cyanogen bromide fragments of subunit c were separated on silica gel 60 precoated TLC plates by using chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5) as solvent. The spots were visualized with nonhydrin spray (0.25% ninhydrin in butanol) or molybdenum spray (Dittmer & Lester, 1964). The modified CB9 fragment (compound X in Figure 5) was scraped off the plate, eluted with chloroform/methanol (2:1), incubated with 50 mM NaOH in methanol for 1 h at 37 °C, and subjected to TLC again as described above. As a control, pure PE was treated with methanolic NaOH in the same way.

Results

EDC Inhibits both ATPase and Proton Channeling Functions of ECF_1–F_0. Reaction of EDC with the F_0 part of membrane-bound or detergent-solubilized ECF_1–F_0 was found to inhibit ATPase activity in a time- and concentration-dependent manner. Figure 1 shows the time course of this inhibition in the presence of 0.5 mM EDC. Circles show the effect of EDC on the ATPase activity of the intact ECF_1–F_0 complex as measured in *E. coli* membranes (panel A) and isolated ECF_1–F_0 (panel B). Triangles show the contribution to the inhibition as a result of modification of the F_1 part alone, as measured by assaying the ATPase activity in the presence of 0.5% LDAO in the assay buffer. Our recent studies have shown that LDAO releases ECF_1 from *E. coli* membranes as well as from isolated ECF_1–F_0 as a four-subunit enzyme (α, β, γ, ϵ) (Lötscher et al., 1984b). When isolated ECF_1 was reacted with EDC and assayed for ATPase activity in the absence and presence of LDAO, no difference in the inhibition profile was observed (Figure 1, panel C). The results in Figure 1 show clearly that inhibition of ATPase activity by EDC at low concentrations of carbodiimide is mainly due to modification of the F_0 portion of the complex and not due to direct interaction of the reagent with ECF_1. EDC does inhibit ECF_1 but at higher concentrations of reagent (Lötscher et al., 1984a). Interestingly, Mg^{2+} was found to prevent the interaction of EDC with the F_1 portion, confirming previous reports (Sartre et al., 1979; Pougeois, 1983). In contrast, the interaction with

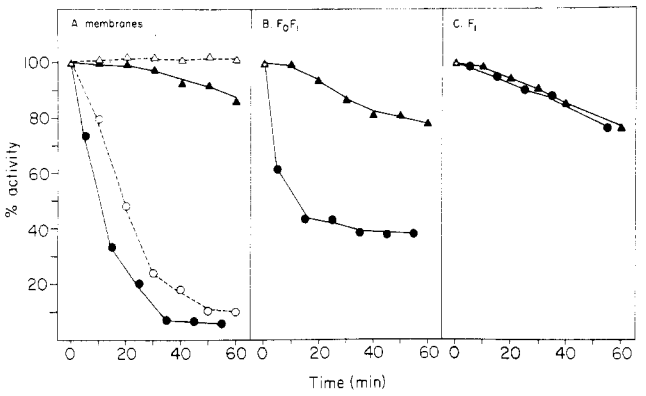

FIGURE 1: Inhibition of ATPase activity in membranes, isolated ECF_1–F_0, and ECF_1 by EDC. Membranes (1.5 mg/mL), ECF_1–F_0 (0.2 mg/mL), and ECF_1 (0.3 mg/mL) in 20 mM Mops, pH 7.0, and 1 mM $MgCl_2$ were reacted with 0.5 mM EDC at 25 °C. At the indicated times, aliquots were withdrawn and immediately assayed for ATPase activity in the absence (circles) and presence (triangles) of 0.5% LDAO. 100% activity corresponds to 3.1 and 10.6 units/mg (membranes), 8.5 and 55 units/mg (ECF_1–F_0), and 29 and 115 units/mg (ECF_1) in the absence and presence of 0.5% LDAO, respectively. The dashed line in panel A represents the EDC inhibition profile obtained in the presence of 10 mM $MgCl_2$.

the F_0 portion was only slightly affected by Mg^{2+} (dashed lines in Figure 1A).

The data shown in Figure 1 were obtained with membrane vesicles from *E. coli* prepared by the French press method of Foster et al. (1980). Such membranes are thought to be insideout in orientation with respect to the intact bacterium. Experiments were also conducted using right-side-out vesicles (Kaback, 1971). These have very low ATPase activity because of the impermeability of the membrane to substrate ATP. They are also impermeable to antibodies. ATPase activity was not inhibited when intact right-side-out vesicles reacted with antibodies against ECF_1 were separated from unbound antibodies by centrifugation, and then assayed for ATPase activity by dissolving the membranes with detergent (LDAO). In contrast, enzyme in French press vesicles could be completely inhibited by reacting the outer surface of these vesicles with anti-ECF_1 antibodies. Although impermeable to ATP and antibodies, right-side-out vesicles were slowly permeable to EDC. In one set of experiments, right-side-out vesicles were reacted with [^{14}C]ETC at relatively high concentration (5 mM compared to 0.5 mM used in the assays in Figure 1). Unreacted reagent was removed before the vesicles were disrupted. ECF_1 isolated from the labeled right-side-out vesicles retained only 35% of control ATPase activity and was labeled by ^{14}C in both the α and β subunits. The leakiness of the *E. coli* membranes to the carbodiimide under our experimental conditions means that no conclusion about the orientation of subunit c (see later) can be drawn from our data.

The results presented in Figure 2 show that EDC reacts with ECF_1–F_0 to block the proton channeling function of the F_0. Trace A shows that the fluorescence of the dye ACMA is quenched (to 80% of the initial fluorescence) by a proton gradient and the accompanying electric potential that is generated by respiration with NADH as substrate or from ATP hydrolysis by the ECF_1F_0. Stripped membranes do not show this effect (trace B). Such results have been interpreted to show that removal of the F_1 opens proton pores through the F_0 which dissipate any proton gradient and electric potential before it can be built up. Trace C shows the effect of DCCD on proton movements through the *E. coli* membrane. Modification of subunit c by this reagent prevented proton translocation through F_0, leading to the establishment of a

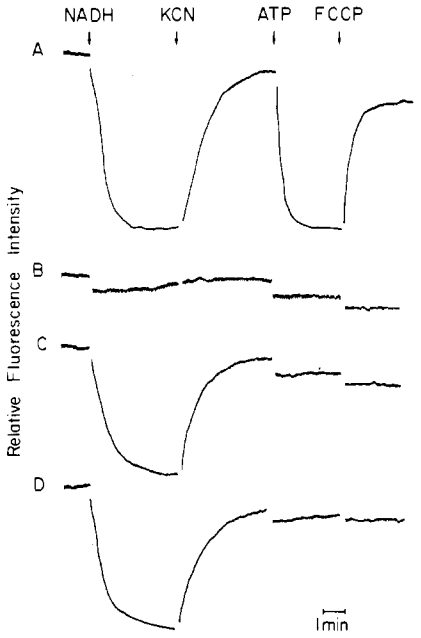

FIGURE 2: Quenching of ACMA fluorescence by membranes treated with EDC and DCCD. The quenching of ACMA fluorescence was measured as described under Experimental Procedures. Trace A, control membranes; trace B, F_1-stripped membranes; trace C, F_1-stripped membranes pretreated with DCCD (250 μM DCCD for 1 h; trace D, F_1-stripped membranes pretreated with EDC (4 mM EDC for 1 h).

proton gradient and electrical potential across the *E. coli* membrane when NADH was oxidized, but preventing the establishment of such a gradient by ATP hydrolysis. Trace D shows that EDC had the exact same effect as addition of DCCD.

Additional evidence that EDC blocks the proton channel of F_0 was obtained in reconstitution experiments. It has been shown that purified ECF_1–F_0 can be reconstituted into phospholipid vesicles by using a cholate/deoxycholate dialysis procedure (Foster & Fillingame, 1979; Friedl et al., 1979). These vesicles can be used to measure ATP-driven proton translocation by the ECF_1–F_0. Stripping of F_1 from the vesicles makes them leaky to protons, such that dissipation of a preformed K^+ ion gradient (with a high concentration of K^+ inside and a low concentration of K^+ outside) by adding valinomycin leads to inward movement of H^+ into the vesicles. This movement of protons can be measured with a pH electrode. EDC was found to have an effect identical with that of DCCD, blocking the inward movement of protons into ECF_1-depleted reconstituted vesicles as shown in Figure 3.

EDC Modifies the C-Terminal Carboxyl of Subunit c. The effect of DCCD on the ATP synthase has been correlated with modification of a single carboxyl of polypeptide c in the F_0 part of the complex. The reaction of DCCD with this residue, Asp-61 in the *E. coli* enzyme, generates an *N*-acylisourea derivative. When [^{14}C]DCCD is used, radioactivity is incorporated into the protein.

Reaction of [^{14}C]ETC with intact membrane vesicles to give 95% inhibition of ATPase activity and complete inhibition of proton pumping function did not lead to a significant incorporation of radioactivity into any of the subunits of F_0 (a, b, or c) as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis after immunoprecipitation of the ECF_1–F_0 complex from the membranes (not shown). Subunit c purified by solubilization in chloroform/methanol (2:1) followed by ether precipitation and chromatography on DEAE-cellulose (Fillingame, 1976) contained no [^{14}C]ETC (Figure 4).

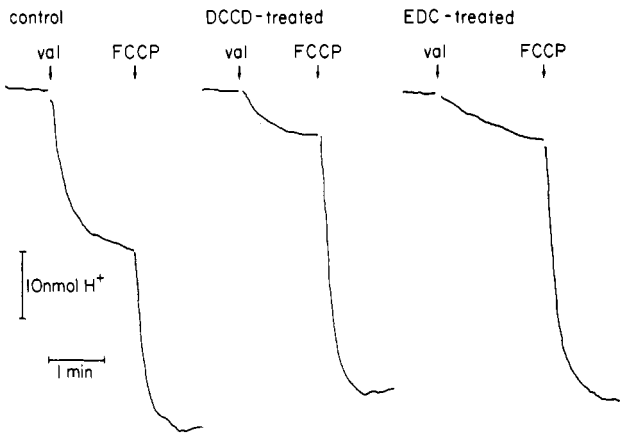

FIGURE 3: Measurement of H^+ conductivity in ECF_1–F_0 vesicles. K^+-loaded ECF_1–F_0 vesicles (containing 50 μg of protein and 8.8 mg of asolectin in 150 μL) were suspended in 3 mL of reaction medium containing 0.2 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid, pH 7.5, 200 mM Na_2SO_4, and 5 mM $MgSO_4$. DCCD- and EDC-treated vesicles were preincubated with 250 μM DCCD and 4 mM EDC, respectively, for 60 min at 25 °C. H^+ uptake was started by adding 20 ng of valinomycin (val) and 2 μM carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone (FCCP) as indicated by the arrows. The movement of H^+ was monitored with a pH electrode.

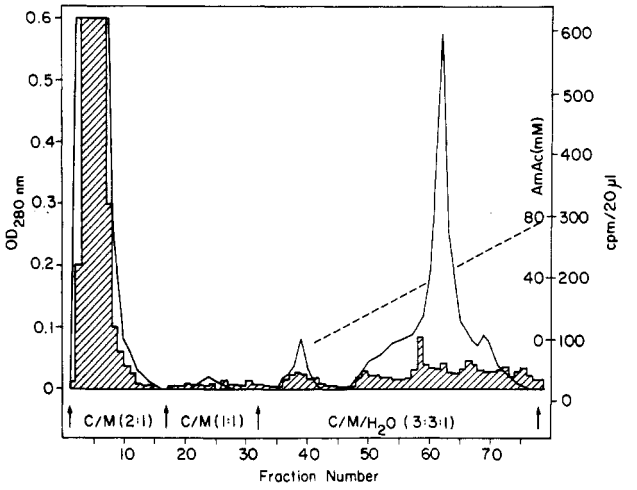

FIGURE 4: Purification of subunit c by DEAE–cellulose chromatography. Membranes (1.1 g) suspended in 20 mM Mops, pH 7.0, and 1 mM $MgCl_2$ (25-mL volume) were incubated with 1.25 mM [^{14}C]ETC (5400 cpm/nmol) for 3 h at 25 °C and then overnight at 4 °C. The membranes were collected by centrifugation and washed once with 20 mM Mops, pH 7.0, and 1 mM $MgCl_2$. Subunit c was extracted and purified according to Fillingame (1976). The elution profile of the DEAE-cellulose column (1.2 cm × 15 cm) is presented. The column was washed with chloroform/methanol (2:1), chloroform/methanol (1:1), and chloroform/methanol/H_2O (3:3:1) as indicated. Subunit c was eluted with an ammonium acetate gradient in chloroform/methanol/H_2O (3:3:1). Fractions of 4.5 mL were collected. The solid line shows the absorption at 280 nm. The histogram with the hatched area indicates the distribution of radioactivity.

Carbodiimides react with carboxyls to generate an unstable *O*-acylisourea. Rearrangement to the stable *N*-acylisourea with covalent incorporation of ^{14}C is only one of the possible reactions. The *O*-acylisourea can also undergo nucleophilic attack by a near-neighbor group such as a primary amino group to generate a covalent cross-link via a peptide bond. This possibility was tested by comparing the peptide maps of subunit c that had been isolated from EDC-treated membranes (4 mM EDC) with a control of subunit isolated from unreacted membranes. Figure 5 shows the chromatogram of CNBr fragments generated from subunit c of EDC-treated and untreated membranes as resolved by HPLC on a reverse-phase

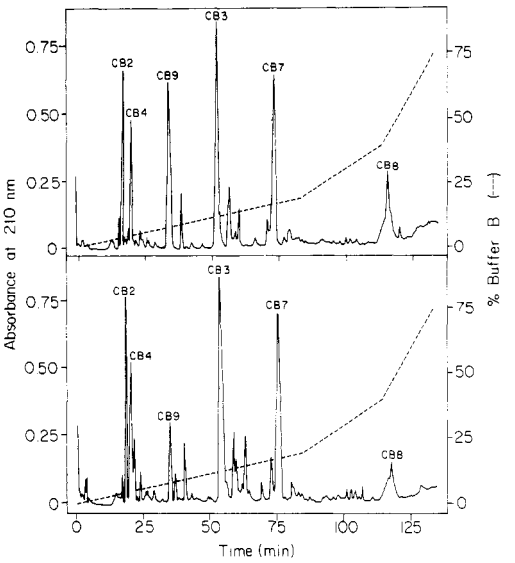

FIGURE 5: Fractionation of cyanogen bromide digested subunit c by HPLC. Subunit c dissolved in formic acid was digested with cyanogen bromide and and subjected to HPLC. The upper and lower panels show the peptide maps of subunit c isolated from control and EDC-treated membranes, respectively. The designation of the fragments (CB1–CB9) follows that used by Wachter et al. (1980a).

Table I: Recovery of Cyanogen Bromide Fragments from HPLC[a]

fragment	yield (nmol) control	yield (nmol) EDC treated
CB2	70.2 (100)[b]	98.0 (100)
CB3	76.7 (109)	114.0 (116)
CB4	66.3 (94)	93.0 (95)
CB6		
CB7	71.6 (102)	96.8 (99)
CB8	18.2 (26)	21.2 (22)
CB9	71.0 (101)	9.5 (10)

[a] The recovery of cyanogen bromide fragments was determined from the peptide maps presented in Figure 4. The recovery of CB2 was arbitrarily taken as 100%. [b] Values in parentheses are the percent recovery.

column. Six major peaks were separated, identified by amino acid analysis as CB2, CB4, CB9, CB3, CB7, and CB8, respectively, in order of elution from the column. Two of the other CNBr fragments, CB1 and CB5, are single Met residues; the third missing fragment from the profile, CB6, is a long hydrophobic peptide not soluble in phosphate bufer. CB6 could be isolated in near pure form (from pure subunit c) as a pellet after the CNBr fragments were solubilized for HPLC analysis in the phosphate buffer system. CB6 could be chromatographed by dissolving the CNBr fragments in 0.2% TFA and eluting with the TFA buffer system, but the resolution of other fragments was less satisfactory under these conditions. The only difference in the chromatograms of EDC-treated and unreacted subunit c in either the phosphate or the TFA buffer system was a dramatic reduction in the amount of CB9 in the profile. No sharp new peaks were seen in either buffer system. The loss of CB9 is clearly indicated by the data in Table I, showing a quantitation of the CB fragments as chromatographed in the phosphate buffer system. Yields of CB8 in the phosphate and TFA buffer systems varied but with no significant difference being observed in EDC-treated and in unreacted subunit c.

Thin-Layer Chromatography of CNBr Fragments Implicates PE as a Nucleophile in Cross-Linking to Subunit c. The results of the HPLC experiments above were interpreted to show that the C-terminal peptide Phe-Ala-Val-Ala-COOH

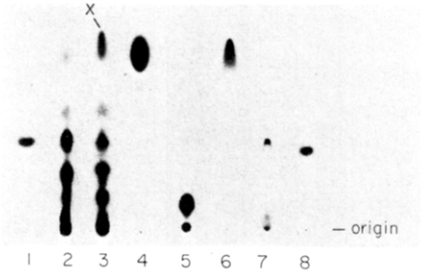

FIGURE 6: Analysis of cyanogen bromide fragments by TLC. TLC analysis was performed as described under Experimental Procedures. The spots were visualized with ninhydrin. The following samples were applied at the origin: lanes 1 and 8, pure CB9 isolated by HPLC; lane 2, cyanogen bromide digested subunit c isolated from control membranes; lane 3, cyanogen bromide digested subunit c isolated from EDC-treated membranes; lane 4, PE; lane 5, PE treated with methanolic NaOH; lane 6, compound X; lane 7, compound X treated with methanolic NaOH.

has been modified by a cross-linking reaction to a species no longer soluble in phosphate buffer and not released as a sharp peak from the HPLC column in TFA-containing buffers. This possibility was confirmed and extended by experiments using thin-layer chromatography to separate CNBr fragments. Figure 6 shows that the major CNBr fragments of subunit c can be separated by TLC (lane 2). Pure CB9 is run as a reference in lanes 1 and 8 and pure PE in lane 4. The CNBr fragments of subunit c isolated from EDC-treated membranes (lane 3) have significantly less of the CB9 but contain a component staining for primary amines and for phosphorus (not shown) that migrates faster than all other fragments (designated as compound X). This new spot was scraped from the plate and subjected to amino acid analysis. Its amino acid composition (F, 0.9; A, 1.85; V, 0.3) identifies the spot as a modified form of CB9. An obvious candidate for the nucleophilic attack of the EDC-activated CB9 was PE, a major component of the *E. coli* membrane (Ingram, 1977). PE contains both a primary amino group and phosphate.

As one test of this possibility, the modified CB9 was scraped from the TLC plate and subjected to mild alkaline alcoholysis, conditions which cleave the ester bonds in phospholipid to release the fatty acid moieties. Lane 7 of Figure 6 shows that treatment of the modified CB9 in this way changed the migration of the peptide to generate a new spot running close to that of unmodified CB9. These experiments were taken as good but indirect evidence that CB9 had become cross-linked via the C-terminal carboxyl to phospholipid by EDC treatment in the *E. coli* membranes.

Subunit c Is Cross-Linked to [^{14}C]PE by EDC Treatment of E. coli Membranes. Direct evidence that subunit c was cross-linked to PE was obtained by using [^{14}C]PE. The radioactive PE was incorporated into *E. coli* vesicles by brief sonication prior to reaction with EDC. Figure 7 shows the chromatogram of subunit c from DEAE-cellulose during purification from EDC-treated (lower panel) and untreated membranes (upper panel). Subunit c was isolated from unmodified membranes essentially free of phospholipid after the DEAE-cellulose step as reported by Fillingame (1976) with all of the [^{14}C]PE eluting from the DEAE-cellulose column in the first chloroform/methanol wash. In marked contrast, subunit c purified in the identical manner from EDC-treated membranes retained a considerable amount of [^{14}C]PE. The possibility that PE reacted with subunit c in the organic solvent (by interaction with an *O*-acylisourea intermediate formed in the membrane) was considered and ruled out. [^{14}C]PE added to the chloroform/methanol extract from EDC-treated membranes was not cross-linked to subunit c.

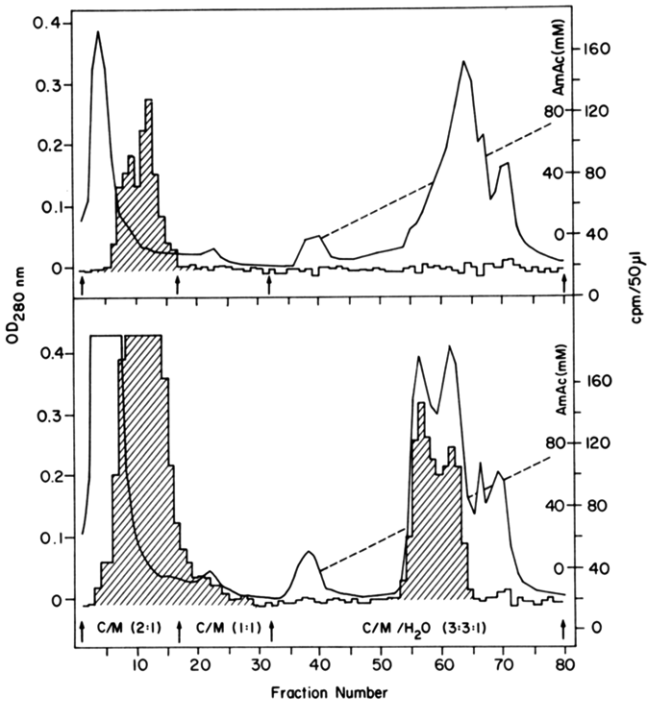

FIGURE 7: DEAE-cellulose chromatography of subunit c isolated from membranes containing [^{14}C]PE. [^{14}C]PE (5 μCi, 0.1 μmol) was incorporated into *E. coli* membrane vesicles (48 mg of protein in 2 mL of 20 mM Mops, pH 7.0, and 1 mM $MgCl_2$) by sonication in a bath sonicator for 10 min. The suspension was then diluted with 48 mL of unlabeled membranes (24 mg/mL) and divided into two parts. One part was reacted with 4 mM EDC for 3 h at 25 °C; the other part was used as a control. Subunit c was extracted and purified on DEAE-cellulose as described in the legend of Figure 3. The upper and lower panels show the elution profiles of subunit c isolated from control and EDC-treated [^{14}C]PE-labeled membranes, respectively. Fractions of 3 mL were collected. The solid line shows the absorption at 280 nm. The histogram with the hatched area indicates the distribution of radioactivity.

The pellet of membrane proteins obtained after solubilization of subunit c was analyzed and found to contain very little [^{14}C]PE compared with subunit c (less than 6% on a milligrams of protein basis). This implies that modification of subunits a and b by cross-linking to PE is negligible and is not responsible for the observed inhibitory effects.

Reaction of Detergent-Solubilized ECF_1–F_0 with EDC. Isolation of ECF_1–F_0 according to Foster & Fillingame (1979) yields a preparation with very little associated phospholipid phosphorus (about 15 mol of P/mol of ECF_0–F_1). Reaction of such preparations dispersed in Tween 80 with EDC resulted in inhibition of ATPase activity, but the contribution from modification of F_0 to this effect was much smaller (only about 50%) as compared with the inactivation profile obtained from intact membranes (see Figure 1, panels A and B). HPLC chromatograms of subunit c isolated from EDC-treated, purified ECF_1–F_0 showed no more than 10% modification of CB9. Apparently, modification of substoichiometric amounts of subunit c by EDC is enough to inhibit ATPase activity, as has been found for DCCD (Fillingame, 1981). The fact that EDC is much less effective in inhibiting isolated (and lipid-depleted) ECF_1–F_0 than membrane-bound enzyme is taken to support the contention that it is the cross-link of CB9 to PE which is responsible for the observed inhibition of both ATPase activity and proton pumping by the ECF_1–F_0 complex.

Discussion

The results presented here show that EDC reacts with the F_0 portion of ECF_1–F_0 to inhibit both the ATPase activity and

the proton channeling function of the protein. The site of modification of ECF_1–F_0 by EDC is clearly different from that of the hydrophobic carbodiimide DCCD, although both reagents have the same effect on the functioning of the enzyme. [^{14}C]DCCD has been found to modify Asp-61 of subunit c of the *E. coli* ATP synthase, with incorporation of radioactivity, presumably through formation of an *N*-acylisourea derivative of the carboxyl group (Wachter et al., 1980).

We find no incorporation of [^{14}C]ETC into the F_0 portion of ECF_1–F_0 in membranes under reaction conditions in which there is 95% loss of ATPase activity and complete blocking of the proton channel through the F_0. This is evident from $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the subunits of the F_0 and is confirmed in the case of subunit c by purification of the subunit by chloroform/methanol extraction.

Our results contrast with the findings of Altendorf and colleagues and Patel & Kaback (1976). According to Altendorf et al. (1980) and Wachter et al. (1980b), EDC reacts with the F_0 of *E. coli* from the outer surface of the *E. coli* membrane and modifies a carboxyl group(s) near the N terminus of subunit c, presumably Glu-2 or Asp-7. This claim cannot be evaluated as it appears in several abstracts, but no details of the experimentation have been published. Patel & Kaback (1976) have also claimed that [^{14}C]ETC is incorporated into polypeptide c after *E. coli* membranes are reacted with this reagent. Their evidence involves purifying subunit c by chloroform/methanol extraction, precipitating the protein by ether, and then subjecting this sample directly to $NaDodSO_4$–polyacrylamide gel electrophoresis. We find [^{14}C]ETC in the band of subunit c when this polypeptide is purified as described by Patel & Kaback (1976). The radioactivity is lost when subunit c is further purified by a DEAE-cellulose step which separates tightly bound phospholipid from the polypeptide. A logical explanation for this is that [^{14}C]ETC reacts with free fatty acids known to be present in a significant amount in *E. coli* membranes (Ingram, 1977). The labeled lipid molecules are then removed on the DEAE-cellulose column.

The results presented here indicate that reaction of ECF_1–F_0 with EDC does lead to modification of polypeptide c. Unambiguous evidence has been obtained that EDC modification of the F_0 results in modification of the C-terminal four amino acid stretch of this subunit. The modification of the C terminus involves addition of material containing phosphorus, and the altered fragment is sensitive to mild alkaline alcoholysis. Subunit c, isolated from *E. coli* membranes containing [^{14}C]PE, contains this radioactive lipid after EDC reaction, but no [^{14}C]PE is bound in the control, unreacted membranes. Taken together, these findings strongly suggest that EDC catalyzes the cross-linking of the C-terminal carboxyl of subunit c to phosphatidylethanolamine, presumably through the amino group of the phospholipid.

The cross-linking of PE to subunit c is the only modification of the F_0 observed as a result of the reaction of EDC with the *E. coli* membrane. We propose that it is this alteration of subunit c which both inhibits the ATPase activity of the ECF_1–F_0 complex and blocks proton movements through the F_0. Other possibilities were considered. Modification of polypeptides a and b does not seem to occur significantly as there is no labeling of these subunits by the stable incorporation of [^{14}C]ETC and not a significant amount of [^{14}C]PE associated with these polypeptides as judged by the total radioactivity in subunit c depleted *E. coli* membranes.

The possibility that inhibition of function is due to modification of carboxyls on subunit c (or subunits a or b) by an unstable *O*-acylisourea must be considered. Such a modification could be stable in the assay medium and thus exert an inhibitory effect but then be released during isolation of the ECF_1–F_0 complex for identification of the sites of labeling. Arguing against this are the results obtained with detergent-solubilized ECF_1–F_0. Enzyme reacted with EDC in detergent solution was much less inhibited than membrane-bound ECF_1–F_0. If an unstable *O*-acylisoura was formed, it is unlikely to have been disrupted on transferring the modified ECF_1–F_0 from the incubation to the assay medium for functional analysis. The relatively low inhibition of activity of ECF_1–F_0 when the EDC reaction is done in detergent is entirely consistent with the proposal made here. PE is largely removed during purification of ECF_1–F_0 and would not be available for cross-linking to subunit c in such preparations.

In summary, evidence is presented that EDC modifies ECF_1–F_0 by inducing the cross-linking of PE to the C-terminal carboxyl group of subunit c, and this reaction inhibits both the proton pumping and ATPase activities of the enzyme. It is interesting to note that the C-terminal carboxyl is not positionally conserved; i.e., subunit c isolated from different organisms is in some cases larger and in others shorter than the polypeptide from *E. coli*. This suggests that the C-terminal carboxyl is not critical for functioning but rather that the conformational constraint imposed by cross-linking to PE is responsible for the observed inhibition.

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Registry No. EDC, 91158-61-1; H^+, 12408-02-5; ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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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†

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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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