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Labeling of the ATP Synthase of *Escherichia coli* from the Head-Group Region of the Lipid Bilayer†

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ABSTRACT: The isolated and membrane-bound forms of the adenosinetriphosphatase of *Escherichia coli* (ECF_1 and ECF_1F_0, respectively) have been reacted with two lysine-specific reagents, sodium hexadecyl 4-[^{3}H]formylphenyl phosphate (HFPP) and sodium methyl 4-[^{3}H]formylphenyl phosphate (MFPP), and with the photoreactive reagent 1,2-[^{3}H]dipalmitoyl-*sn*-glycerol 3-[[[(4-azido-2-nitrophenyl)amino]ethyl]-phosphate] (arylazidoPE). HFPP and arylazidoPE are amphipathic molecules, inserting by their hexadecyl moieties (one and two chains, respectively) into the lipid bilayer, with the reactive groups intercalated among the phospholipid head groups. MFPP is the water-soluble analogue of HFPP. The labeling patterns of ECF_1F_0 obtained with HFPP and arylazidoPE were very similar; in both cases the a and b subunits of the F_0 part were the most heavily labeled polypeptides of the complex. Models of subunit a, arranged in six transmembrane helices, place most of the lysines in the head-group region, available for reaction with HFPP. Subunits α and β of the ECF_1 part were very poorly labeled in comparison to the a and b subunits, together incorporating only 4% as much HFPP and 7.5% as much arylazidoPE as the two F_0 subunits together on a protein mass basis. Trypsin cleavage studies localized any labeling of the α subunit by arylazidoPE to the N-terminal 15 residues of this polypeptide. When MFPP was used, the α and β subunits were very much more reacted than the F_0 subunits. This implies that most of the mass of the α and β subunits in ECF_1F_0 is above the membrane and not in contact with the bilayer surface. One subunit of ECF_1, the δ subunit, was heavily labeled by HFPP and MFPP in free ECF_1 but was shielded from reaction with both of these probes and with arylazidoPE in ECF_1F_0 preparations. The implication is that the δ subunit, a link between ECF_1 and F_0, is shielded by protein–protein interactions, at least in the lipid head-group region of the bilayer.

The adenosine 5′-triphosphate (ATP) synthase of *Escherichia coli* (EF_1F_0) is organized into two major domains, the F_1 part, extrinsic to the membrane lipid bilayer and containing the catalytic sites, and an F_0 part, intrinsic to the membrane bilayer continuum and involved in proton translocation. ECF_1 contains five different subunits, α, β, γ, δ, and ϵ, in the molar ratio 3:3:1:1:1. F_0 contains three different polypeptides, a, b, and c, in the molar ratio 1:2:10 or 12 [reviewed in Senior and Wise (1983), Walker et al. (1984), and Bragg (1984)]. The F_1 part of the ATP synthase is very similar in all organisms, with the same number of subunits and with considerable sequence conservation of the α and β polypeptides (Walker et al., 1984). F_1, with a molecular weight of 380 000, is a large globular structure. X-ray studies of the rat liver enzyme indicate that the protein is an ellipsoid with dimensions of around 110 × 80 Å (Amzel et al., 1982). How the F_1 is oriented in the F_1F_0 complex is not known.

Electron microscopy studies of the ATP synthases from bacteria, mitochondria, and chloroplasts show F_1 associated with the membrane part (F_0) through an extension or stalk (Soper et al., 1979; Kagawa et al., 1976; Gogol et al., 1987). Reconstitution studies indicate that both the δ and ϵ subunits are important for the interaction of ECF_1 with F_0 (Sternweis, 1978). Also, protease digestion of subunit b in ECF_1-depleted membranes has been shown to prevent rebinding of ECF_1 to F_0 (Perlin et al., 1983; Hermolin et al., 1983). Thus the δ, ϵ, and b subunits may all contribute to the stalk that links F_1 and F_0.

The arrangement of subunits in ECF_1F_0 has been probed previously by chemical labeling (Hoppe & Sebald, 1984; Bragg, 1984), cross-linking (Aris & Simoni, 1983; Hermolin et al., 1983; Bragg & Hou, 1980), antibody binding, and protease digestion experiments (Dunn et al., 1980; Smith & Sternweis, 1982). In this study we have used amphipathic reagents to label those parts of the protein at the lipid head group–water interface of the membrane. One reagent is 1,2-[^{3}H]dipalmitoyl-*sn*-glycerol 3-[[[(4-azido-2-nitrophenyl)amino]ethyl]phosphate] (arylazidoPE); the other is the novel protein modifying reagent sodium hexadecyl 4-[^{3}H]-formylphenyl phosphate, introduced recently by Keana, Griffith, and associates (McMillen et al., 1986). Our results are interpreted in terms of the proximity of the F_1 to the F_0

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part of the ATP synthase.

Experimental Procedures

Sodium methyl 4-[^{3}H]formylphenyl phosphate (MFPP) (5.5 mCi/mmol) and sodium hexadecyl 4-[^{3}H]formylphenyl phosphate (HFPP) (8.0 mCi/mmol) were synthesized according to the procedure of McMillen et al. (1986).

Synthesis of ArylazidoPE. 1,2-[^{3}H]Dipalmitoyl-*sn*-glycerol 3-[[[(4-azido-2-nitrophenyl)amino]ethyl]phosphate] (arylazidoPE) was synthesized essentially according to the method of Chakrabarti and Khorana (1975) and Griffith et al. (1986). 1,2-[^{3}H]Dipalmitoyl-*sn*-glycero-3-phosphorylcholine [2.94 mg, 4 μmol, 200 mCi/mmol (New England Nuclear)] was converted to 1,2-[^{3}H]dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine with 0.32 g of ethanolamine hydrochloride in 0.75 mL of 0.5 M NaOAc (pH 5.2), 0.1 mL of 1 M $CaCl_2$, and 0.6 mL of diethyl ether by using 5.3 mg of phospholipase D and stirring overnight at 32 °C. The product was purified on a silica gel column in chloroform–methanol–water (70:25:4 by volume), then 1.87 mg of the material (2.7 μmol) was dissolved in 50 μL of chloroform at 45 °C, and 1.47 mg of 4-fluoro-3-nitrophenyl azide (8.1 μmol) in 7 μL of chloroform and 4 μL of triethylamine was added. This reaction mixture was kept at 41 °C for 5 days. A total of 0.95 μmol of the final product, 1,2-[^{3}H]dipalmitoyl-*sn*-glycerol 3-[[[(4-azido-2-nitrophenyl)amino]ethyl]phosphate], with a specific activity of 200 mCi/mmol was obtained after purification on a silica gel column (Chakrabarti & Khorana, 1975) and a Sephadex LH20 (Pharmacia) column (chloroform–methanol, 3:1 by volume).

Source of Cells and Preparation of Membranous ECF_1F_0. The overproducing *E. coli* strain AN1460 was kindly provided by Dr. A. E. Senior, University of Rochester Medical Center. ECF_1 was prepared by a modification of the procedure of Wise et al. (1984) to be described elsewhere. ECF_1F_0 was purified and reconstituted as described by Foster and Fillingame (1979, 1980). Minor modifications included (i) the omission of the ammonium sulfate fractionation, (ii) the use of egg lecithin (0.25 mg/mL) in the sucrose gradient step instead of *E. coli* phospholipid (1 mg/mL), and (iii) the reconstitution of ECF_1F_0 obtained from this gradient without further addition of lipid.

The reconstitution step was carried out as follows: Enzyme from the sucrose gradient was dispersed by adding 10% sodium deoxycholate and 100 mM $MgSO_4$ to a final concentration of 0.38% and 1.2 mM, respectively. This solution was dialyzed at 4 °C against 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 5 mM $MgSO_4$, 1 mM dithioerythritol (DTE) and 10% glycerol for 36 h with two changes of buffer and then against 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 8.0), 5 mM $MgSO_4$, 1 mM DTE, and 10% glycerol overnight. Vesicles were collected by centrifugation at 45 000 rpm in a Beckman Ti60 rotor for 1 h at 4 °C.

Materials and Assays. Protein was determined according to the method of Lowry et al. (1951) as modified by Markwell et al. (1978) or Peterson (1977).

The phosphorus content was determined as described by Lowry and Tinsley (1974) as modified by McMillen et al. (1986). The ATPase activity of ECF_1 and ECF_1F_0 was assayed according to the method of Lotscher et al. (1984).

For $NaDodSO_4$ gel electrophoresis, ECF_1 and ECF_1F_0 samples were dissolved in 2% $NaDodSO_4$, 50 mM DTE, 5% glycerol, and 0.12 M Tris (pH 6.8). Slab gels (1.5 mm thick) were run as described by Laemmli (1970) with a 3% polyacrylamide stacking gel and a 10–18% polyacrylamide separating gel, both containing 0.16% $NaDodSO_4$. Staining with Coomassie brilliant blue R and destaining were carried out according to the method of Downer et al. (1976). Gels containing radioactively labeled protein were sliced on a Mickel gel slicer. The slices were dissolved in 0.5 mL of 15% H_2O_2 at 50 °C for 10 h, mixed with 4 mL of Ready-Solv EP (Beckman), and counted in a Beckman LS 7500 scintillation counter.

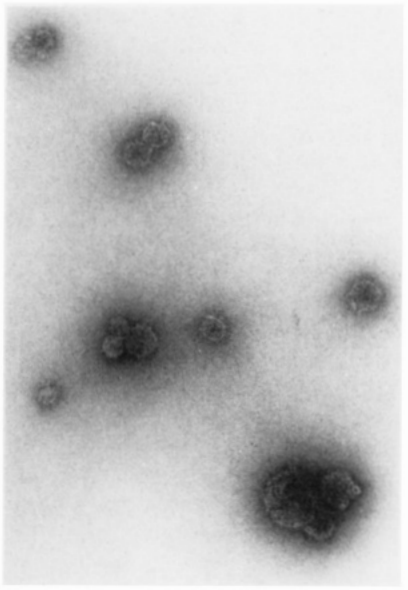

FIGURE 1: (Top) Negatively stained electron micrograph of liposomes containing ECF_1F_0. The enzyme preparation was stained with phosphotungstate (pH 7.0). (Bottom) Structures of the labels used in this study: (A) hexadecyl 4-[^{3}H]formylphenyl phosphate (HFPP); (B) 1,2-[^{3}H]dipalmitoyl-*sn*-glycerol 3-[[[(4-azido-2-nitrophenyl)-amino]ethyl]phosphate] (arylazidoPE); (C) methyl 4-[^{3}H]formylphenyl phosphate (MFPP).

Electron microscopy was performed with a Phillips EM 300 transmission electron microscope. Samples were negatively stained with phosphotungstate (pH 7.0).

Covalent Labeling of ECF_1 and ECF_1F_0 with the Water-Soluble and the Amphipathic Aldehyde. MFPP and HFPP were placed as an organic solution in a glass vial and dried under nitrogen for 15 min. A protein solution or suspension of ECF_1 and F_1F_0 respectively, was added in concentrations of 0.6–1.4 mg/mL in the case of the amphipathic reagent and 2.8–6.9 mg/mL in the case of the water-soluble reagent. These mixtures were equilibrated for 1 h at room temperature with occasional vortexing. Sodium cyanoborohydride was added in three portions over 1.5 h up to a final concentration of 10–30 mM for HFPP and 75–115 mM in experiments on MFPP. Reactions were quenched by addition of a large excess of glycine (0.1–0.6 M, pH 8.0).

Photolabeling of ECF_1F_0 with the ArylazidoPE. Two milligrams of ECF_1F_0 from the sucrose gradient step was

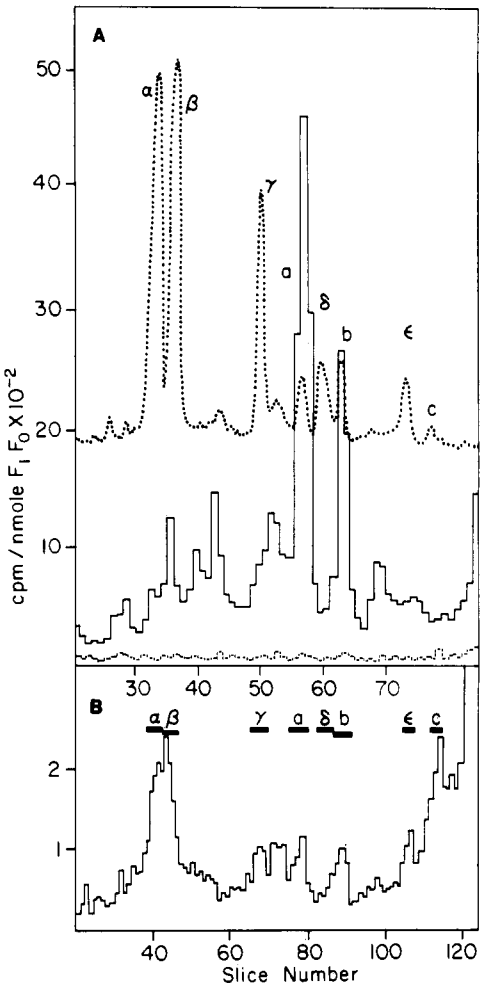

FIGURE 2: Labeling of ECF_1F_0 with the aldehydes HFPP and MFPP. (A) ECF_1F_0 (220 μg) was labeled with 60 nmol of [^{3}H]HFPP (430000 cpm) in a volume of 200 μL by adding 3 × 5 μL of 0.4 M $NaCNBH_3$. After the reaction was quenched with 22 μL of 2 M glycine, dissociation buffer was added and the sample was run on a $NaDodSO_4$–polyacrylamide gel (10–18% gradient). Radioactivity in gel slices was determined and expressed as cpm/nmol of protein (bar graph). The dotted line represents the densitometer tracing of the Coomassie blue stained gel. The dashed line shows a control in which no $NaCNBH_3$ was used. (B) ECF_1F_0 (220 μg) was labeled with 1.40 μmol of [^{3}H]MFPP (6 × 10^6 cpm) in a volume of 50 μL by adding 3 × 3 μL of 0.5 M $NaCNBH_3$.

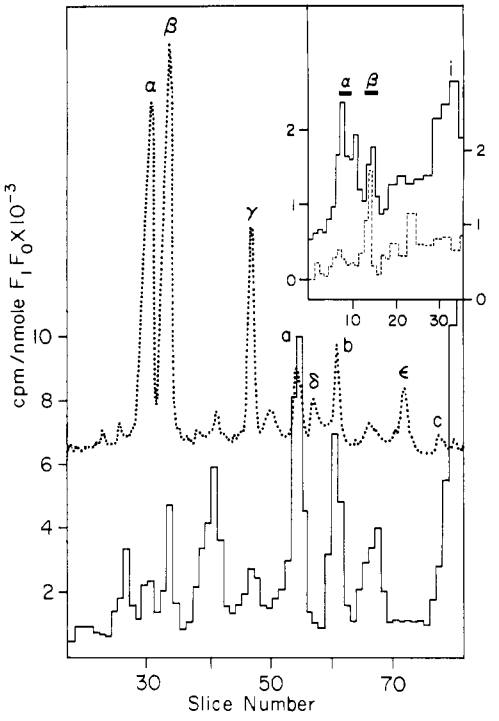

FIGURE 3: Photolabeling of ECF_1F_0 with the arylazidoPE. Vesicles containing egg lecithin, ECF_1F_0 (150 μg), and 8.2 nmol of arylazidoPE (1.5 × 10^6 cpm) were irradiated for 40 min. Dissociation buffer was added and the sample was run on a $NaDodSO_4$–polyacrylamide gel (10–18% gradient). (Insert) Trypsin digestion of photolabeled ECF_1F_0. EF_1F_0 (36 μg) was photolabeled with arylazidoPE (1 nmol, 25-min irradiation). Trypsin (0.36 μg) was added and the sample incubated for 1 h at room temperature. The proteolysis was stopped by adding phenylmethanesulfonyl fluoride (final concentration 1 mM) and pancreatic trypsin inhibitor in a ratio to trypsin of 4:1 (w/w). The sample was run on a 10% $NaDodSO_4$–polyacrylamide gel for twice the time required for the front to reach the bottom of the gel (dashed line). A control was treated the same way but trypsin was omitted (solid line).

added in the dark to 80 nmol of arylazidoPE, brought to 0.38% sodium deoxycholate and 1.2 mM $MgSO_4$ and dialyzed as described above, with 5 mM sodium phosphate (pH 8.0), 50 mM KCl, 5 mM $MgSO_4$, and 10% glycerol as final buffer. After centrifugation at 45 000 rpm in a Beckman Ti60 rotor for 1 h at 4 °C, the vesicles were resuspended with the same buffer to a concentration of 1.33 mg of ECF_1F_0/mL and irradiated at room temperature for 40 min in a Pyrex cuvette with an 18-W 366-nm UV lamp (UVP, Inc., Model UVL-56, BLAK-RAY Lamp).

For gel filtration experiments, ECF_1F_0 (0.67 mg) was photolabeled with arylazidoPE (21.2 nmol) in vesicles as described above, solubilized with 30 mM lysolecithin (from egg yolk, Sigma) and 3 mM sodium deoxycholate, and applied on a Sepharose CL-6B-200 (Sigma) column (1 cm × 90 cm) in 0.67 mL. The column was eluted with 50 mM Tris-HCl (pH 7.5), 1 mM $MgSO_4$, 1 mM DTE, 10% glycerol, and 0.2 mM lysolecithin. Aliquots of the column fractions were precipitated with 4 volumes of acetone at −20 °C, dissolved in 30 μL of dissociation buffer, and run on a $NaDodSO_4$–polyacrylamide gel (10–18% gradient) as described above.

RESULTS

Two preparations were used in this study, ECF_1 prepared according to a modification of the method of Wise et al. (1984) with five different subunits and ECF_1F_0 isolated by the method of Foster and Fillingame (1979) and containing eight different components. ECF_1F_0 was reconstituted for labeling experiments in vesicles with egg phosphatidylcholine. These vesicles, made with a protein to lipid ratio of 1:1.5 (w/w), are shown in the negatively stained electron micrograph in Figure 1 (top). The reconstituted enzyme was 95% inhibited by 10 μM dicyclohexylcarbodiimide in 1 h at room temperature.

Initial labeling experiments were conducted with HFPP and MFPP [Figure 1 (bottom)], two formylphenyl phosphates that react with proteins at lysine residues by formation of a Schiff base. This adduct can be converted to a stable covalent complex by reduction with cyanoborohydride. HFPP was added to ATP synthase containing vesicles in the ratio 145:1100:1 molecules of reagent:lipid molecules:molecule of ECF_1F_0, a ratio that gave significant labeling of the protein (1–2.5 mol/mol) but with minimal disruption of the lipid bilayer by the fatty acid derivative. MFPP was added in higher concentrations, i.e., 3400:1100:1.

The labeling of ECF_1F_0 by HFPP and MFPP is compared in Figure 2. The amphipathic probe, designed to react with proteins only from the head-group region of the lipid bilayer, labeled the a and b subunits of the F_0 part of the ATP synthase very heavily in comparison with subunits of the F_1 part, the labeling of α, β, γ, and ϵ all being in the range of the background labeling of small amounts of impurities in the prepa-

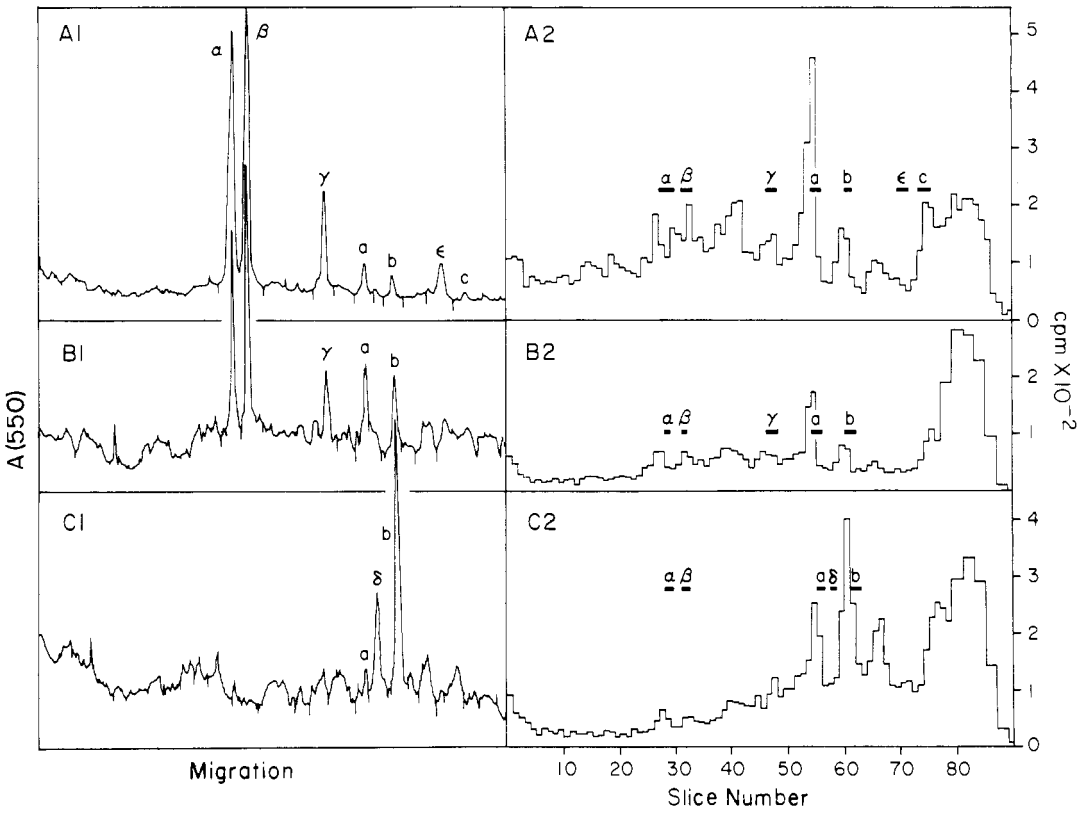

FIGURE 4: Photolabeled (21.2 nmol of arylazidoPE) ECF_1F_0 vesicles (0.67 mg of protein) solubilized with lysolecithin and sodium deoxycholate (30 and 3 mM, respectively) and run on a Sepharose CL-6B-200 column (1 cm × 90 cm) at room temperature at 5 mL/h. Fractions (1.5 mL) were collected and 0.3-mL aliquots precipitated with acetone and run on a $NaDodSO_4$–polyacrylamide gel (10–18% gradient). (A1–C1) Densitometric traces at 550 nm of Coomassie blue stained lanes from pools of the column fractions 27–28 (A1), 31–32 (B1), and 35–36 (C1). (The irregular base line for scans B1 and C1 represents noise because of the small amounts of proteins on the gels rather than multiple impurities.) (A2–C2) Bar graphs representing radioactivity distribution in lanes from pools of the column fractions 25–30 (A2), 31–32 (B2), and 33–38 (C2).

ration. No peak of radioactivity corresponding to labeling of the δ subunit was observed. In contrast, MFPP, the water-soluble analogue of HFPP, labeled the α and β subunits of F_1 much more heavily than subunits a and b of the F_0 part, the distribution of radioactivity now being approximately in proportion to the number of lysine residues in the different subunits [α = 3 copies with 24 lysines each = 72; β = 3 × 20 = 60; a = 1 × 10 = 10; b = 2 × 15 = 30 (Walker et al., 1984)]. There was significant labeling of the γ and ϵ subunits, but again no labeling of the δ subunit by MFPP, although this subunit contains eight lysines.

As a second approach to identifying polypeptides in contact with the lipid head groups, the ECF_1F_0 complex was reacted with arylazidoPE. This amphipathic reagent is different in two important ways from HFPP. The reactive group is a photoactivated nitrene, with broader specificity than the formylphenyl phosphate, reacting with any nucleophilic side chain in the protein (Bayley & Staros, 1984; Griffith et al., 1986). Also, the arylazidoPE is a two-chain phospholipid and is, therefore, better anchored in the membrane than the single-chain fatty acid derivative.

The labeling of ECF_1F_0 by arylazidoPE is shown in Figure 3. The relative labeling of subunits by this probe was very similar to that obtained with HFPP, with the a and b subunits the most heavily labeled and the α and β subunits very poorly labeled in comparison. As with HFPP, it was difficult to resolve the labeling of ECF_1 subunits from background because of the significant reactivity of the probe with small amounts of the impurities in the ECF_1F_0 preparations. (The gels in Figures 2 and 3 were loaded with more than 150 μg of protein: impurities are not seen in gel profiles when 20–50 μg of protein are applied, as is normal in identifying subunit compositions of ECF_1F_0 preparations.)

Two additional kinds of experiments were conducted to decide the extent of labeling of ECF_1 subunits. First, protease digestion was used. The insert of Figure 3 shows the labeling profile in the α,β subunit region of the gel of ECF_1F_0 that had been reacted first with arylazidoPE and then treated with trypsin. Trypsin cleavage has been shown to cleave the α subunit and release the 15 N-terminal amino acids both in isolated ECF_1 (Dunn et al., 1980) and in ECF_1F_0 (M. Gavilanes-Ruiz and R. A. Capaldi, unpublished results). It also cleaves the β subunit by removing a small fragment from the C-terminus (M. Gavilanes-Ruiz and R. A. Capaldi, unpublished results). This protease step removed almost all of the labeling in the region of the α subunit and removed the labeled impurity of M_r ~39000 but left a peak of radioactivity in the position of the β subunit on the gel. In other experiments, ECF_1F_0 was treated with trypsin prior to reacting with arylazidoPE. Again there was little or no labeling in the region of the α subunit, but a small peak of radioactivity was seen in the position of the β subunit (result not shown). These protease digestion experiments indicate that the major part of the α subunit is not labeled significantly and the β subunit is labeled only poorly (relative to the a and b subunit of the F_0 part).

This was further confirmed by fractionation studies of labeled ECF_1F_0. In initial experiments, the ECF_1 part was stripped from the F_0 part by the standard procedures of low ionic strength and ethylenediaminetetraacetic acid treatment, but the extent of stripping was low in the reconstituted vesicles. The alternative method adopted was to dissolve the

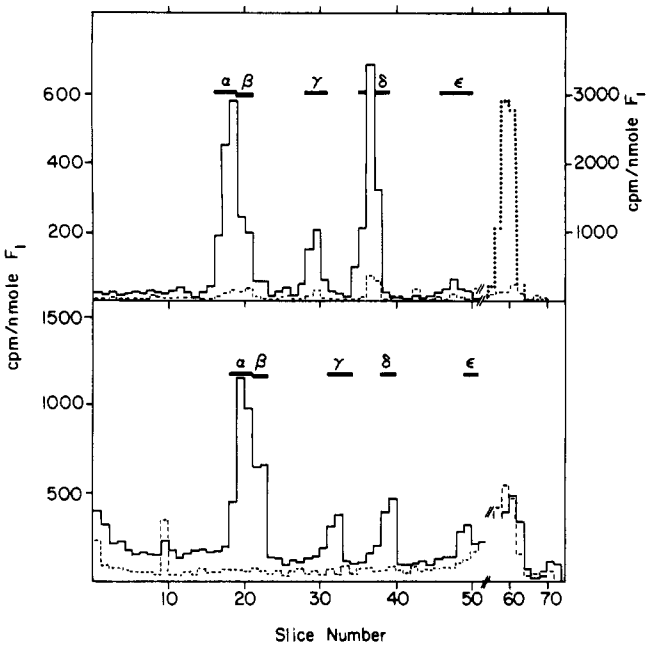

FIGURE 5: Labeling of free ECF_1 with the aldehydes HFPP and MFPP. (A) ECF_1 (276 μg) was labeled with 60 nmol of [^{3}H]HFPP (430000 cpm) in the presence of 440 nmol of egg lecithin by adding 3 × 3 μL of 0.2 M $NaCNBH_3$ in a volume of 200 μL. The dashed line shows a control in which no $NaCNBH_3$ was used. (B) ECF_1 (88 μg) was labeled with 0.93 μmol of [^{3}H]MFPP (4 × 10^6 cpm) by adding 3 × 2 μL of 0.5 M $NaCNBH_3$ in a volume of 20 μL. The dashed line shows a control, where ECF_1 was added after the reaction of the label MFPP with glycine and $NaCNBH_3$.

ECF_1F_0-containing membranes after labeling with arylazidoPE by treatment with deoxycholate and lysophosphatidylcholine (lysoPC) and then to elute the solubilized enzyme through a column in 0.2 mM lysoPC. ECF_1F_0 eluted from this column in three overlapping peaks shown by subunit composition and for labeling by arylazidoPE in Figure 4. The first fractions of enzyme to elute contained mostly ECF_1 depleted of the δ subunit. The next fractions contained ECF_1 subunits and were enriched in a and b subunits. The last fractions contained mainly δ and b subunits. Some of the trace impurities eluted before the ECF_1F_0 fractions, but others still ran in the enzyme-containing fractions. A comparison of the labeling profiles of the different fractions emphasizes the heavy labeling of subunits a and b in relation to other components of the ECF_1F_0 complex. Thus the fraction enriched in ECF_1 has only a small amount of subunit a in comparison with intact ECF_1F_0 (compare the densitometric trace in Figure 4A with those in Figures 2 or 3), and yet subunit a is the most labeled polypeptide by far, with α, β, γ, and ε subunits only poorly reacted with arylazidoPE. The labeling profile of the fraction enriched in δ and b subunits (Figure 4C), likewise, shows labeling of subunit a, although this polypeptide is present in very small amounts compared to δ or b subunits. There is heavy labeling of the b subunit of F_0 but no significant labeling of the δ subunit.

This lack of labeling of the δ subunit by HFPP, MFPP, or arylazidoPE is not a result of any lack of reactive groups for the probes. As shown in Figure 5, when isolated ECF_1 was bound to lipid vesicles in the absence of F_0, the δ subunit was labeled by both HFPP and MFPP and, in fact, was more heavily labeled than either the α or β subunit by HFPP.

Discussion

The ATP synthase of *E. coli*, like all F_1F_0 type ATPases, is made up of two domains, the F_1 part (ECF_1) extrinsic to the membrane continuum and an F_0 part containing the proton channel and intercalating within the lipid bilayer. The proximity of the F_1 part to the lipid bilayer remains a matter of debate. Negatively stained electron micrographs [e.g., Fernandez-Moran et al. (1964) and Soper et al. (1979)] show F_1 separated from the lipid bilayer part by a stalk. However, some biochemical data [e.g., Hackney (1980) and Hoppe et al. (1984)] and models such as that proposed by Bragg and Hou (1980) place F_1 closely opposed to F_0.

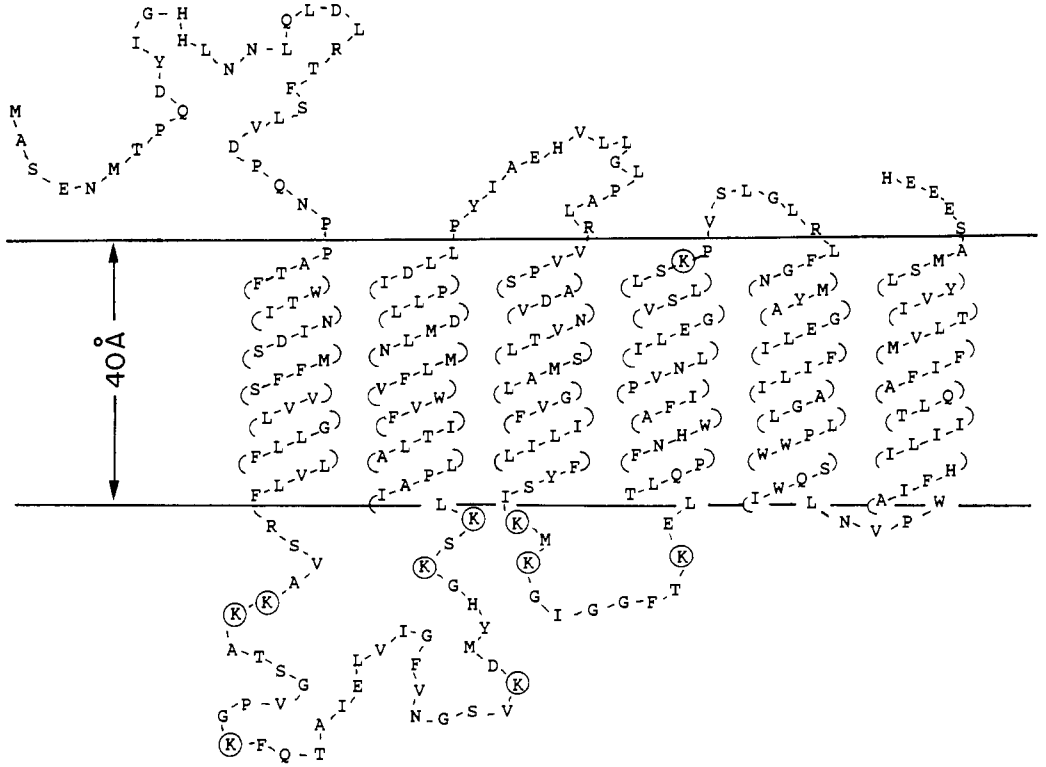

FIGURE 6: Model for the folding of the a subunit of the F_0 part modified from Walker et al. (1984).

We have recently examined the ATP synthase of *E. coli* reconstituted into liposomes of phosphatidylcholine, using electron microscopy of specimens in a thin layer of amorphous ice (Gogol et al., 1987). These micrographs show the ECF_1 and F_0 parts separated by a stalk of 50-Å length and 20-Å diameter, and because they were taken in ice and without perturbants such as phosphotungstate or uranyl acetate, the pictures rule out one objection to the earlier work, that the stalk is an artifact caused by the negative stain used to contrast protein and solvent.

The chemical labeling experiments described here offer another approach to examining the proximity of the F_1 part of the ATP synthase to the membrane bilayer. The two probes used, HFPP and arylazidoPE, would be expected to react with any protein within approximately 7 and 17 Å, respectively, of the glycerol backbone region of the lipid bilayer. Two of the polypeptides of the ECF_1F_0 complex, subunits a and b of the F_0 part, are labeled most heavily in comparison to other components of the complex by both HFPP and arylazidoPE. A model of subunit a is presented in Figure 6. This shows the polypeptide traversing the membrane bilayer a total of 6 times via >20-residue hydrophobic amino acid sequences. It can be seen that 6 of the 10 lysines in subunit a are close to the ends of these putative transmembrane sequences. Like subunit a, the b subunit is now established to be transmembranous (Hoppe & Sebald, 1984) and can thus be expected to interact with lipid head groups in the bilayer, as observed experimentally.

The poor labeling of the ECF_1 part by both HFPP and arylazidoPE has important structural implications. If a major part of the ECF_1 was closely opposed to the F_0 domain, there should have been significant labeling of the α and β subunits, but this was not the case. The labeling of the α and β subunits together by HFPP and arylazidoPE was only 4% and 7.5%, respectively, of that of the a and b subunits together on a mass basis (i.e., $3\alpha + 3\beta$ M_r around 300000; a + 2b M_r 60000). The labeling of the α and β subunits of ECF_1 proved to be no greater than that of trace impurities, presumably of intrinsic membrane proteins in the ECF_1F_0 preparations. This poor labeling cannot be explained by there being few reactive groups for the probes, as both subunits have lysines for HFPP labeling and nucleophilic side chains (arylazidoPE) distributed along their length and thus presumably distributed widely across the surface of the ECF_1 complex. Instead, the poor labeling of α and β subunits must be a function of the topology of the ECF_1F_0 complex and supports a structure in which ECF_1 is separated from F_0 by a stalk as revealed in electron microscopy studies (Soper et al., 1979; Fernandez-Moran et al., 1964; Gogol et al., 1987).

There may be some limited interaction of the α and β subunits with the bilayer, given that there are small peaks of radioactivity migrating with the subunits on gels after labeling by HFPP and arylazidoPE. In the case of the α subunit, this labeling can be localized to the N-terminus as trypsin cleavage to remove the N-terminal 15 residues (Dunn et al., 1980; M. Gavilanes-Ruiz and R. A. Capaldi, unpublished results) releases the bound label.

Both the γ and ϵ subunits of F_1 were labeled only poorly when ECF_1F_0 was reacted with HFPP or arylazidoPE, while the δ subunit was not labeled by either probe or by the water-soluble reagent MFPP. This contrasts with the results obtained when ECF_1 was allowed to bind to lipid vesicles without F_0 present when the δ subunit reacted with both HFPP and MFPP. In fact, the δ subunit was the most heavily labeled subunit of F_1 by HFPP in this case.

There is considerable evidence that the δ subunit is required for binding of ECF_1 to F_0 (Futai et al., 1974; Smith & Sternweis, 1977, 1982), and this subunit should, therefore, be a part of any stalk (Sternweis, 1978). The fact that it is not labeled can best be explained if the δ subunit is shielded at the bilayer surface by protein–protein interactions. The cryptic location of at least a part of subunit δ is supported by protease digestion studies. We have found that the δ subunit is cleaved rapidly by trypsin in ECF_1 but is shielded from digestion in ECF_1F_0 (M. Gavilanes-Ruiz and R. A. Capaldi unpublished results). An obvious candidate for interaction with the δ subunit is subunit b, also a part of the stalk (Cox et al., 1984), present in two copies and relatively heavily labeled by the amphipathic probes.

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Covalent Coupling of 4-Thiouridine in the Initiator Methionine tRNA to Specific Lysine Residues in *Escherichia coli* Methionyl-tRNA Synthetase†

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ABSTRACT: A new method has been developed to couple a lysine-reactive cross-linker to the 4-thiouridine residue at position 8 in the primary structure of the *Escherichia coli* initiator methionine tRNA ($tRNA^{fMet}$). Incubation of the affinity-labeling $tRNA^{fMet}$ derivative with *E. coli* methionyl-tRNA synthetase (MetRS) yielded a covalent complex of the protein and nucleic acid and resulted in loss of amino acid acceptor activity of the enzyme. A stoichiometric relationship (1:1) was observed between the amount of cross-linked tRNA and the amount of enzyme inactivated. Cross-linking was effectively inhibited by unmodified $tRNA^{fMet}$, but not by noncognate $tRNA^{Phe}$. The covalent complex was digested with trypsin, and the resulting tRNA-bound peptides were purified from excess free peptides by anion-exchange chromatography. The tRNA was then degraded with T1 ribonuclease, and the peptides bound to the 4-thiouridine-containing dinucleotide were purified by high-pressure liquid chromatography. Two major peptide products were isolated plus several minor peptides. N-Terminal sequencing of the peptides obtained in highest yield revealed that the 4-thiouridine was cross-linked to lysine residues 402 and 439 in the primary sequence of MetRS. Since many prokaryotic tRNAs contain 4-thiouridine, the procedures described here should prove useful for identification of peptide sequences near this modified base when a variety of tRNAs are bound to specific proteins.

Recent studies from this laboratory have involved development of new methods for the covalent coupling of specific protein–nucleic acid complexes, with a view to isolation and sequencing of peptides at nucleic acid binding sites. To date, we have focused our attention on the interaction of *Escherichia coli* methionine tRNAs with *E. coli* methionyl-tRNA synthetase (MetRS).[1] High-resolution X-ray crystallographic data is available for a biologically active monomeric form of MetRS (Zelwer et al., 1982), providing an opportunity to interpret the results of cross-linking studies in terms of the known three-dimensional structure of the enzyme. Affinity-labeling derivatives of $tRNA^{fMet}$ carrying cross-linkers at the 5′ terminus, the dihydrouridine loop, and the anticodon have been coupled to MetRS in high yield, and specific peptides attached to each site have been identified (Schulman et al., 1981a,b; Valenzuela et al., 1984; Valenzuela & Schulman, 1986; Leon & Schulman, 1987a). In addition, we have made use of the presence of the minor base 3-(3-amino-3-carboxypropyl)uridine in the elongator methionine tRNA to attach a site-specific cross-linker to the variable loop of this tRNA and have determined the sequence of the major MetRS peptide coupled to this site (Leon & Schulman, 1987b). The initiator tRNA also contains a unique minor base, 4-thiouridine, which can be used to prepare a site-specific affinity-labeling tRNA derivative carrying a cross-linker on the inside of the L-shaped three-dimensional structure of the tRNA. This region of tRNAs has been suggested to form part of a general binding domain for aminoacyl-tRNA synthetases (Rich & Schimmel, 1977) and has been shown to directly interact with cognate

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[1] Abbreviations: $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; $tRNA_m^{Met}$, *E. coli* elongator methionine tRNA; MetRS, *E. coli* methionyl-tRNA synthetase; SBrAB, *N*-succinimidyl [(bromoacetyl)amino]-benzoate; SBrAB-$tRNA^{fMet}$, $tRNA^{fMet}$ modified by coupling SBrAB to the 4-thiouridine residue at position 8 from the 5′ terminus; [$^{35}S_8$]-$tRNA^{fMet}$, $tRNA^{fMet}$ labeled with ^{35}S in the sulfur atom of the 4-thiouridine residue at position 8; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DEAE, *N*,*N*-diethylaminoethyl; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; NHS, *N*-hydroxysuccinimide; ODS, octadecylsilane; PTH, phenylthiohydantoin; PITC, phenyl isothiocyanate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; RNase, ribonuclease.

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