Coupling Factor 1 from *Escherichia coli* Lacking Subunits δ and ϵ: Preparation and Specific Binding to Depleted Membranes, Mediated by Subunits δ or ϵ[†]

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ABSTRACT: A procedure for the preparation of coupling factor 1 (F_1) from *Escherichia coli* lacking subunits δ and ϵ is described. Using chloroform and dimethyl sulfoxide, we can isolate F_1 containing only subunits α, β, and γ [$F_1(\alpha\beta\gamma)$] directly from membrane vesicles in 10-mg quantities. Pure and active subunits δ and ϵ were prepared from five-subunit F_1 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After addition of these subunits, $F_1(\alpha\beta\gamma)$ is as active in reconstituting ATP-dependent transhydrogenase as five-subunit F_1. The ATPase activity of $F_1(\alpha\beta\gamma)$ is inhibited by subunit ϵ in a 1:1 stoichiometry to the same extent (≈90%) and with the same affinity (K_i = 0.2–0.8 nM) as reported earlier [Dunn, S. D. (1982) *J. Biol. Chem. 257*, 7354–7359]. In the presence of either δ or ϵ, $F_1(\alpha\beta\gamma)$ binds to F_1-depleted membrane vesicles and to liposomes containing the membrane sector (F_o) of the ATP synthase to an extent commensurate with the F_o content. The binding ratios $\epsilon/F_1(\alpha\beta\gamma)$ and probably also $\delta/F_1(\alpha\beta\gamma)$ are close to unity. The specific, δ- or ϵ-deficient $F_1{\cdot}F_o$ complexes presumably formed show ATPase activities sensitive to subunit ϵ but not to dicyclohexylcarbodiimide, and no energy-transfer capabilities. Binding studies at different pH values suggest that F_1–F_o interactions in the presence of both subunits δ and ϵ are similar to a combination of those mediated by δ or ϵ alone. Salts containing mono-, di-, or trivalent cations promote specific binding of $F_1(\alpha\beta\gamma)$ by δ or ϵ at concentrations of about 0.1, 0.01, and 0.01 M, respectively, suggesting a general electrostatic effect.

ATP synthases from bacteria, chloroplasts, and mitochondria are complex multimeric enzymes consisting of a catalytic segment, F_1, attached to a proton-conducting, membrane-bound segment, F_o. In most cases, F_1 detached from the membrane exhibits ATPase activity and contains five subunits in a stoichiometry widely accepted to be $\alpha_3\beta_3\gamma\delta\epsilon$ (Senior & Wise, 1983). F_o is a *N,N'*-dicyclohexylcarbodiimide (DCCD)[1]-sensitive proton channel that may also serve as a highly cooperative proton trap (Junge et al., 1984). The morphology of the ATP synthase, the forces that hold the $F_1{\cdot}F_o$ complex together, and the role of the individual subunits are of obvious interest for the understanding of energy transfer between F_o and F_1. Subunits α and β are directly involved in hydrolysis and formation of ATP (Rao & Senior, 1987; Futai et al., 1988). In *Escherichia coli* and chloroplasts, $F_1(\alpha\beta\gamma)$ is the minimal structure capable of hydrolytic activities (Futai, 1977; Patrie & McCarty, 1984). Thus, subunit γ appears to stabilize the correct arrangement of α and β. Subunits δ and ϵ play important, little understood roles in establishing a physical link between $F_1(\alpha\beta\gamma)$ and F_o and creating the topology necessary for energy transfer in the $F_1{\cdot}F_o$ complex. Subunit δ from *E. coli* and chloroplast F_1 is a rod-shaped polypeptide long enough to penetrate both $F_1(\alpha\beta\gamma)$ and F_o (Sternweis & Smith, 1977; Schmidt & Paradies, 1977). Chloroplast δ and OSCP, the mitochondrial homologue of δ, have been reported to bind to F_1-depleted F_o (Roos & Berzborn, 1982; Tzagoloff, 1970). Therefore, a direct role of subunit δ in connecting F_1 to F_o appears likely in the *E. coli* system as well. Subunit ϵ binds tightly to $F_1(\alpha\beta\gamma)$, interacting with the catalytic subunit β (Tozer & Dunn, 1987) and with γ (Dunn, 1982). It inhibits the ATPase activity of free, but not of membrane-bound, F_1 (Sternweis & Smith, 1980), probably by decreasing the rate of product release (Dunn et al., 1987). The presence of both δ and ϵ is required for the catalysis of ATP-dependent energy transduction (Smith & Sternweis, 1977; Sternweis & Smith, 1977).

Divalent cations are necessary for the functioning of the $F_1{\cdot}F_o$ complex and for maintaining the attachment of F_1 to the membrane. Chelation of magnesium ions by EDTA suffices to release F_1 from bacterial and chloroplast membranes, and rebinding requires the presence of divalent cations (Muñoz, 1982). Two magnesium binding sites have been identified in isolated F_1 from both *E. coli* and mitochondria (Senior et al., 1980). Substitution of serine-174 by phenylalanine in the β subunit results in the loss of the magnesium-dependent, but not the calcium-dependent, ATPase activity of *E. coli* F_1 (Kanazawa et al., 1980; Noumi et al., 1984). In the interaction of F_1 and F_o, a specific role for magnesium as a bridge between carboxyl groups of subunits δ and b, and ϵ and b, has been proposed (Cox et al., 1984). To our knowledge, no data on the role of the magnesium binding sites or on the relationship between functional and structural effects of magnesium in the $F_1{\cdot}F_o$ complex from *E. coli* are available.

In the present study, with the use of new preparations of $F_1(\alpha\beta\gamma)$ and subunits δ and ϵ, several aspects of the binding of F_1 and F_o were investigated. We show that either subsunit δ or subunit ϵ alone is capable of mediating the specific attachment of $F_1(\alpha\beta\gamma)$ to the membrane. From the study of pH effects, we conclude that this type of binding represents

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; $F_1(\alpha\beta\gamma)$, F_1 containing subunits α, β, and γ; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OSCP, oligomycin sensitivity conferring protein; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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individual contributions of δ or ϵ to the binding of complete F_1 to F_o. The influence of mono-, di-, and trivalent cations shows that general electrostatic interactions are also important in the stability of the $F_1 \cdot F_o$ complex.

Materials and Methods

Materials

DMSO, p.a., was from Merck (Darmstadt), tris(ethylenediamine)cobalt(III) chloride hydrate was from Aldrich Chemical Co., and tris(ethylenediamine)chromium(III) chloride was from Alfa Products. All other chemicals were the purest commercially available grades. ACMA and *E. coli* strains AN1460 and KY7485 were generous gifts from Dr. R. Kraayenhof (Free University, Amsterdam, The Netherlands), Dr. A. E. Senior (University of Rochester), and Dr. K. Altendorf (University of Osnabrück, West Germany), respectively.

Methods

Growth of E. coli. Cells of strains K12 and ML308/225 were grown in minimal media with 0.2% and 0.4% glucose, respectively (Davies & Mingioli, 1950). AN1460 and KY7485 were grown as described (Gibson et al., 1977; Senda et al., 1983). All strains were harvested in the late exponential phase.

Preparation of Purified F_1. Complete F_1 was prepared as described (Vogel & Steinhart, 1976) with modifications as follows. The cells were suspended in a buffer containing 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 2.5 mM mercaptoethanol, 0.1 mM EDTA, 1 mM PMSF, and 10 μg of DNase/mL, and broken in a Ribi press. After removal of cell debris at 20000*g*, the vesicles were sedimented at 150000*g* for 120 min, resuspended in 20 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 2.5 mM mercaptoethanol, and 0.1 mM EDTA, and sedimented again. For a complete release of F_1, the vesicles were incubated twice at low ionic strength, once for 1 h at room temperature and a second time overnight at 4 °C. After poly(ethylene glycol) fractionation, the enzyme was further purified by ion-exchange chromatography and gel filtration as described below for $F_1(\alpha\beta\gamma)$.

$F_1(\alpha\beta\gamma)$ was prepared from strains ML308/225 or K12 by a method using chloroform/DMSO for the release of ATPase.

Step 1. Vesicles prepared as described above from 200–250 g of cells (wet weight) in 650 mL of buffer were suspended in a buffer containing 0.25 M sucrose, 10 mM MOPS (pH 6.8), and 2.5 mM mercaptoethanol, sedimented, resuspended in a small volume of the same buffer at high protein concentration (60 mg/mL), frozen in liquid nitrogen, and kept at −70 °C.

Step 2. The vesicles were diluted to 30 mg/mL with a buffer containing 0.25 M sucrose/10 mM MOPS (pH 6.8) and supplemented with 25% DMSO, 5 mM mercaptoethanol, 1 mM EDTA, and 1 mM ATP. Two-milliliter portions of the vesicle suspension were rapidly mixed for 30 s at room temperature with an equal volume of chloroform. After separation of the phases in a clinical centrifuge, the aqueous layer was centrifuged at 50000*g* for 1 h at room temperature.

Step 3. $F_1(\alpha\beta\gamma)$ in the supernatant was purified at 4 °C by ion-exchange chromatography on DEAE-Sepharose CL-6B (2.5 × 20 cm) equilibrated with 50 mM MOPS (pH 7.0), 20% methanol, and 2.5 mM mercaptoethanol. When a linear NaCl gradient (0–0.6 M in the equilibration buffer) was used, nearly pure $F_1(\alpha\beta\gamma)$ was eluted at around 0.2 M.

Step 4. To remove minor impurities, the F_1-containing fractions were concentrated on ice to 5–10 mL by ultrafiltration (Amicon PM 10) and subjected to gel filtration (Bio-gel A 1.5m, 2.6 × 95 cm) at 4 °C. $F_1(\alpha\beta\gamma)$ elutes at around 260 mL using the equilibration buffer of step 3. The active fractions were combined, repeatedly concentrated at room temperature by ultrafiltration, diluted with 0.1 M Tris·HCl, pH 8, finally diluted with an equal volume of 0.5 M sucrose, frozen in liquid nitrogen, and stored at −70 °C. The activity of this material is unchanged after 24 months of storage. The yield is about 35 mg of $F_1(\alpha\beta\gamma)$ from 250 g of cells. Instead of DMSO, 10% of alcohols such as methanol, ethanol, or 2-propanol can be added. The yield is, however, lower, and ϵ is not always completely removed.

Preparation of Subunits δ and ϵ. Subunits δ and ϵ were prepared from complete F_1 (Vogel & Steinhart, 1976) by preparative gel electrophoresis, elution, and renaturation according to the general procedure of Hager and Burgess (1980) as follows. F_1 (25 mg) was subjected to electrophoresis (Laemmli, 1970) in 36 6-mm-diameter cylindrical SDS–polyacrylamide gels (14% separation gel). The protein bands were visualized in ice-cold 0.25 M KCl, and those corresponding to δ and ϵ were cut out, rinsed with distilled water, crushed with a Teflon pestle, and eluted with 20–30 mL of a buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 0.5% SDS, 0.1 mM EDTA, and 1 mM DTE. After agitation of the slurry for 3 h, the gel material was forced through glass wool stuffed 50-mL syringes and stored in the cold for a second extraction. The eluate was dialyzed with one change of buffer for 15 h against 25 volumes of a buffer containing 10 mM MOPS (pH 7.0) and 1 mM DTE, first at room temperature to prevent precipitation of SDS and later at 4 °C. The volume was reduced to 1–1.5 mL (Amicon PM 10), and the protein was precipitated in test tubes by the addition of 5 volumes of cold acetone (−20 °C). After at least 14 h at −20 °C, the solutions were centrifuged at room temperature for 20 min in a clinical centrifuge. The supernatant was discarded, and most of the remaining acetone was allowed to evaporate. The pellets were dissolved in 20 μL of 6 M guanidine hydrochloride per test tube and diluted 50-fold with a renaturing buffer containing 50 mM Tris·HCl (pH 8.0), 20% (v/v) glycerol, 150 mM NaCl, 0.5 mM DTE, and 0.1 mM EDTA. Insoluble material was removed by centrifugation. The clear solution was dialyzed for 8 h against the renaturing buffer and for 24 h against 100 volumes of 50 mM MOPS (pH 7), with one change of buffer. If necessary, the solutions were concentrated by ultrafiltration (Amicon PM 10). The average yields were 30–40% for δ and 40–50% for ϵ. Both subunits were pure and active in reconstitution, and ϵ also in the inhibition of soluble $F_1(\alpha\beta\gamma)$ ATPase activity (see Figure 1–3).

Binding of Subunit ϵ to $F_1(\alpha\beta\gamma)$. In the inhibition of F_1-ATPase activity by ϵ, the binding stoichiometry, $n = \epsilon/F_1(\alpha\beta\gamma)$, and the inhibitory dissociation constant, K_i, can be determined from a plot of the ϵ concentration necessary for half-inhibition, $I_{1/2}$, vs the $F_1(\alpha\beta\gamma)$ concentration. That the y intercept and the slope are $K_i^{1/n}$ and $n/2$, respectively, can be derived as follows, with subscripts (b, f, tot) having the usual meaning. At half-inhibition

$$[F_1]_b = [F_1]_f = \tfrac{1}{2}[F_1]_{tot} \qquad [\epsilon]_{tot} = I_{1/2}$$

The dissociation constant is then

$$K_i = ([\epsilon]_{tot} - n[F_1]_b)^n[F_1]_f/[F_1]_b = (I_{1/2} - \tfrac{1}{2}n[F_1]_{tot})^n$$

and

$$I_{1/2} = K_i^{1/n} + \tfrac{1}{2}n[F_1]_{tot}$$

Preparation of F_o-Containing Liposomes. F_o prepared according to Schneider and Altendorf (1984) was incorporated

into soybean phospholipids as described (Sone et al., 1977).

F_1·F_o Binding Experiments. Binding of $F_1(\alpha\beta\gamma)$ to vesicles mediated by δ and/or ϵ was determined by centrifugation in a desk-top ultracentrifuge (Airfuge, Beckman) at room temperature. $F_1(\alpha\beta\gamma)$ (0.1–0.2 mg/mL) was incubated for 10 min with δ and/or ϵ and vesicles in a total volume of 100–175 μL in cellulose propionate centrifugation tubes. The membranes were sedimented by 10-min centrifugation at 140000*g* (30 psi air pressure), and the supernatants were analyzed for remaining F_1 content. This was done mostly by determination of ATPase activity, occasionally also by assaying ATP-dependent transhydrogenase activity in the presence of additional subunits (Table I). When subunit ϵ, an inhibitor of ATPase activity, was present in the binding experiment, at least a 3-fold excess of ϵ was added to the ATPase assay to ensure uniform inhibition. As a control, binding of F_1 was determined from the F_1 protein content in the supernatants by SDS–polyacrylamide gel electrophoresis. The staining intensities of the protein bands corresponding to the α and β subunits were compared with calibration curves obtained with pure F_1. Binding data obtained by this method corresponded closely to those (Figure 7) derived from enzymatic activities (data not shown).

Stoichiometry of Subunits δ and ϵ in Binding Experiments. The ratio of bound $F_1(\alpha\beta\gamma)$ to added subunit (Figure 5, right panel) can be calculated from relevant dissociation constants as follows. Assuming that subunits x (δ or ϵ) bind to $F_1(\alpha\beta\gamma)$ in a 1:1 ratio, the dissociations of x from free $F_1(\alpha\beta\gamma x)$ and of bound $F_1(\alpha\beta\gamma x)$ from $F_1(\alpha\beta\gamma x)$·F_o are characterized by the constants

$$K_D = [F_1(\alpha\beta\gamma)][x]/[F_1(\alpha\beta\gamma x)]$$

and

$$K_V = [F_1(\alpha\beta\gamma x)][F_o]/[F_1(\alpha\beta\gamma x)\cdot F_o]$$

The ratio bound $F_1(\alpha\beta\gamma x)$/added subunit is then

$$[F_1(\alpha\beta\gamma x)\cdot F_o]/[x]_{tot} = 1 - L/(L + 1)$$

where

$$L = (K_D/[F_1(\alpha\beta\gamma)] + 1)(K_V/[F_o])$$

With small dissociation constants K_D and K_V, the ratio [bound $F_1(\alpha\beta\gamma x)$/added subunit] will be close to unity at the beginning of the titration when the concentration of $F_1(\alpha\beta\gamma)$ is largest (see right panel of Figure 5 for ϵ and $\delta + \epsilon$). Weaker binding of x to $F_1(\alpha\beta\gamma)$ and/or of $F_1(\alpha\beta\gamma x)$ to the membrane results in values smaller than 1 (Figure 5, right panel, δ) suggesting larger x/F_1 stoichiometries even in cases where the correct value is unity.

Analytical Procedures. Vesicle protein was determined by a Biuret procedure (Gornall et al., 1949), F_1, δ, and ϵ were quantified according to Read and Northcote (1981) using Serva Blue G 250, and F_o protein was estimated by a modified Lowry method (Dulley & Grieve, 1975). The concentration of F_o in vesicles was determined either by reconstitution experiments (AN1460, K12) or by comparison of the Coomassie-staining intensities of subunit b in SDS-polyacrylamide gel electrophoresed F_o–liposomes (see Table I) with standard preparations.

Enzymatic Activities. ATPase activity was determined spectroscopically in the presence of an ATP-regenerating system (Pullman et al., 1960) at 30 °C. ATP-driven NADP–NADH transhydrogenase activity was measured according to Futai et al. (1974) at 37 °C. Spectral responses of oxonol VI were measured as described (Kiehl & Hanstein, 1984) with ATP (2.5 mM) and NADH (2 mM) as the energy source.

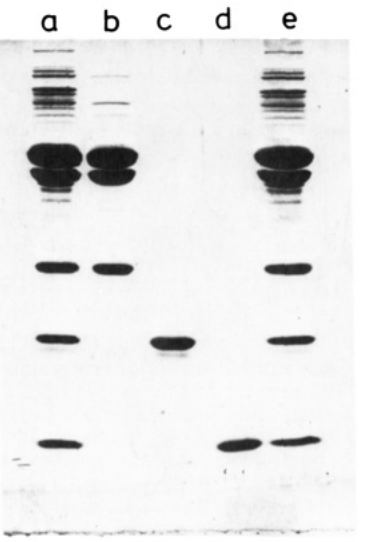

FIGURE 1: SDS–polyacrylamide gel electrophoresis of chloroform–DMSO $F_1(\alpha\beta\gamma)$, δ, and ϵ. Lanes a and e, 19.5 μg of complete F_1; lane b, 20 μg of chloroform–DMSO $F_1(\alpha\beta\gamma)$; lane c, 1.9 μg of δ; lane d, 1.8 μg of ϵ.

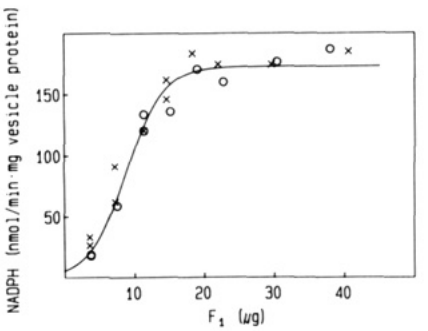

FIGURE 2: Reconstitution of ATP-driven NADH–NADP transhydrogenase activity. F_1-depleted vesicles from *E. coli* K12 (215 μg) were incubated with increasing concentrations of complete F_1 (circles) or $F_1(\alpha\beta\gamma)$ and 1.1 μg of δ + 1.4 μg of ϵ (×). The actual amounts of $F_1(\alpha\beta\gamma)$ were increased by a factor of 1.10 in order to compensate for the molecular weight difference between the two enzymes used. For other conditions, see Methods. The sigmoidal curve is due to the fact that increasing reconstitution of F_1·F_o enhances both proton translocation and coupling efficiency by decreasing proton leakiness (Smith & Sternweis, 1975).

Results

Preparation of $F_1(\alpha\beta\gamma)$, δ, and ϵ. $F_1(\alpha\beta\gamma)$ prepared by the chloroform–DMSO method and purified by ion-exchange and gel permeation chromatography is an electrophoretically homogeneous enzyme devoid of subunits δ and ϵ (Figure 1). The presence of functional δ and ϵ was tested in experiments where the formation of complete F_1 from this preparation of $F_1(\alpha\beta\gamma)$ and added ϵ or δ could be detected. Within the experimental error margin (≈2%), no ATP-driven transhydrogenase, an activity dependent on the presence of $F_1(\alpha\beta\gamma\delta\epsilon)$, was found when $F_1(\alpha\beta\gamma)$ was assayed in the presence of F_1-depleted vesicles and additional ϵ or δ. With both subunits and $F_1(\alpha\beta\gamma)$ present, however, this activity can be restored to the same extent as with complete F_1 (Figure 2), indicating that the new preparations of $F_1(\alpha\beta\gamma)$, δ, and ϵ are fully active. The ATPase activity (45–59 units/mg of protein) of $F_1(\alpha\beta\gamma)$ is inhibited to about 90% by a 3-fold excess of ϵ. An analysis of inhibition data at increasing concentrations of ϵ and $F_1(\alpha\beta\gamma)$ indicates that inhibitory ϵ binds in a 1:1 stoichiometry and in a K_i range of 0.2–0.7 nM (Figure 3).

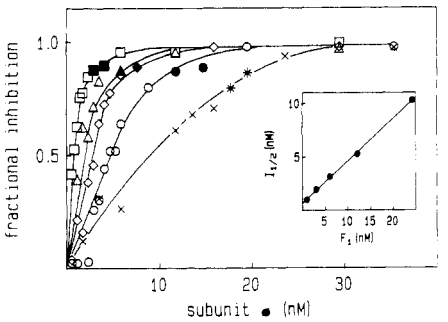

FIGURE 3: Inhibition of $F_1(\alpha\beta\gamma)$ by subunit ϵ. $F_1(\alpha\beta\gamma)$ and ϵ were incubated for 2 min in the assay buffer before starting the reaction with the addition of ATP and phosphoenolpyruvate. $F_1(\alpha\beta\gamma)$ (μg/mL, nM): (squares) 0.35, 1.01; (triangles) 1.05, 3.02; (diamonds) 2.1, 6.04; (circles) 4.2, 12.1; (×) 8.4, 24.2. Other conditions as described under Methods. Inset: Plot of $I_{1/2}$ vs $[F_1(\alpha\beta\gamma)]$. The slope is 0.40 ± 0.07. Values of 0.5 or 1.0 are expected for $\epsilon/F_1(\alpha\beta\gamma) = 1$ or 2, respectively (see Methods). The intercept, K_i, is 0.67 ± 0.09 nM. Calculations of K_i from individual points (closed symbols and *) yielded 0.35 ± 0.17 nM, in good agreement with a value of 0.29 nM reported by Dunn (1982).

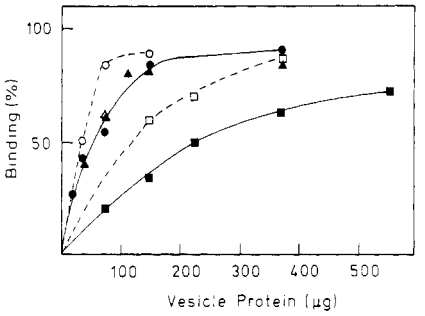

FIGURE 4: Binding of $F_1(\alpha\beta\gamma)$ to F_1-depleted membrane vesicles mediated by subunits δ and ϵ. $F_1(\alpha\beta\gamma)$ (84 μg/mL), δ (14 μg/mL), ϵ (11 μg/mL), and increasing concentrations of F_1-depleted vesicles of strain KY7485 in Tris·HCl (50 mM, pH 8.0) (closed symbols) or MOPS (50 mM, pH 6.5) (open symbols) and $MgCl_2$ (10 mM) were assayed for binding. δ, triangles; ϵ, squares; $\delta + \epsilon$, circles.

Binding of $F_1(\alpha\beta\gamma)$. F_1-depleted membrane preparations from wild-type (K12) and overproducing strains of *E. coli* (AN1460, KY7485), and F_o incorporated into liposomes, have been used to study binding of $F_1(\alpha\beta\gamma)$ by F_o. In previous reports (Futai et al., 1974; Smith & Sternweis, 1975, 1977; Sternweis, 1978), the attachment of F_1 to F_o could be observed only when all five ATPase subunits were present. Unexpectedly, with the present preparation of $F_1(\alpha\beta\gamma)$, significant binding to F_o-containing vesicles is induced by either δ or ϵ alone. It is seen in Figure 4 that approximately 90% of $F_1(\alpha\beta\gamma)$ can be bound when excess F_1-depleted particles are present. Although none of the typical activities of a complete ATP synthase complex, $F_1(\alpha\beta\gamma\delta\epsilon)\cdot F_o$, could be detected (see below), the binding observed is specific for F_o (Table I).

Binding of $F_1(\alpha\beta\gamma)$ induced by δ and ϵ is not additive. Figure 5 (left) shows that even at low subunit concentrations there is less binding in the presence of both subunits than expected from the individual contributions of δ and ϵ. The apparent stoichiometry of $F_1(\alpha\beta\gamma)/\epsilon$ and $F_1(\alpha\beta\gamma)/(\delta + \epsilon)$ (1.05 and 1.16; Figure 5, right) is unity as expected (Sternweis, 1978). The value for $F_1/\alpha\beta\gamma)/\delta$ (0.6) corresponding to 1.7 copies of $\delta/F_1(\alpha\beta\gamma)$, is in agreement with results obtained by Smith and Sternweis (1977) using $F_1(\alpha\beta\gamma\epsilon)$ and δ. However, these numbers do not necessarily imply higher stoichiometries. Curves calculated with varying dissociation constants show that weak binding of subunit δ to $F_1(\alpha\beta\gamma)$ and of $F_1(\alpha\beta\gamma\delta)$ to the vesicles can also account for these data (Figure 5, right panel).

Table I: Binding of $F_1(\alpha\beta\gamma)$ by F_o-Containing Membranes As Mediated by Subunits δ and ϵ[a]

membrane	max binding (% of total F_1)		subunit/F_1 (mol/mol) at half-max binding	
	δ	ϵ	δ	ϵ
AN1460	90	93	0.85	0.5
K12	64	68	1.05	0.5
F_o–liposomes	80	50	0.95	0.6
liposomes	6	0		

[a] F_1-depleted vesicles (AN1460 protein, 563 μg; K12 protein, 1720 μg), $F_1(\alpha\beta\gamma)$ (20 μg), and increasing concentrations of δ or ϵ were assayed for binding in 160 μL of a buffer containing 50 mM Tris·HCl, pH 8, and 10 mM $MgCl_2$. Binding by F_o–liposomes (≈3500 μg) was assayed with $F_1(\alpha\beta\gamma)$ (10 μg) and increasing amounts of δ or ϵ (in the control, 1.9 μg of δ and 1.4 μg of ϵ) in 115 μL of the same buffer containing 10% (v/v) glycerol. The F_1/F_o ratio was 0.30, 0.29, and 0.20 in AN1460, K12, and liposomes, respectively.

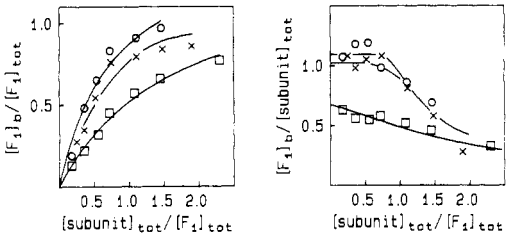

FIGURE 5: Binding of $F_1(\alpha\beta\gamma)$ to F_o mediated by δ, ϵ, and $\delta + \epsilon$. $F_1(\alpha\beta\gamma)$ (78 μg/mL) and an approximately 4-fold excess of F_1-depleted particles (strain KY7485, 2.93 mg/mL) were assayed for binding in the presence of increasing concentrations of δ, ϵ, or $\delta + \epsilon$ in 50 mM MOPS, pH 6.6, and 10 mM $MgCl_2$. (Left) Bound F_1 vs molar concentration ratio of total subunit to total F_1. (Right) Molar concentration ratios bound F_1/total subunit vs total subunit/total F_1. Subunit δ, squares; ϵ, (×); $\delta + \epsilon$, circles. When both δ and ϵ were used, they were applied in an equimolar ratio, and the amount of only one subunit was used in the calculation of the abscissa values. The curve for δ in the right panel has been calculated by using dissociation constants of 1.5 μM and 60 nM for the binding of δ to $F_1(\alpha\beta\gamma)$ and of $F_1(\alpha\beta\gamma)$ to vesicles, respectively, assuming a molar ratio $\delta/F_1(\alpha\beta\gamma) = 1$ (see Methods).

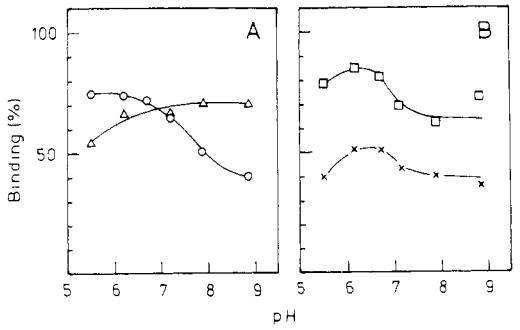

FIGURE 6: Effect of pH on the binding of $F_1(\alpha\beta\gamma)$ to F_o-containing vesicles. $F_1(\alpha\beta\gamma)$ (84 μg/mL), F_1-depleted vesicles (strain KY7485), δ (14 μg/mL), and/or ϵ (11 μg/mL) were assayed for binding at different pH values in the presence of MES (25 mM), Tris (25 mM), and $MgCl_2$ (10 mM). Vesicle concentrations: (A) 1.86 mg/mL (ϵ, circles); 0.74 mg/mL (δ, triangles); (B) 0.74 mg/mL ($\delta + \epsilon$, squares); 0.37 mg/mL ($\delta + \epsilon$, ×).

Effect of pH. The pH dependence of binding of $F_1(\alpha\beta\gamma)$ induced by δ, ϵ, and $\delta + \epsilon$, already apparent in Figure 4, is shown in Figure 6 over a range covering the stability of the enzyme. For ϵ, the optimum is at pH 6 or below, while binding induced by δ increases toward alkaline pH values (Figure 6A). The independent contributions of δ and ϵ can be clearly discerned in the binding induced by both subunits. In Figure 6B, the pH dependency appears to be a composite of the individual curves. These data suggest that δ- or ϵ-induced binding of $F_1(\alpha\beta\gamma)$ to F_o occurs at sites similar to or identical with those

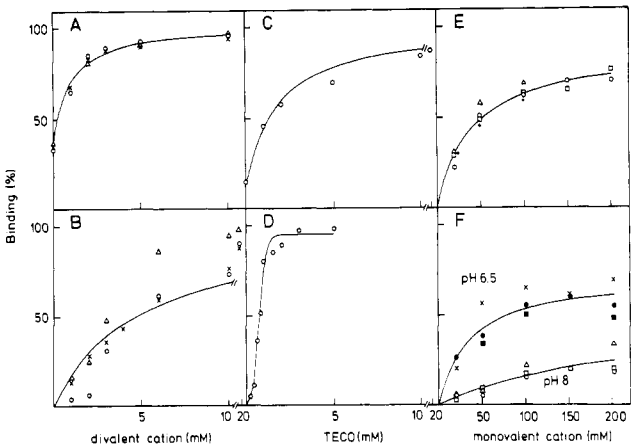

FIGURE 7: Effect of cations on the binding of $F_1(\alpha\beta\gamma)$ to F_o-containing vesicles in the presence of subunits δ or ϵ. F_1-depleted vesicles, $F_1(\alpha\beta\gamma)$, and δ (upper panels) or ϵ (lower panels) were assayed for binding at increasing concentrations of di-, tri-, and monovalent cations. (A and B) Divalent cations ($MgCl_2$, ×; $MnCl_2$, triangles; $CaCl_2$, circles); F_1-depleted vesicles, 3.5 mg/mL (A, strain AN1460; B, strain KY7485); $F_1(\alpha\beta\gamma)$, 130 μg/mL; δ, 19 μg/mL (A); ϵ, 14 μg/mL (B); 50 mM Tris·HCl, pH 8. Binding in the absence of added cations (A) due to endogenous magnesium ions (~0.3 mM) can be abolished by 2 mM EDTA. (C and D) Tris(ethylenediamine)cobalt(III) chloride: TECO (the effect of the corresponding chromium salt is quantitatively similar); F_1-depleted particles, 3.72 mg/mL (strain KY7485); $F_1(\alpha\beta\gamma)$, 100 μg/mL, δ, 15 μg/mL (C); ϵ, 7 μg/mL (D); 50 mM Tris·HCl, pH 8 (C), or a mixture of Tris·HCl, 50 mM, pH 8, and MES, 50 mM, pH 6, final pH 6.6 (D). (E and F) Monovalent cations (LiCl, triangles, NaCl, squares; KCl, circles, RbCl, +; CsCl, ×); F_1-depleted particles (strain KY7485), 3.5 mg/mL; $F_1(\alpha\beta\gamma)$, 125 μg/mL; δ, 19 μg/mL (E); ϵ, 14 μg/mL; EDTA, 2 mM; Tris·HCl, 50 mM, pH 8 (open symbols), or a mixture of Tris·HCl, 50 mM, pH 8, and MES, 50 mM, pH 6, final pH 6.5 (closed symbols and ×).

involved in the binding of complete F_1.

Effect of Ions. Magnesium ions promote strong binding of complete F_1 to F_o which can be easily reversed by chelation. It was of interest, therefore, to investigate the role of ions in the binding of subunit-deficient F_1 mediated by δ or ϵ.

In Figure 7A,B, it is seen that millimolar concentrations of divalent cations are necessary for binding and that magnesium, manganese, and calcium chloride are nearly equally effective.

Highly charged uncomplexed cations such as iron(III) and chromium(III) precipitate at neutral pH, and lanthanum(III) causes F_1 precipitation. With chelated cobalt(III) and chromium(III) salts, it is possible to show that they can substitute for divalent cations in the binding of $F_1(\alpha\beta\gamma)$ and δ or ϵ (Figure 7C,D).

Alkali salts at concentrations 10-fold higher than those necessary with divalent cations cause appreciable binding of $F_1(\alpha\beta\gamma)$ in the presence of δ or ϵ at near-optimal pH (Figure 7E,F). Since full binding does not seem to occur at the highest salt concentrations used, a population of $F_1(\alpha\beta\gamma)$ might exist which is unable to bind under such conditions. This possibility has been tested in experiments where $F_1(\alpha\beta\gamma)$ and ϵ were repeatedly incubated with new batches of F_1-depleted particles. In the presence of 200 mM LiCl or KCl, at least 90% of $F_1(\alpha\beta\gamma)$ became membrane bound after four incubations (data not shown).

After binding of $F_1(\alpha\beta\gamma)$ and δ or ϵ, F_1-depleted membranes do not recover energy-transducing activities such as ATP-driven transhydrogenase or ATP-dependent membrane energization as measured by ACMA fluorescence quenching or spectral response to oxonol VI. This has been ascertained under assay conditions where up to 40% of $F_1(\alpha\beta\gamma)$ was bound (data not shown). However, in the presence of a full set of F_1 subunits, NADH-dependent ACMA fluorescence

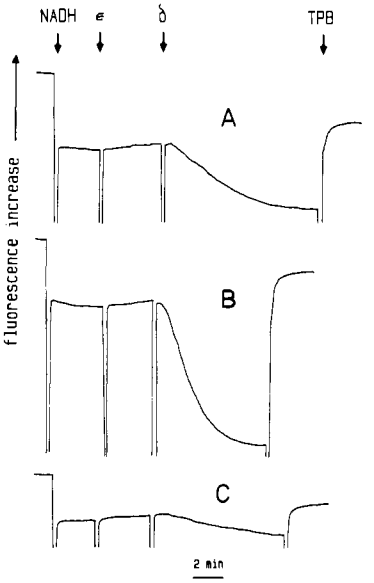

FIGURE 8: ACMA fluorescence quenching of membranes reconstituted in the presence of mono-, di-, and trivalent cations. To F_1-depleted vesicles (ML308/225, 0.62 mg) and $F_1(\alpha\beta\gamma)$ (25.6 μg) in 2 mL of a buffer containing ACMA (0.75 μM), MOPS (50 mM, pH 7.0), and (A) $CaCl_2$ (20 mM), (B) NaCl (200 mM), or (C) tris(ethylenediamine)cobalt(III) chloride (5 mM) were added NADH (0.75 mM), ϵ (2.1 μg), δ (3.5 μg), and sodium tetraphenylborate (TPB, 5 μM). Excitation and emission wavelengths, 410 and 490 nm, respectively.

Table II: DCCD Sensitivity of the ATPase Activity in Partially Reconstituted Membrane Vesicles[a]

	ATPase act.[b] (μM ATP/min)	
	-DCCD	+DCCD
vesicles	41	3
$F_1(\alpha\beta\gamma)$	36	32
vesicles + $F_1(\alpha\beta\gamma)$	77 (77)	48 (35)
vesicles + $F_1(\alpha\beta\gamma)$ + δ	78 (77)	46 (48)
$F_1(\alpha\beta\gamma)$ + ϵ	7	7
vesicles + $F_1(\alpha\beta\gamma)$ + ϵ	51 (48)	8 (10)
vesicles + $F_1(\alpha\beta\gamma)$ + δ + ϵ	90	5

[a] Vesicles from strain AN1460 (8 mg/mL), partially depleted in F_1, were incubated with limiting amounts of $F_1(\alpha\beta\gamma)$ (150 μg/mL), 3-fold excess of δ (38 μg/mL), and ϵ (32 μg/mL). Aliquots for ATPase assays were taken in the presence or absence of 50 μM DCCD. [b] Values in parentheses are calculated from component data assuming that bound $F_1(\alpha\beta\gamma\delta)$ and $F_1(\alpha\beta\gamma\epsilon)$ remain DCCD insensitive.

quenching can be demonstrated with mono-, di-, and trivalent cations (Figure 8), indicating that correct binding of F_1 is possible with all types of salts tested.

DCCD Sensitivity of Bound $F_1(\alpha\beta\gamma)$. The inhibition of the ATPase activity by DCCD is not demonstrably energy linked but nevertheless closely associated with the capability of the F_1·F_o complex to pump protons (Solioz, 1984). It is seen in Table II that no DCCD sensitivity has been detected in reconstituted systems lacking δ and/or ϵ. In addition, these data confirm that in the complete system, subunit ϵ no longer inhibits the ATPase activity (Sternweis & Smith, 1980).

Discussion

Bacterial and chloroplast F_1-ATPase preparations containing only subunits α, β, and γ represent the smallest well-defined subunit arrangements still possessing full ATPase activity. In *E. coli*, methods to remove subunits δ and ϵ from $F_1(\alpha\beta\gamma\delta\epsilon)$ include limited proteolysis with trypsin (Bragg & Hou, 1975), interaction with antibodies (Sternweis, 1978; Dunn, 1986), and HPLC ion-exchange chromatography (Finel et al., 1984). In

a search for methods suitable for large-scale preparation of $F_1(\alpha\beta\gamma)$, treatments with octyl glucoside or glycerol and ethanol similar to those applied to chloroplast F_1 (Patrie & McCarty, 1984; Richter et al., 1984) were tried and found not to be successful with the *E. coli* enzyme. Recently, a method to prepare δ-deficient *E. coli* enzyme in good yield has been developed in our laboratory (Tuttas-Dörschug, 1987), using a modified chloroform extraction originally designed for mitochondrial F_1 (Beechey et al., 1975). With the same procedure applied to a membrane suspension containing 25% DMSO, a three-subunit ATPase has now been obtained in 10-mg quantities which is fully active in reconstitution (Figure 2) and rebinding tests (Figures 4–6). $F_1(\alpha\beta\gamma)$ is potentially valuable for kinetic studies, because δ and ϵ are not firmly enough attached to F_1 to allow the preparation of a completely homogeneous five-subunit ATPase or its maintenance in dilute solution. For the same reason, $F_1(\alpha\beta\gamma)$ may also be useful for growing crystals suitable for X-ray crystallography. By analogy to the chloroplast system (Mitra & Hammes, 1988), $F_1(\alpha\beta\gamma)$ from *E. coli* is expected to retain many of the general features found in the five-subunit enzyme, while also exhibiting interesting subunit-specific differences (Dunn et al., 1987).

In the present work, the binding of F_1 to the membrane segment F_o of the ATP synthase has been studied by using this preparation of $F_1(\alpha\beta\gamma)$ and isolated subunits δ and ϵ. Specific attachment of F_1 to the membrane in the absence of either δ or ϵ has been known in the chloroplast system (Andreo et al., 1982; Roos & Berzborn, 1982; Patrie & McCarty, 1984). It has now been possible to demonstrate this also in the *E. coli* system, probably because the binding components have been used at concentrations that were significantly higher than those applied in earlier studies (Futai et al., 1974; Smith & Sternweis, 1975, 1977; Sternweis, 1978). Two lines of evidence indicate that this type of binding is specific. Most importantly, under the conditions used, it occurs only with vesicles containing F_o (Table I). Second, when comparing the binding of $F_1(\alpha\beta\gamma)$ induced by δ or ϵ with that of complete F_1, the pH dependency of the latter appears to be a composite of the former two (Figure 6). Thus, it is likely that $F_1(\alpha\beta\gamma\delta)$ and $F_1(\alpha\beta\gamma\epsilon)$ bind at sites overlapping with those occupied by $F_1(\alpha\beta\gamma\delta\epsilon)$. Furthermore, the molar ratios $\epsilon/F_1(\alpha\beta\gamma)$ and probably also $\delta/F_1(\alpha\beta\gamma)$ are unity (Figure 5) as expected (Senior & Wise, 1983).

Crucial for a detectable interaction of F_1 and F_o is the presence of cations in at least millimolar concentrations. Surprisingly, not only Mg^{2+} and other divalent cations but also alkali and chelated trivalent cations were effective in promoting structural binding (Figure 7). While binding to Mg^{2+}-specific sites may be important for the stability of the $F_1 \cdot F_o$ complex (Senior et al., 1980), the data presented here argue for an additional, more general role of cations. The most likely explanation would involve electrostatic adsorption of cations to the membrane, leading to a decrease of the negative surface potential and a reorientation of the phospholipid head groups (Seelig et al., 1987). Similar conclusions have been presented for the effect of cations on the binding of mitochondrial (Sandri et al., 1983) and chloroplast F_1 (Telfer et al., 1980) to the corresponding depleted membranes.

The present results show that either δ or ϵ is sufficient to mediate binding of $F_1(\alpha\beta\gamma)$. This is in accord with studies on *E. coli* mutants lacking subunits δ or ϵ (Futai et al., 1988) and binding data from the closely related chloroplast system (Andreo et al., 1982). Since binding of an enzyme as large as $F_1(\alpha\beta\gamma)$ requires considerable energy (30–40 kcal/mol; Janin & Chotia, 1978), other interactions between F_1 and F_o such as those involving subunit β (Aris & Simoni, 1983) may also play a role in stabilizing the $F_1 \cdot F_o$ complex.

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Registry No. ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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Interfacial Properties and Critical Micelle Concentration of Lysophospholipids†

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ABSTRACT: The critical micelle concentration (cmc) of several lysophospholipids and of a lysophospholipid analogue was determined from surface tension measurements using the maximum bubble pressure method and/or ^{31}P NMR. The use of the maximum bubble pressure method has now been extended to micromolar concentrations of surfactant, and the experimental parameters that effect its use have been explored. Surface activity was found to vary with changes in the chain length and in the headgroup polarity of the lysophospholipid. The cmc's for 1-decanoyl-, 1-dodecanoyl-, 1-tetradecanoyl-, and 1-hexadecanoyl-*sn*-glycero-3-phosphocholine are 7.0, 0.70, 0.070, and 0.007 mM, respectively. The cmc's for 1-decanoyl- and 1-dodecanoyl-*sn*-glycero-3-phosphoethanolamine are 4.4 and 0.33 mM, respectively. The cmc for dodecylphosphocholine, a lysophospholipid analogue, was found to be 1.1 mM. The cmc's for 1-tetradecanoyl- and 1-hexadecanoyl-*sn*-glycero-3-phosphoglycerol were found to be 3.0 and 0.60 mM, respectively, in pure water. In 0.1 M Tris-HCl (pH = 8.0), their cmc's are 0.16 and 0.018 mM, respectively. Surface tension and adsorption density values determined at the cmc are reported for each compound. The relationship of dynamic surface tension and lipid purity is discussed. These studies provide information about the micellization and interfacial properties of several biologically important lysophospholipids.

Lysophospholipids comprise a very important subclass of phospholipids that exhibit unique physical and biological properties not found in their parent phospholipids [for review, see Stafford and Dennis (1988)]. Unlike diacylphospholipids, which naturally form bilayers, lysophospholipids are more soluble, and they can disrupt the structure of biological membranes. Although lysophospholipids make up less than 5 mol % of the total phospholipids in a normal cell, higher lysophospholipid concentrations are associated with certain disease states, cell fusion and cell lysis [for review, see Weltzien (1979)]. Very little information has been available on the physical properties of lysophospholipids, other than 1-hexadecanoyllysophosphatidylcholine (Stafford & Dennis, 1988). Only recently have the physical properties of phospholipids and lysophospholipids such as PE[1] (Yeagle & Sen, 1986), lyso-PE (Tilcock et al., 1986), and PG (Macdonald & Seelig, 1987; Seelig et al., 1987; Eklund et al., 1987) been studied in relation to cellular metabolism and membrane structure.

It is well-known that increased phospholipase activity can occur upon the formation of a lipid–water interface [for review, see Dennis (1983)]. In our studies of human amnionic lysophospholipase (Jarvis et al., 1984) and of a lysophospholipase from the macrophage-like cell line P388D_1 (Zhang & Dennis, 1988), we originally observed that the enzymatic activity of these enzymes toward micellar lipids was apparently much greater than their enzymatic activity toward monomeric lipids. In order to study the relationship between the formation of an interface and enzymatic activity, it is necessary to know the cmc of each lipid substrate. cmc's have been reported for

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[1] Abbreviations: cmc, critical micelle concentration; lyso-PC, 1-acyl-*sn*-glycero-3-phosphocholine; lyso-PE, 1-acyl-*sn*-glycero-3-phosphoethanolamine; lyso-PG, 1-acyl-*sn*-glycero-3-phosphoglycerol; PG, 1,2-diacyl-*sn*-glycero-3-phosphoglycerol; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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