c. Consider changes in chemical shifts for axes c and the axis perpendicular to axis c associated with variations of angle β by ±7°. Chemical shift variation induced by a change in β by ±7° is estimated to be $(\sigma_{33} - \sigma_i)[\cos(\beta - 7) - \cos(\beta + 7)] = 21$ ppm for the component along the axis perpendicular to the axis c and $(\sigma_{33} - \sigma_i)[\sin(\beta - 7) - \sin(\beta + 7)] = 12$ ppm for the component along the c axis. Here σ_i is the isotropic chemical shift, $(\sigma_{11} + \sigma_{22} + \sigma_{33})/3$. In other words, the variation of angle β emphasizes the change in the chemical shift for the parallel component more than for the perpendicular. A difference of 9 ppm observed between the line widths for the parallel and perpendicular spectra can be quantitatively interpreted in terms of a variation of angle β by ±7°.

The hypothesis mentioned above may also be supported by the facts that the spectra of the perpendicularly oriented fibers do not exhibit doublet patterns even at low temperatures and that the spectra of the parallel-oriented fibers exhibit a wide range of chemical shift dispersion for the C-form LiDNA at 25 °C and for NaDNA at -80 °C at which temperatures both DNA molecules are essentially rigid. Thus, it can be concluded that all of our ^{31}P NMR spectra from DNA fibers are interpreted consistently in terms of the presence of significant variations in the backbone conformation of DNA; the magnitude of the variation in phosphodiester orientations may reach as much as ±20° from an average conformation. Yet it remains to be established to what extent variation in the backbone conformation can be allowed in interpreting X-ray diffraction data from DNA fibers.

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Characterization of the Inhibitory (ϵ) Subunit of the Proton-Translocating Adenosine Triphosphatase from *Escherichia coli*†

Paul C. Sternweis‡ and Jeffrey B. Smith*

ABSTRACT: The inhibitory subunit (ϵ) of the F_1 adenosine triphosphatase (ATPase) was purified to homogeneity from the ML 308-225 and K12(λ) strains of *Escherichia coli*. No tryptophan or cysteine was detected in the subunit from either strain. The highly active ϵ from both strains was found to be a globular protein with a Stokes' radius of 18-19 Å. Circular dichroism spectra suggested an α-helix content of ~40%. The molecular weight of ϵ was ~15 000-16 000 by sedimentation equilibrium centrifugation in the presence and absence of guanidinium hydrochloride, molecular sieve chromatography, and gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea. The $s_{20,w}$ of ϵ was ~1.6 s^{-1}. Inhibition of the purified F_1 ATPase by ϵ displayed noncompetitive kinetics with a K_i of ~10 nM. The inhibition of the ATPase was rapidly reversed by diluting the enzyme-ϵ mixture. [^{125}I]ϵ which was incorporated into ECF_1 was readily displaced by unlabeled ϵ. ϵ had no significant effect on the ATPase activity of "native" or reconstituted everted membrane vesicles under a variety of assay conditions. Combining the ϵ-inhibited F_1 ATPase with its hydrophobic portion in everted membrane vesicles reconstituted the reversible proton-translocating ATPase and restored nearly full ATPase activity. These results suggest that ϵ inhibits the enzyme only when the F_1 ATPase becomes detached from its hydrophobic subunits.

Subunit ϵ[1] is the smallest or fifth subunit of the F_1 ATPase from mitochondria, chloroplasts, and bacteria (Senior, 1973; Pedersen, 1975; Wilson & Smith, 1978). Homogeneous ϵ from *Escherichia coli* (Smith & Sternweis, 1977) and chloroplasts

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[1] Abbreviations used: F_1, that portion of the proton-translocating ATPase which is peripheral to the membrane; F_0, that portion of the enzyme which is an integral component of the membrane; ECF_1, F_1 from *E. coli*; MF_1, F_1 from mitochondria; α, β, γ, δ, and ϵ, individual subunits of F_1 in order of decreasing size; ATPase, adenosine triphosphatase; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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(Nelson et al., 1972) strongly inhibits the respective F_1 ATPase, suggesting a regulatory role for ε in proton transport or oxidative phosphorylation.

The first protein inhibitor of an ATPase was isolated from bovine heart mitochondria by Pullman & Monroy (1963). Recently, Cintrón & Pedersen (1979) described the purification of a potent inhibitor protein from rat liver mitochondria. Experiments with submitochondrial particles implicated the inhibitor in the regulation of energy transfer between the respiratory chain and F_0F_1 (Asami et al., 1970; van de Stadt et al., 1973).

The inhibitor from bovine heart mitochondria not only blocks the ATPase activity of the particles but also depresses the ATP–P_i exchange reaction and other ATP-dependent energy-transfer reactions (Pullman & Monroy, 1963; Ernster et al., 1973; Lang & Racker, 1974). On the other hand, neither ATP synthesis nor any of the other reactions driven by energy derived from respiration were affected by the inhibitor (Pullman & Monroy, 1963; Asami et al., 1970). However, some recent observations have led Harris and coworkers to suggest that the *initial* rates of phosphorylation by chloroplasts and submitochondrial particles are governed by the large portion of ATPase molecules which are "switched off" by the ATPase inhibitor protein (Harris & Crofts, 1978; Harris et al., 1979).

The F_1 inhibitor from bovine heart mitochondria is probably distinct from the fifth MF_1 subunit as there appear to be qualitative differences in their amino acid compositions (Senior, 1973). Knowles & Penefsky (1972) showed that the fifth subunit of the bovine heart F_1 weakly inhibited F_1 ATPase activity. However, since poly(lysine) and cytochrome *c* are known to be weakly inhibitory toward the F_1 ATPase from *Alcaligenes faecalis* (Adolfsen & Moudrianakis, 1976), the significance of the weak inhibition by the fifth subunit of the bovine heart F_1 remains unclear. Consequently, the role of the fifth F_1 subunit in the regulation of the proton-translocating ATPase is still putative.

ε from ECF_1 was recently shown by Sternweis (1978) to be required along with subunit δ for the attachment of ECF_1 to the more hydrophobic subunits of the enzyme in the plasma membrane and therefore the reconstitution of oxidative phosphorylation and ATP-driven transhydrogenation in everted membrane vesicles. Here we report some structural and functional properties of ε which was purified to homogeneity from two strains of *E. coli*.

Experimental Procedures

Preparations. The ML 308-225 and K12(λ) strains of *E. coli* were grown as previously described (Smith & Sternweis, 1977). A detailed description of the method we used for purifying ECF_1 and its ε subunit can be found in Smith & Sternweis (1977).

The chloramine-T method was used for iodinating purified ε from the ML strain. About 1 mCi of carrier-free iodine-125 (New England Nuclear) was added to 20 μL of 0.5 M potassium phosphate (pH 7.5) containing 75 μM KI and ~10 μg of ε. The chloramine-T (100 μg in 5 μL of water) was added with mixing, and the reaction was immediately stopped with 0.1 mL of sodium metabisulfate (2.5 mg/mL). After the addition of 5 μL of KI (25 mg/mL) as carrier, free iodide was separated from ε by passing the mixture through a Sephadex G-50 column (20 × 1.5 cm). About half of the label coeluted with the protein peak.

For incorporation of the radiolabeled subunit into ECF_1, 2 mg of δ-deficient, four-subunit enzyme (α, β, γ, and ε) from the K12(λ) strain was cold inactivated as previously described (Larson & Smith, 1977) and mixed with 0.3 mL of [^{125}I]ε (6×10^7 cpm). Then the enzyme was reactivated by dialysis (Larson & Smith, 1977) which removed ~60% of the ^{125}I, indicating that it was probably not covalently bound to ε. The enzyme was precipitated by adding solid ammonium sulfate to 65% saturation, applied to a Bio-Gel A-1.5m column (85 × 1.5 cm), and eluted with the same buffer used for purifying ECF_1 [50 mM Tris-HCl, pH 7.3, 2 mM EDTA, 1 mM ATP, 1 mM DTT, and 10% (v/v) glycerol]. It eluted in a single peak of ATPase activity which contained ~15% of the applied radioactivity. The remainder of the ^{125}I eluted in a second peak at the same volume as free ε. No radioactivity eluted in the included volume of the column. The fractions containing the enzyme were combined, concentrated by ultrafiltration (Amicon, UM-10 membrane), and chromatographed again on the same column; ATPase and ^{125}I eluted as single coincident peaks with a constant specific radioactivity of 10000–11000 cpm/unit of ATPase.

Analytical Procedures. ATPase activity was assayed by measuring the liberation of inorganic phosphate from MgATP as previously described (Futai et al., 1974). A unit of ATPase activity is defined as 1 μmol of P_i liberated per min under these assay conditions. The cited references contain descriptions of the methods used for assaying oxidative phosphorylation (Hertzberg & Hinkle, 1974) and ATP-driven reduction of NADP by NADH (Smith & Sternweis, 1977) and quantitating protein (Lowry et al., 1951).

The inhibition of the ATPase by ε was assayed as previously described (Smith et al., 1975; Smith & Sternweis, 1977). Briefly, about 10 or 20 μL of ε (about 50 μg/mL) in purification buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 mM DTT) was mixed with about 0.01 unit of purified ECF_1 (~100 units/mg) at room temperature in 0.1 mL of the above buffer. After a few minutes 0.2 mL of 2 mM Tris-HCl, pH 8, containing 20 μg/mL defatted bovine serum albumin was added, followed by 0.3 mL of a 37 °C solution containing 50 mM Tris-HCl, pH 8, 4.0 mM $MgCl_2$, and 8.0 mM NaATP (Sigma). The serum albumin prevents the loss of ATPase activity which may occur when the enzyme is very dilute. After 10 min at 37 °C the ATPase reaction was terminated and the amount of inorganic phosphate liberated was measured.

Polyacrylamide gel electrophoresis was carried out in three ways: the Weber & Osborn (1969) method using sodium dodecyl sulfate; the Swank & Munkres (1971) method using 8 M urea and sodium dodecyl sulfate as denaturants; a neutral pH discontinuous system developed by T. Jovin. For the latter system, the running gel (5% acrylamide) contained 44 mM Tris adjusted to pH 7.5 with HCl and the stacking gel (3% acrylamide) contained 44 mM Tris adjusted to pH 6.6 with H_3PO_4. The running buffer contained 33 mM Tris and 40 mM Tricine. The ratio of acrylamide to *N,N'*-methylenebis(acrylamide) was 30:1, and no denaturant was used. The running gels (8 cm) were made in tubes (5 mm i.d.) with a 1-cm stacking gel. Electrophoresis was carried out at 25 °C and 2 mA/tube until the phenol red tracking dye came to within ~1 cm of the bottom of the tube. The gels were then removed from the glass tubes, and a fine wire was inserted to mark the position of the tracking dye before staining them with Coomassie blue.

Amino acid analysis of ε was done with a Beckman Model 120C analyzer at high sensitivity. About 100 μg of ε was hydrolyzed with 3 N mercaptoethanesulfonic acid (Pierce) at 100 °C for varying times (Penke et al., 1974). Cysteine content was determined as cysteic acid following performic

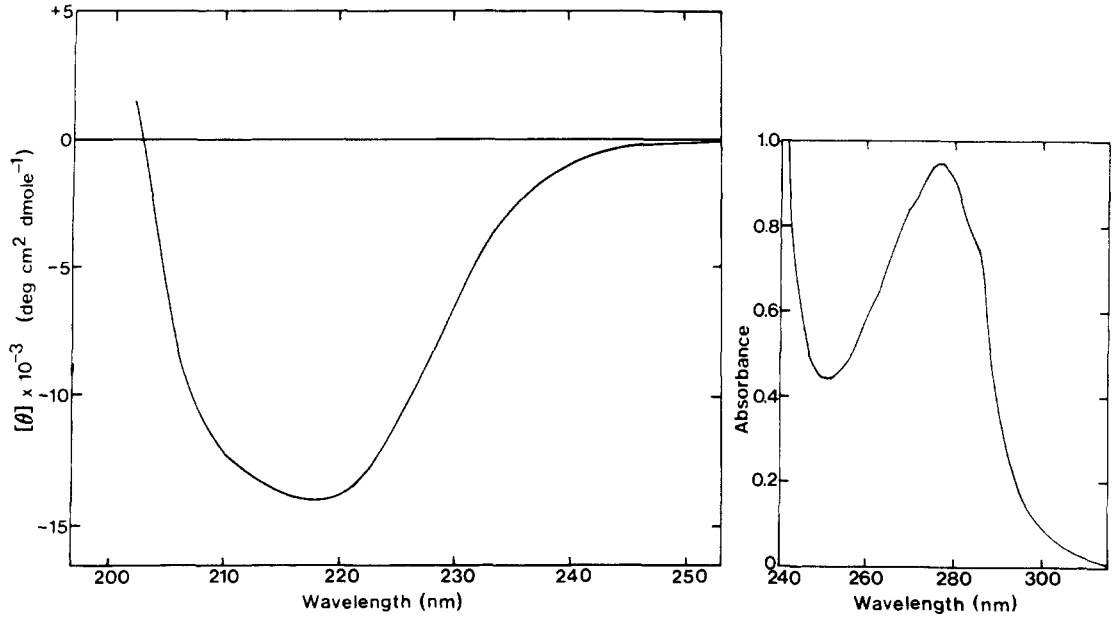

FIGURE 1: Ultraviolet absorption and circular dichroism spectra of ϵ. The absorption spectrum (right) was obtained at room temperature in 100 mM phosphate, pH 7.0, by using a Cary dual-wavelength spectrophotometer. The CD spectrum (left) was obtained with a Cary 60 recording spectropolarimeter at a protein concentration of 1.3 mg/mL and a cell path length of 1 mm. ϵ was derived from the ML 308-225 strain.

acid oxidation (Moore, 1963).

Sedimentation analysis of ϵ was carried out with a Beckman Model E ultracentrifuge equipped with absorption optics and a computer-linked scanning system for data collection and analysis (Crepeau et al., 1974).

Results

Subunit ϵ was purified from both the ML 308-225 and K12(λ) strains of *E. coli* after treating the purified ECF_1 with pyridine to dissociate ϵ from the enzyme (Smith & Sternweis, 1977). For larger quantities of the subunit (1–2 mg), several preparations were combined, concentrated by ultrafiltration (UM-10, Amicon), and chromatographed a second time on a G-75 (Sephadex) column, to remove minor impurities. Various subunit preparations were considered sufficiently pure if only a single band was present after gel electrophoresis in the presence of $NaDodSO_4$. Moreover, ϵ from K12(λ) migrated as a single, sharp band when 10 μg was electrophoresed in the absence of any denaturant at neutral pH by using a discontinuous system (see Experimental Procedures). No significant differences have been observed in the structural or functional properties of ϵ from the two strains of *E. coli*. The F_1 ATPase from both strains is equally sensitive to inhibition by ϵ from both strains.

Molecular Weight. Previously, the size of ϵ was estimated to be 12 000 by sodium dodecyl sulfate gel electrophoresis of purified ECF_1 (Bragg & Hou, 1972; Futai et al., 1974; Hanson & Kennedy, 1973). The purified ϵ gave this value as well (Table I). However, this method appears to underestimate the size of ϵ by ~25%. Electrophoresis in the presence of $NaDodSO_4$ and 8 M urea gave a value of 15 000–16 000 for ϵ from both *E. coli* strains, as did equilibrium centrifugation and gel filtration chromatography (Table I). Since this value was obtained by sedimentation in the presence and absence of guanidinium hydrochloride as well as molecular sieve chromatography, the active inhibitory subunit appears to be composed of a single polypeptide chain, probably with a globular shape. The Stokes' radius of ϵ was estimated by comparison with the sieving properties of reference proteins (Ackers, 1964) to be 18–19 Å, as compared to a minimum calculated radius of 17 Å by assuming a spherical shape.

Table I: Molecular Weight of the ϵ Subunit

method	strain[a]	M_r	trials	av error
Sephadex G-75[b]	ML	15 700	2	300
	K12	16 500	2	350
$NaDodSO_4$ gel electrophoresis[c]				
Weber-Osborn	ML	11 700	3	400
	K12	12 000	1	
Swank-Munkres	ML	16 700	1	
	K12	16 500	1	
sedimentation equilibrium[d]				
in phosphate buffer	ML	15 900	4	300
	K12	15 300	2	450
in phosphate buffer + 6 M guanidine hydrochloride	ML	13 900	1	

[a] ϵ obtained from either *E. coli* strain ML 308-225 or K12(λ) is denoted ML or K12, respectively. [b] Described previously (Smith & Sternweis, 1977). [c] The methods described by Weber & Osborn (1969) and Swank & Munkres (1971) were used as described for K12 ϵ or adapted for use with 1-mm slab gels for analysis of ML ϵ. Results were obtained with gels formed from 12.5% acrylamide and either 0.4 or 0.8% *N,N'*-methylenebis(acrylamide) for the $NaDodSO_4$ or $NaDodSO_4$–urea systems, respectively. [d] Sedimentation equilibrium was performed as described under Experimental Procedures. The partial specific volume was calculated from the amino acid composition (Table II) by the method described by Cohn & Edsall (1943). The calculated value of 0.737 $cm^3\ g^{-1}$ was used for measurements in guanidine hydrochloride as well as for measurements of the native subunit.

In the ultracentrifuge ϵ showed homogeneous sedimentation boundaries and gave an $s_{20,w}$ of 1.6 s^{-1}.

Optical Properties. The ultraviolet absorption spectrum of ϵ (Figure 1) suggested that tyrosine was the dominant chromophore in the molecule and that tryptophan was probably absent. Maximum absorption was at 276 nm with an extinction coefficient $\epsilon_{1cm}^{1\%}$ of 4.0 compared to 3.8 at 280 nm.

Fluorescence spectra of ϵ gave a maximum emission peak at 305 nm with the highest fluorescence occurring with excitation at 280–285 nm. This is characteristic of tyrosine fluorescence and provides additional evidence that the subunit lacked tryptophan.

The circular dichroism spectrum of ϵ (Figure 1) had a single rotational minimum at ~218 nm. The amplitude of the

Table II: Amino Acid Analysis of the ε Subunit

	strain ML 308-225		strain K12	
amino acid	mol %	suggested residue composition/ 15 900 M_r	mol %	suggested residue composition/ 15 900 M_r
Lys	6.18	9	6.77	10
His	3.53	5	4.65	7
Arg	3.30	5	3.92	6
Asp	6.64	10	5.57	8
Thr	4.37	7	4.28	6
Ser	6.56	10	6.09	9
Glu	15.14	23	15.15	22
Pro	3.22	5	3.03	4
Gly	9.16	14	8.94	13
Ala	12.26	18	12.30	18
Val	6.19	9	6.11	9
Met	3.62	5	3.44	5
Ile	6.36	9	6.76	10
Leu	8.26	12	8.54	13
Tyr	2.91	4	2.83	4
Phe	1.94	3	1.62	2
		148		146
% nonpolar	41.8		41.8	

minimum suggests a moderate degree of secondary structure, and analysis by the method of Chen et al. (1974) predicts a structure with ~40% α helix.

Amino Acid Content. The amino acid composition of ε from both strains was very similar (Table II). The subunit contained neither tryptophan nor cysteine. The overall composition revealed no unusual properties and appears somewhat similar to the composition of ε obtained from the F_1 of mitochondria (Knowles & Penefsky, 1972; Brooks & Senior, 1972) and chloroplasts (Nelson et al., 1972). Obvious dissimilarities are the presence of one cysteine in the subunit from the eucaryotic F_1 molecules (Senior, 1975; Nelson et al., 1972) and the absence of tyrosine in the ε subunit from chloroplasts. On the other hand, the amino acid composition of ε differs markedly from that of the mitochondrial ATPase inhibitor which is composed of over 25% basic amino acids and lacks threonine, proline, and methionine (Brooks & Senior, 1971).

Recently, Nieuwenhuis & Bakkenist (1977) reported the amino acid composition of the ε subunit of the ATPase from the A428 strain of *E. coli*. The subunit was purified by sodium dodecyl sulfate gel electrophoresis, appeared to lack tyrosine, and had different ratios of other amino acids compared to ε from the two strains we studied.

Kinetics and Reversal of ε Inhibition. Previously we showed that ε is a potent inhibitor of the hydrolytic activity of purified ECF_1 (Smith et al., 1975). Figure 2 shows that the inhibition was noncompetitive with respect to the substrate, ATP, and the K_i was estimated to be ~10 nM, which agrees well with the concentration of ε previously reported to half-maximally inhibit the enzyme (~15 nM; Smith & Sternweis, 1977).

If the interaction between ε and the core subunits (α, β, and γ) of ECF_1 is governed by an association–dissociation equilibrium, then inhibition should be reversed by lowering the concentration of the free subunit. Figure 3 shows that diluting the ε–ECF_1 mixture prior to the ATPase assay reversed the inhibition of the ATPase by ε. First, ECF_1 was inhibited maximally (80%) by a high ε concentration. Then a sample of the mixture was diluted 50-fold and assayed at various times after dilution. The half-time for the reversal of ε inhibition was of the order of 1 min at 37 °C, which was the duration of the ATPase assay. The 15% inhibition remaining after dilution is consistent with the amount of inhibition expected

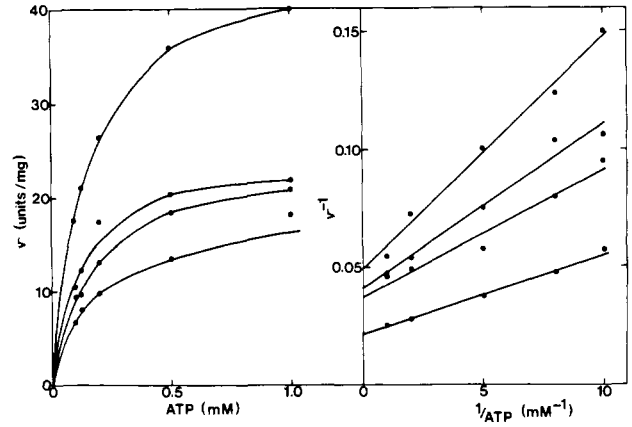

FIGURE 2: Effect of ATP concentration on the inhibition of the ECF_1 ATPase by ε. Purified ECF_1 (K12) and ε (K12) were diluted with 2 mM Tris-HCl, pH 8, containing 10 μg/mL bovine serum albumin. The reaction mixture (0.6 mL) contained the following components besides the indicated level of ATP: 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 8, 5 mM KCl, 0.1 mg/mL bovine serum albumin, 17 mM phosphoenolpyruvate, 5 μg of pyruvate kinase. The ATPase reaction was started by enzyme addition (0.2 or 0.4 μg in 0.1 mL) and stopped after 15 or 30 min at 37 °C as previously described (Futai et al., 1974). (Left) Top curve, no ε; next three curves, 0.04, 0.08, and 0.17 μg of ε per mL, respectively. (Right) Double-reciprocal plot of the same data.

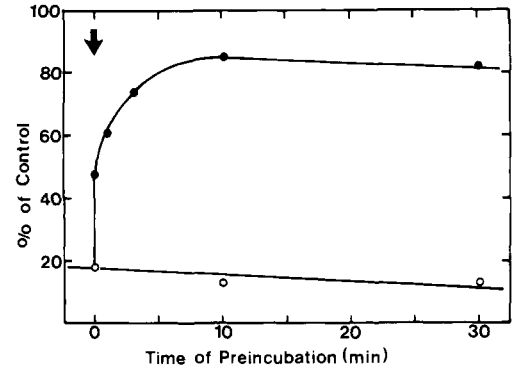

FIGURE 3: Reversal of ε inhibition by dilution. Two units of ECF_1 (ML) (20 μg) was added to 0.40 mL of 2 mM Tris (pH 8), 1 mM ATP, 10 μg/mL BSA, and 10 μg/mL ε (ML) and incubated for 10 min at 37 °C. A control sample was treated in the same manner but contained no ε. At time 0 (arrow), 10-μL samples were diluted into 0.5 mL of 2 mM Tris–BSA either with (○) or without (●) 6–7 μg/mL ε and incubated at 37 °C. At the times indicated, 0.1 mL of 120 mM Tris–HCl (pH 8), 24 mM ATP, and 12 mM $MgCl_2$ was added to a sample and the reaction was allowed to proceed for 1 min at 37 °C.

by the final ε concentration (0.15 μg/mL). No reversal of inhibition occurred when the enzyme was diluted 50-fold into buffer containing the same high concentration of ε (Figure 3).

The kinetics of ε inhibition and its reversibility suggest that the subunit is in rapid equilibrium with the enzyme. To obtain structural evidence for an equilibrium binding between ε and the enzyme, we prepared ^{125}I-labeled ε and incorporated it into four-subunit ECF_1 (missing subunit δ). Highly purified ECF_1 containing only α, β, γ, and ε was dissociated into individual subunits by cold inactivation (Larson & Smith, 1977) and mixed with iodinated ε before reconstituting nearly full ATPase activity by dialysis against MgATP. Then the ECF_1 containing labeled ε was purified by gel filtration chromatography. After ECF_1 containing [^{125}I]ε was mixed with unlabeled ε, the mixture was applied to a gel filtration column. Figure 4 shows that unlabeled ε readily displaced the iodinated ε from ECF_1 (part B of Figure 4). No radioactivity eluted in the inclusion

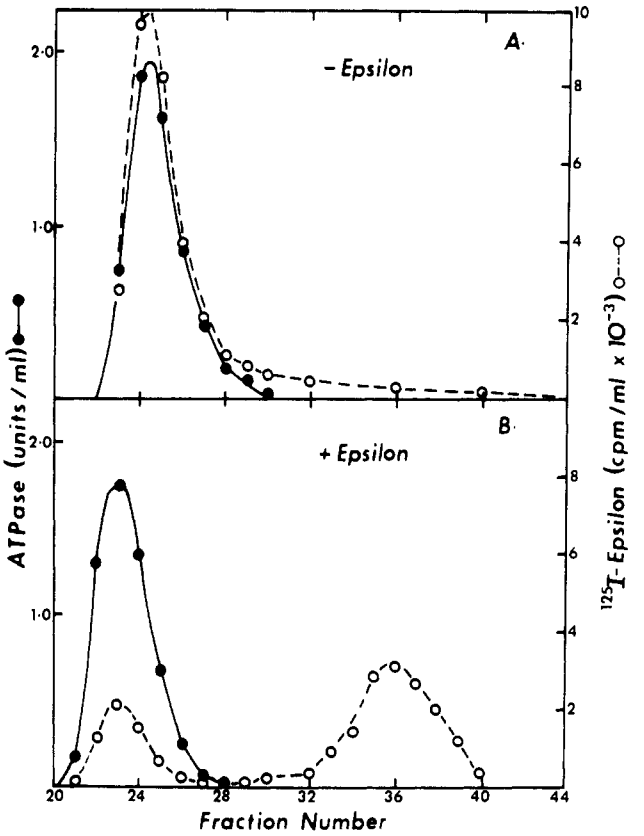

FIGURE 4: Exchange of ECF_1-bound ϵ with free ϵ in solution. (A) Eight units of [^{125}I]ϵ ECF_1 (sp act. ~7000 cpm/unit) was applied to a Sephadex G-75 column (65 × 1 cm) and eluted at room temperature with a flow rate of 4–5 mL/h. The elution buffer consisted of 40 mM Tris-HCl (pH 7.3), 1 mM EDTA, 0.1 mM ATP, 0.1 mM DTT, and 10% glycerol. (B) Eight units of [^{125}I]ϵ ECF_1 (sp act. ~5000 cpm/unit) was mixed with ~40 μg of pure ϵ from the ML strain and incubated at 37 °C for 10 min (both ATPase and ϵ were in a 0.2-mL volume of the buffer in which they were purified). After incubation, the mixture was applied to Sephadex G-75 and eluted as described in (A).

volume. The iodinated ϵ that was displaced eluted at the same volume as purified ϵ, which behaves as a globular protein with a molecular weight of 16 000 on this column (Table I). The three other subunits of ECF_1 (α, β, and γ) are 2 or more times larger than ϵ and would not be expected to coelute with ϵ, suggesting that most of ^{125}I displaced by unlabeled ϵ was tightly associated with ϵ. The exchanged [^{125}I]ϵ eluted from the column in a symmetrical peak, indicating that most of it was released from the enzyme during the 10-min incubation before being applied to the column. This result suggests that the [^{125}I]ϵ which was incorporated into ECF_1 by reconstituting it from subunits is in rapid equilibrium with ϵ in the medium. Without added ϵ (part A of Figure 4) most of the [^{125}I]ϵ remained bound to the enzyme, except for a small amount that trailed behind the ATPase. Thus, some iodinated ϵ dissociated from ECF_1 in the absence of free ϵ as expected.

Although unlabeled ϵ displaced most of the ^{125}I from ECF_1, some ^{125}I did remain bound to ECF_1. The portion of the ^{125}I that was not displaced from ECF_1 by unlabeled ϵ may or may not have been associated with ϵ, since the labeled ϵ contained noncovalently bound ^{125}I and some may have been transferred to other F_1 subunits.

Effect of Membrane Attachment on ϵ Inhibition. In the native form ECF_1 is attached to the cytoplasmic side of the bacterial cell membrane via the hydrophobic F_0 subunits of the enzyme, and the δ (Smith & Sternweis, 1977) and ϵ subunits are required for binding F_1 to F_0 (Sternweis, 1978).

Table III: Attachment of ECF_1 to the F_0 Portion of the Enzyme in Everted Membrane Vesicles Reverses the Inhibition of ATPase Activity by ϵ[a]

additions			ATPase act. (nmol of P_i liberated per min)		inhibn by ϵ (%)
ECF_1 missing δ	depleted membranes (DM)	subunit δ	no ϵ	plus ϵ	
–	+	–	7.7	7.7	0
+	–	–	7.1	3.3	54
+	+	–	14.9 (7.2)	9.8 (2.1)	71
+	+	+	14.9 (7.2)	15.0 (7.3)	0

[a] ATPase activity was assayed for 10 min at 37 °C in the presence of 15 mM Tris-HCl, pH 8, 1 mM ATP, and 2.5 mM $MgCl_2$. The activity in parentheses is that portion due to ECF_1 and was obtained by subtracting the activity due to residual membrane-bound enzyme in the depleted membranes (DM) from the total activity. The ATPase activity of DM was 7.7 nmol/min and was not significantly affected by the presence of ECF_1 or the δ or ϵ subunits. A 5-min incubation at 37 °C preceded the ATPase assay to allow time for the binding of ECF_1 to the membrane. The specific activity of ECF_1 in the presence of 2.5 mM $MgCl_2$ and 1 mM ATP is ~5 units/mg compared to 100 units/mg at 4 mM ATP and 2 mM $MgCl_2$. The excess of Mg compared to ATP, however, is needed for the binding of ECF_1 to DM. ECF_1 missing the fourth (δ) subunit was purified from the K12(λ) strain; the other components (ϵ, δ, and everted membrane particles depleted of ECF_1) were of ML origin.

ϵ has no significant effect on the ATPase when it is membrane bound (Table III and unpublished experiments), in contrast to inhibition of purified ECF_1 (Smith et al., 1975). ϵ failed to significantly effect the membrane-bound ATPase activity of "native" or reconstituted everted membrane vesicles either in the presence or absence of Mg^{2+} and/or ATP when tested at acidic, neutral, or alkaline pH (unpublished experiments). The fact that the membrane-bound enzyme was not inhibited by ϵ suggested that attaching ϵ-inhibited ECF_1 to the membrane might reverse the inhibition. The results in Table III indicate that reattaching ECF_1 to the membrane did in fact reverse ϵ inhibition. For this experiment we used ECF_1 missing subunit δ. In the absence of δ, mixing the ϵ-inhibited enzyme with F_1-depleted membranes neither reattached F_1 to F_0 nor reversed the inhibition by ϵ (Table III). In the presence of δ, the ϵ-inhibited enzyme became membrane bound and full ATPase activity was restored. Subunit δ itself had no effect on the ATPase activity of membrane-bound or purified ECF_1 (unpublished experiments). The reversal of ϵ inhibition when ECF_1 binds to the membrane suggests that the membrane-bound F_0F_1 complex is in an active state and not inhibited by the endogenous ϵ subunit.

Discussion

The results obtained in this investigation have shown that the highly active inhibitory (ϵ) subunit of the proton-pump ATPase from *E. coli* is a single polypeptide chain with a globular shape and a molecular weight between 15 000 and 16 000. Previously, a stoichiometry of one ϵ per ECF_1 was suggested by reconstitution experiments with purified ϵ and ECF_1 missing ϵ (Sternweis, 1978). ϵ inhibition of the purified ATPase was noncompetitive and had an inhibitory constant in the nanomolar range (Figure 2). The inhibition was reversed by diluting the ECF_1–ϵ mixture, and studies with [^{125}I]ϵ provided physical evidence for an association–dissociation equilibrium between ϵ and ECF_1 (Figures 3 and 4). ϵ by itself does not appear to bind to the hydrophobic F_0 portion of the enzyme in F_1-depleted membranes (Sternweis, 1978). Hence, the in vitro reconstitution of ECF_1 missing ϵ to depleted membranes probably proceeds in the following manner. First,

there is an initial complex formation between ε and the rest of ECF_1, which would inhibit its ATPase activity. Then, this complex binds to the membrane, restoring ATPase activity. The reversal of ε inhibition presumably results from an alteration in the tertiary structure of the enzyme induced by the F_0–F_1 interaction. Upon detachment of ECF_1 from the membrane the ATPase would again become inhibited by ε.

Recently, Laget & Smith (1979) obtained evidence that the endogenous ε in purified ECF_1 does in fact inhibit its ATPase activity. The inhibition by the endogenous ε was previously overlooked because diluting the enzyme prior to the assay reversed the inhibition. Curiously, in the membrane-bound form the *E. coli* enzyme does not appear to be in an ε-inhibited state, nor does added ε inhibit the enzyme in membrane vesicles under a variety of assay conditions including the presence of excess Mg^{2+} or ATP and acidic (6.5) or alkaline pH (8) (unpublished experiments). Moreover, attaching ε-inhibited ECF_1 to ECF_1-depeleted vesicles reversed the ATPase inhibition (Table III).

As yet there is no evidence that ε is regulatory in vivo. In fact, the available evidence does not exclude the possibility that ε inhibition results from the artificial detachment of ECF_1 from F_0 during ECF_1 purification. However, even if ε only inhibits the ATPase when it is free of the F_0 portion, this might have physiological significance by protecting cellular ATP during the enzyme's biosynthesis.

Alternatively, if ε does not have a direct regulatory role in vivo, there may be a cytoplasmic protein whose level modulates the effect of ε on the holoenzyme. The mitochondrial F_1 inhibitor, which is probably distinct from the fifth or ε subunit of MF_1 (Senior, 1973), inhibits the mitochondrial ATPase both in the membrane-bound and soluble forms (Pullman & Monroy, 1963; Horstman & Racker, 1970). To further clarify this issue, it would be helpful to know if the fifth F_1 subunit in mitochondria is required for the inhibition of MF_1 by the inhibitor protein or if there is an additional ATPase inhibitor in *E. coli* besides ε.

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