ε Subunit of *Escherichia coli* F_1-ATPase: Effects on Affinity for Aurovertin and Inhibition of Product Release in Unisite ATP Hydrolysis†

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ABSTRACT: The ε subunit of *Escherichia coli* F_1-ATPase is a tightly bound but dissociable partial inhibitor of ATPase activity. The effects of ε on the enzyme were investigated by comparing the ATPase activity and aurovertin binding properties of the ε-depleted F_1-ATPase and the ε-replete complex. Kinetic data of multisite ATP hydrolysis were analyzed to give the best fit for one, two, or three kinetic components. Each form of F_1-ATPase contained a high-affinity component, with a K_m near 20 μM and a velocity of approximately 1 unit/mg. Each also exhibited a component with a K_m in the range of 0.2 mM. The velocity of this component was 25 units/mg for ε-depleted ATPase but only 4 units/mg for ε-replete enzyme. The ε-depleted enzyme also contained a very low affinity component not present in the ε-replete enzyme. In unisite hydrolysis studies, ε had no effect on the equilibrium between substrate ATP and product ADP·P_i at the active site but reduced the rate of product release 15-fold. These results suggest that ε subunit slows a conformational change that is required to reduce the affinity at the active site, allowing dissociation of product. It is suggested that inhibition of multisite hydrolysis by ε is also due to a reduced rate of product release. ε-depleted F_1-ATPase showed little of no modulation of aurovertin fluorescence by added ADP and ATP. Aurovertin fluorescence titrations in buffer containing ethylenediaminetetraacetic acid (EDTA) revealed that ε-depleted enzyme had high affinity for aurovertin (K_d < 0.1 μM) regardless of the presence of nucleotides. In contrast, ε-replete F_1-ATPase had similar high affinity in the presence of nucleotides but much lower affinity, K_d = 3 μM, in the absence of ADP or ATP. In the presence of MgADP, the affinity for aurovertin was lower in the absence of ε (K_d = 0.35 μM) than in its presence (K_d = 0.2 μM). The maximal fluorescence of aurovertin bound to ε-replete F_1-ATPase was substantially lower in the absence of nucleotides or in the presence of Mg^{2+} than in the presence of nucleotide and EDTA, but only small effects on maximal fluorescence were observed for ε-depleted enzyme. Aurovertin titrations of multisite ATPase activity gave $I_{0.5}$ of 0.09 μM in the absence of ε and 0.04 μM in the presence of saturating ε. These results indicate that the ε subunit alters the conformation of the β subunit in a manner that depends on the presence of Mg^{2+} and nucleotides.

The F_1F_o-ATPase has a central role in the bioenergetics of *Escherichia coli*, interconverting the energy stored in ATP with that of the protonmotive force across the cell membrane. The F_o portion, an integral membrane protein composed of three types of subunits, catalyzes the movement of protons across the membrane. The F_1 portion is a peripheral membrane complex composed of five different subunits, designated α through ε in order of decreasing molecular weight, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. ATPase activity is expressed by $\alpha_3\beta_3\gamma$; no single pure subunit has ATPase activity. The active sites are believed to be located on the β subunits or at α–β interfaces [for recent reviews, see Vignais and Satre (1984) and Senior (1985)].

Substantial evidence supports the hypothesis developed by Boyer and co-workers (Rosen et al., 1979; Cross et al., 1984) that F_1-ATPase has multiple catalytic sites, which exist in alternating conformations and positions in the catalytic cycle during steady-state catalysis. Furthermore in this model, the release of product from one site is promoted by the binding of substrate to the next site. Thus if sufficient ATP is present to bind to more than one of the sites, rapid multisite hydrolysis occurs (Cross et al., 1982). However, if only one of the catalytic sites is occupied, the overall rate of hydrolysis is slowed by a factor of 10^4–10^6. This hydrolysis, which is not promoted by binding of subsequent molecules of ATP, is termed unisite hydrolysis (Grubmeyer et al., 1982).

The ε subunit of the complex is a partial noncompetitive inhibitor of the multisite ATPase activity of soluble F_1-ATPase (Sternweis & Smith, 1980). ε has been shown to bind strongly to the isolated γ subunit (Dunn, 1982), and cross-linking experiments with F_1-ATPase show that it can be linked to either γ (Bragg & Hou, 1980) or one of the β subunits (Lotscher et al., 1984a). The dissociation constant for the interaction of ε with ATPase is in the nanomolar range, and the form of the enzyme with ε bound has about 10% of the activity of ε-depleted F_1-ATPase (Sternweis & Smith, 1980; Dunn, 1982, 1986). Due to the tight binding, ε remains with F_1-ATPase during purification procedures that are performed at relatively high protein concentrations but may dissociate during activity assays that are performed on much more dilute solutions (Laget & Smith, 1979). As the enzyme concentration in assays increases, the proportion of F_1-ATPase in the ε-inhibited form also increases, leading to notable nonlinearity of activity with enzyme concentration. This property of existing in multiple forms with different activities causes considerable complications in the study of *E. coli* F_1-ATPase. For example, kinetic analyses are hard to interpret, and results

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obtained by methods requiring high protein concentrations, such as most techniques for the investigation of structure, cannot properly be compared with results obtained by methods that work at low concentrations, such as enzyme activity.

Methods have been developed for the preparation of ϵ (Smith & Sternweis, 1977; Dunn, 1982) and of ϵ-depleted ATPase (Sternweis, 1978; Dunn & Futai, 1980; Dunn, 1986), but only limited comparisons of the activity of the ϵ-depleted F_1-ATPase and the residual activity of the ϵ-saturated enzyme have appeared (Sternweis, 1978; Laget & Smith, 1979). In this paper, we present studies of the effects of the ϵ subunit on unisite and multisite ATP hydrolysis, aurovertin inhibition, and aurovertin fluorescence.

Experimental Procedures

Preparation of F_1-ATPase, ϵ-Depleted F_1-ATPase, and ϵ Subunit. F_1-ATPase (Dunn et al., 1985) and ϵ subunit (Dunn, 1982) were prepared from *E. coli* strain AN1460 by methods described previously. F_1-ATPase was depleted of ϵ subunit by ϵ-4 monoclonal antibody affinity chromatography as described (Dunn, 1986), except that a 4-mL column containing 24 mg of ϵ-4 monoclonal antibody was used and 20 mg of F_1-ATPase was treated in each batch. The ϵ-depleted enzyme was chromatographed on a column of Sephacryl S-400 (85 × 1.5 cm) in 50 mM Tris-HCl,[1] pH 7.4, 10% glycerol, 1 mM EDTA, and 1 mM ATP to remove any dissociated material and stored at −80 °C in this buffer. The preparation was judged to be more than 95% depleted of ϵ subunit, on the basis of scans of SDS–polyacrylamide gels stained with Coomassie blue R-250.

Preparation of Aurovertin D. Calcarisporium arbuscula NRRL 3705 was obtained from the U.S. Department of Agriculture, Agricultural Research Service, North Regional Center, Peoria, IL 61604. The organism was grown on medium F14 (Baldwin et al., 1964) at 23 °C for 3 weeks. Aurovertins were purified from the growth medium by a modification of the method of Linnett and Beechey (1979). The medium was extracted 3 times with one-fifth volume of chloroform. The extracts were pooled and dried under reduced pressure. Aurovertins were extracted from the residue with a few milliliters of methanol, which was then dried, and the crude aurovertins were redissolved in a small volume of chloroform. Aurovertins B and D were purified by preparative thin-layer chromatography on 0.75 mm thick silica gel plates. The solvent was hexane–ethyl acetate (3:7). The two major bands were scraped from the plates, extracted with methanol, and rechromatographed. The aurovertins were identified as aurovertins B and D by analytical thin-layer chromatography (Osselton et al., 1974). The aurovertins were stored in methanol at −20 °C and were protected from light. The concentration of aurovertin D was determined with a molar extinction coefficient of 36000 at 368 nm (Issartel et al., 1983).

Multisite ATP Hydrolysis Assays. Multisite ATP hydrolysis was assayed by incubating the enzyme in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 4 mM ATP, 18 mM phosphoenolpyruvate, 25 mM KCl, 0.2 mg/mL pyruvate kinase, and 20 μg/mL BSA at 30 °C. The assay volume was 0.25 mL. Hydrolysis was stopped by addition of 0.05 mL of 20% sodium dodecyl sulfate; then, the liberated phosphate was determined colorimetrically (Taussky & Shorr, 1983). In cases of ϵ supplementation, ϵ-depleted F_1-ATPase and ϵ were incubated together for 10 min before they were added to the reaction mixture.

For determination of multisite kinetic constants, the ATP content was varied from 3 μM to 20 mM, and $MgCl_2$ was present at 6 mM excess over ATP. Assays were performed in triplicate. Data were analyzed, without averaging, by nonlinear regression, fitting directly to the relationship of velocity to substrate concentration with the SAS NLIN program. Each data set was analyzed assuming either one, two, or three kinetic components, and the significance of improvement of fit when additional components were allowed was determined by *F* test. Data were analyzed both without weighting and with weighting proportional to 1/[S], which produces a better apparent fit to an Eadie–Hofstee plot.

Unisite ATP Hydrolysis Assays. Unisite ATP hydrolysis was assayed at 20 °C in "unisite reaction buffer" containing 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 1 mM potassium phosphate, and 0.1 mg/mL BSA. F_1-ATPase was freed of ATP in the storage buffer by repeated column centrifugation through Bio-Gel P-10, which was equilibrated with 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA. ATPase was added, with rapid mixing using a magnetic stirbar, to [γ-^{32}P]ATP in unisite reaction buffer. At various times thereafter, samples were analyzed in duplicate by the methods described by Grubmeyer et al. (1982). Total [^{32}P]P_i was determined on 50-μL samples by quenching catalysis with 6% $HClO_4$, 4 mM ATP, 2 mM $MgCl_2$, and 1 mM sodium phosphate and then precipitating P_i as described by Sugino and Miyoshi (1964). ATP that had been hydrolyzed or was committed to hydrolysis was determined by adding 1 mM unlabeled ATP 15 s before the acid quench and then determining [^{32}P]P_i. ^{32}P Bound to F_1-ATPase was determined by column centrifugation of 50-μL aliquots on 1-mL columns of Bio-Gel P-10 that were equilibrated with unisite reaction buffer. Recovery of enzyme in this procedure was 70%. All assays were performed in duplicate and averaged.

The rate of product release from the active site was determined by a modification of the procedure described by Penefsky (1986). F_1-ATPase and [γ-^{32}P]ATP were mixed at concentrations of 1 μM each in unisite reaction buffer. ϵ subunit was included at 2 μM in some experiments. After 1 min the enzyme was separated from unbound ^{32}P by column centrifugation on Bio-Gel P-10 equilibrated with the unisite reaction buffer. The eluate was placed at 20 °C, and in some cases ϵ subunit was added to a concentration of 0.5 μM to assure saturation, and then, a zero time point of bound ^{32}P was taken by the column centrifugation method. At various times bound ^{32}P was determined until more than 80% of it had dissociated. The data were corrected for enzyme-bound ^{32}P that did not dissociate even after addition of unlabeled ATP and plotted on semilogarithmic paper.

Experiments to determine the equilibrium of ATP to ADP·P_i at the active site were set up in the same manner. After freeing the enzyme of unbound ^{32}P and again supplementing with 0.5 μM ϵ to maintain ϵ saturation if it was desired, the bound [^{32}P]P_i was measured by column centrifugation of samples into the $HClO_4$ quench solution, with a bent needle on the syringe tip to facilitate mixing, and then [^{32}P]P_i was determined. [γ-^{32}P]ATP bound to catalytic sites was determined as the difference between the bound [^{32}P]P_i and the [^{32}P]P_i obtained after column centrifugation into buffer containing unlabeled 1 mM ATP followed by quenching with acid. The measurements were repeated after one half-time of product dissociation to ensure that equilibrium had been reached.

[1] Abbreviations: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Aurovertin Fluorescence. Fluorescence studies were conducted with an Eppendorf filter fluorometer, with excitation at 366 nm and emission at wavelengths over 400 nm. The buffer was 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA, containing 50 μg/mL BSA. Stepwise titrations were performed with 1-mL samples by adding aurovertin in methanol from a 10-μL Hamilton syringe with a repeating dispenser. Final methanol concentrations never exceeded 2%. After each addition, 5 min was allowed for equilibration before the sample was placed in the excitation beam. Data were analyzed as described by Dunn and Futai (1980).

Other Methods. The protein concentrations of F_1-ATPase solutions were determined by the Bradford (1976) method. The concentrations of ε solutions were determined by the method of Lowry et al. (1954). BSA was used as a standard. SDS–polyacrylamide gels were run as described by Laemmli (1970).

Results

Kinetics of Multisite Hydrolysis by F_1-ATPase. The effect of ATP concentration on the rate of hydrolysis was determined for ε-depleted F_1-ATPase and the same preparation in the presence of a saturating concentration of ε subunit. Data were analyzed with the SAS NLIN program as described under Experimental Procedures. Results are shown as Eadie–Hofstee plots in Figure 1. Attempts to fit the data with one kinetic component were unsuccessful due to the evident curvature. In both cases, the improvment in fit obtained by using a two-component analysis was statistically significant at the 99.5% confidence level.

Data obtained for ε-replete F_1-ATPase (Figure 1A) were fit well by models having two components. An unweighted analysis yielded the following parameters (± standard error of the estimate): K_{m1} = 18 ± 5 μM; V_1 = 1.12 ± 0.27 units/mg; K_{m2} = 150 ± 13 μM; V_2 = 3.98 ± 0.26 units/mg. These components define the line drawn in the figure. Relatively small changes in the derived parameters were found if the data were weighted in inverse proportion to [ATP]: K_{m1} = 15 ± 5 μM; V_1 = 0.91 ± 0.17 unit/mg; K_{m2} = 138 ± 13 μM; V_2 = 4.15 ± 0.15 units/mg. Regardless of weighting, no statistically significant improvement of fit was obtained with a three-component analysis.

Analysis of the data for ε-depleted F_1-ATPase (Figure 1B) indicated that a three-component model (solid curve) was required for optimal fit. The model included a high-affinity component with K_{m1} = 18 ± 33 μM and V_1 = 0.74 ± 0.98 unit/mg, a medium-affinity component with K_{m2} = 248 ± 21 μM and V_2 = 25.4 ± 0.7 units/mg, and a low-affinity component with K_{m3} = 28.7 ± 6.8 mM and V_3 = 27.8 ± 3.3 units/mg. The large standard errors in the estimation of the high-affinity component arise from its very small contribution to maximal velocity and the fact that a substantial alteration in one of its parameters can be offset by compensatory changes in the other parameters. While this component is somewhat poorly defined, the data indicate strongly that it does exist. The fit obtained with two components (broken line) lacks such a high-affinity component and is a much poorer fit (99.5% confidence level). At the higher velocities, there was essentially no difference between the fits for two and three components.

Although the low-affinity component appears to be well defined from the statistical analysis, it was found to vary substantially between independent determinations. Whether this is a true MgATP binding site or a general anion activation site that overcomes the inhibition brought about by the excess Mg^{2+} is not clear. In support of the latter possibility, when assays were carried out at 4 mM ATP, the ε-depleted F_1-ATPase was activated 3.8-fold by 40 mM Na_2SO_3 while the ε-replete enzyme was activated only 1.7-fold. Thus ε renders the enzyme less sensitive to anion activation. It is evident from Figure 1A that the low-affinity component is not present in the ε-replete enzyme.

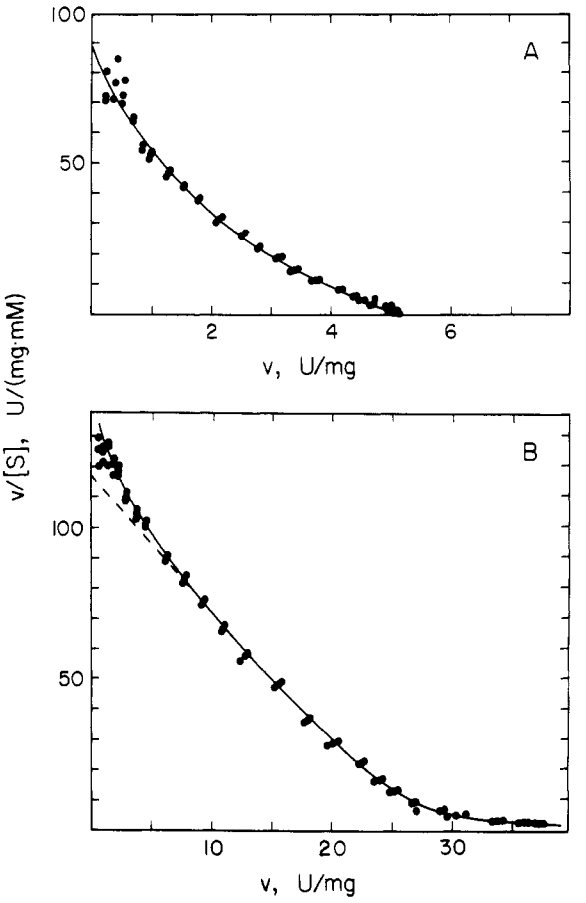

FIGURE 1: Kinetic analysis of the effect of ε on multisite ATP hydrolysis by F_1-ATPase. The rate of ATP hydrolysis was determined at MgATP concentrations ranging from 3 μM to 20 mM in the presence of an ATP-regenerating system as described under Experimental Procedures. Assays were performed in triplicate, and each point is shown. (A) ε-replete F_1-ATPase. ε-depleted F_1-ATPase and 110-fold molar excess of ε were incubated together in 50 mM Tris-HCl, pH 7.4, 50 μg/mL BSA, and 36 μM ATP at 30 °C for 10 min to ensure saturation of the enzyme with ε; then ATP hydrolysis was measured as described under Experimental Procedures. The final concentrations of ε and F_1-ATPase were 150 and 1.3 nM, respectively. (B) ε-depleted F_1-ATPase was incubated in 50 mM Tris-HCl, pH 7.4, 50 μg/mL BSA, and 36 μM ATP at 30 °C for 10 min and then assayed at an enzyme concentration of 0.3 nM. The solid line describes the best fit for a three-component system. The broken line describes the best fit for a two-component system.

Effect of the ε Subunit on Unisite ATP Hydrolysis. The hydrolysis of ATP was also followed under conditions where F_1-ATPase was in molar excess over [γ-^{32}P]ATP, so that most F_1 molecules had no more than one molecule of ATP bound and promotion could not occur. In the experiments shown in Figure 2, the progress of the reaction was followed by determining at various times the total amount of [^{32}P]P_i formed (●), the total amount of [^{32}P]P_i formed plus [γ-^{32}P]ATP that could be promoted to hydrolyze by added ATP (▲), and the amount of ^{32}P bound to F_1 (■).

The results shown in Figure 2 are basically similar to those obtained by other workers with mitochondrial (Grubmeyer et al., 1982) and *E. coli* (Duncan & Senior, 1985) F_1-ATPase. In short, although much of the added [γ-^{32}P]ATP is rapidly hydrolyzed and released by multisite hydrolysis during the mixing phase, some becomes bound to enzyme in tight catalytic

Table I: ϵ Subunit Inhibits Product Release in Unisite ATP Hydrolysis

	form of ATPase		
	as purified	ϵ depleted	ϵ depleted plus ϵ[a]
bound ATP/bound P_i	2[b]	3[b]	3[b]
$T_{1/2}$ of ^{32}P release (min)	60	5	80
K_{rel} (s^{-1})[c]	6×10^{-4}	9×10^{-3}	5×10^{-4}

[a] ϵ was added at 0.7 μM excess over ϵ-depleted F_1-ATPase 10 min before the substrate. [b] All values were between 2 and 3. [c] Rate constant for release of $[^{32}P]P_i$ from F_1·ADP·$[^{32}P]P_i$.

sites where it exists in a reversible equilibrium between [γ-^{32}P]ATP and ADP·$[^{32}P]P_i$. The bound [γ-^{32}P]ATP is observable as the increase in P_i after promotional ATP is added. At times shortly after mixing, we consistently observed lower amounts of ^{32}P bound to ϵ-replete F_1-ATPase than to ϵ-depleted F_1-ATPase, presumably due to differences in the amount of multisite catalysis that occurred during the mixing phase. Nevertheless, it is evident that the ^{32}P that became bound to the ϵ-replete enzyme in a unisite situation was released less rapidly than that which became bound to ϵ-depleted enzyme (note the difference in time scales).

In further experiments, the enzyme was briefly incubated with [γ-^{32}P]ATP and then freed of unbound ^{32}P by column centrifugation. The ratio of ATP to ADP·P_i at the catalytic site and the rate of product release were subsequently determined as described under Experimental Procedures. The ratio of ATP to ADP·P_i was between 2 and 3 regardless of the presence of ϵ (Table I). In contrast, ϵ had a large effect on the dissociation event itself, reducing the rate constant for product release, k_{rel}, by a factor of about 15. In agreement with the observations of Duncan and Senior (1985), we found that the hydrolysis and release of a small proportion of the bound [γ-^{32}P]ATP was not promotable by 1 mM ATP, indicating that it was bound in noncatalytic sites.

One concern in experiments of this type is that the substrate ATP must remain bound to the enzyme for the interpretations to be valid. If ATP was released, it could bind to the promotional site of another F_1-ATPase molecule. The following experiments were performed to test the ability of hexokinase to trap the ^{32}P of released substrate as glucose 6-$[^{32}P]$phosphate. F_1-ATPase and [γ-^{32}P]ATP were mixed at concentrations of 1 μM each. After 1 min, the enzyme and bound substrate were separated from free ^{32}P by column centrifugation. The eluate was mixed with buffer containing 12 mM glucose or 12 mM glucose plus 1 mg/mL hexokinase. After incubation for 20 min (ϵ-depleted F_1) or 180 min (ϵ-replete F_1), the solutions were analyzed for $[^{32}P]P_i$ and glucose 6-$[^{32}P]$phosphate (Grubmeyer et al., 1982). Of the ^{32}P that was initially bound to the enzyme, approximately 10% was converted to glucose 6-$[^{32}P]$phosphate in either the presence or absence of ϵ subunit, while nearly all of the rest was released as $[^{32}P]P_i$. Higher concentrations of hexokinase were not more effective. In other experiments in which the time course of formation of glucose 6-$[^{32}P]$phosphate was followed, the rate of formation appeared to be proportional to the concentration of ^{32}P bound to F_1-ATPase. Thus, some substrate release occurred, but the rate was low compared to the rate of release of product $[^{32}P]P_i$. In contrast, Duncan and Senior (1985) did not detect substrate release by normal *E. coli* F_1-ATPase, presumably due to slightly different conditions.

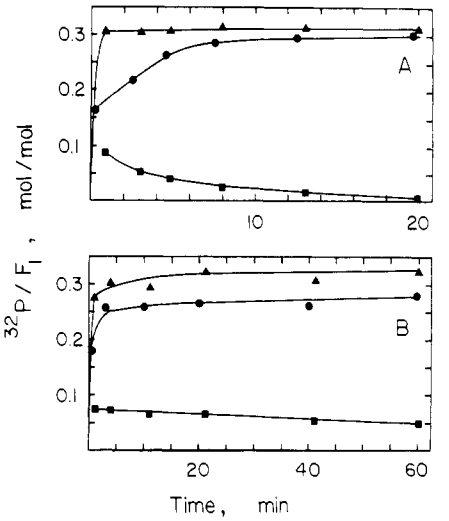

FIGURE 2: Effect of ϵ subunit on unisite ATP hydrolysis by F_1-ATPase. (A) ϵ-depleted F_1-ATPase was freed of excess nucleotide by column centrifugation and then mixed with [γ-^{32}P]ATP at final concentrations of 1 and 0.3 μM, respectively, in unisite reaction buffer. Periodically duplicate samples were removed for determination of $[^{32}P]P_i$ (●), [γ-^{32}P]ATP which had been hydrolyzed or was committed to hydrolysis (▲), and ^{32}P bound to enzyme (■) by methods described under Experimental Procedures. (B) Procedures were similar to those described for panel A, except that the ϵ-depleted F_1-ATPase was mixed with a 50% molar excess of pure ϵ before adding it to the [γ-^{32}P]ATP.

Effect of the ϵ Subunit on Aurovertin Fluorescence. Aurovertin fluorescence has been used in many studies of conformational effects in F_1-ATPase. The fluorescence of mixtures of aurovertin and F_1-ATPase generally increases when ADP or ATP are added and is then quenched if Mg^{2+} is added (Satre et al., 1980). These changes are the result of ligand-induced changes in both the affinity of one of the β subunits of F_1-ATPase for aurovertin and the fluorescence yield of aurovertin bound to this β subunit (Issartel et al., 1983).

In our first tests we found that nucleotides had little or no effect on aurovertin fluorescence with ϵ-depleted ATPase, although the expected responses were seen with our regular preparation of F_1-ATPase and with the ϵ-depleted enzyme to which pure ϵ had been added (data not shown). Titrations were then performed to determine the effect of ϵ on the affinity for aurovertin and the maximal fluorescence of bound aurovertin under several different conditions (Table II). The

Table II: Effect of ϵ on Aurovertin D Binding and Fluorescence[a]

	form of ATPase					
	as purified		ϵ depleted		ϵ depleted plus ϵ[b]	
conditions	K_d (μM)	F_{max}[c]	K_d (μM)	F_{max}[c]	K_d (μM)	F_{max}[c]
no ligand	0.07	6	0.08	130	3	84
	3	74				
5 mM ATP	0.08	150[d]	0.08	150[d]	0.09	140[d]
0.5 mM ADP	0.06	130	0.04	150	0.07	140
0.5 mM ADP, 3 mM $MgCl_2$	0.2	71	0.3	150	0.2	72

[a] Aurovertin titrations were performed as described under Experimental Procedures. The F_1-ATPase concentration was 0.02 μM. [b] ϵ was added to a final concentration of 0.2 μM. [c] Maximal fluorescence is expressed in arbitrary units. [d] Maximal fluorescence of the high-affinity component only. All titrations in the presence of ATP showed an additional component with lower maximal fluorescence and $K_d > 2$ μM.

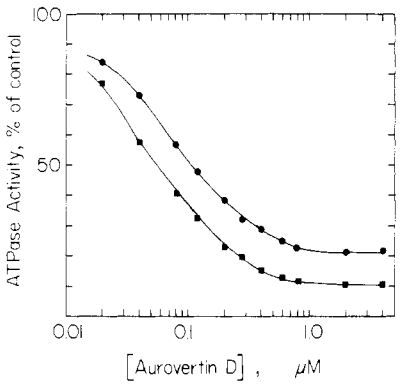

FIGURE 3: Inhibition of ATPase activity by aurovertin D. Inhibition of the ATPase activity of ϵ-depleted F_1-ATPase (●) and ϵ-replete F_1-ATPase (■) by various concentrations of aurovertin D was determined. ϵ-replete enzyme was produced by incubation of the ϵ-depleted enzyme with 0.3 μM ϵ subunit for 10 min before the assay was started. ϵ-depleted F_1-ATPase received a control incubation. Activities are presented as percentage of the activity expressed by that form of enzyme in the absence of aurovertin.

alteration of properties induced by removal of ϵ was also shown to be reversible by addition of pure ϵ.

Analysis of aurovertin titrations of regular ATPase in the absence of ligands yielded curved double-reciprocal plots that could be resolved into a high-affinity component (K_d = 0.07 μM), which contributed less than 10% of total fluorescence, and a much lower affinity component (K_d = 3 μM), which was responsible for most of the fluorescence enhancement. The presence of two components under these conditions has been previously reported by Issartel and co-workers (Issartel et al., 1983). The high-affinity component was not seen in the ϵ-depleted F_1 that had been reconstituted with excess ϵ and was eliminated from normal ATPase by addition of ϵ. In the ϵ-depleted enzyme, only a high-affinity component (K_d = 0.08 μM) with an enhanced fluorescence was seen.

Addition of ATP and ADP to regular F_1-ATPase raised the affinity for aurovertin and its maximal fluorescence to values similar to those in the ϵ-depleted enzyme. In contrast, the nucleotides had only small effects on the properties of the ϵ-depleted F_1-ATPase. Regarding the ATP titrations, in all cases a lower affinity component, with a K_d in the range of several micromolar, was also observed. The maximal fluorescence values shown reflect only the high-affinity site.

An effect of ϵ can also be seen by comparing results obtained in the presence of Mg^{2+} and ADP with those observed when only ADP was present. For the normal ϵ-replete form, Mg^{2+} reduced maximal fluorescence by about 50%, but little effect was seen for the ϵ-depleted form of the enzyme. However, in this case, the affinity was higher in the presence of ϵ than in its absence. Thus in either the presence of Mg^{2+} or in the absence of any added ligands, the ϵ-depleted and ϵ-replete F_1-ATPases had notably different aurovertin binding properties.

Effect of ϵ on Inhibition of ATPase Activity by Aurovertin. As aurovertin is a partial inhibitor of multisite ATPase activity, it was of interest to determine if the ϵ-replete and ϵ-depleted forms were equally sensitive to inhibition and to determine the concentrations required for half-maximal inhibition in the presence of Mg^{2+} and ATP in the hydrolysis assay. Figure 3 shows the activity expressed in the presence of various concentrations of aurovertin D by these two forms of the enzyme. In each case, activity is expressed as percentage of that shown by the same form in the absence of aurovertin. The ϵ-depleted enzyme exhibited maximal inhibition of 80%, with half-maximal inhibition at 0.07 μM aurovertin D. In contrast, the ϵ-replete enzyme exhibited maximal inhibition of 90%, with half-maximal inhibition at 0.04 μM aurovertin D.

Discussion

The results presented above indicate the very major effects of the ϵ subunit on the conformation and catalytic properties of *E. coli* F_1-ATPase. It is apparent that studies conducted under conditions where a mixture of the ϵ-replete and ϵ-depleted forms exist will give inaccurate data.

The ϵ subunit has been shown to interact with one the β subunits (Lotscher et al., 1984a). Inhibition of ATPase activity may arise directly through this interaction (Lotscher et al., 1984b). Recently, it has been shown that each of the β subunits, which are believed to contain the catalytic and promotional sites, has unique reactivities in chemical modification studies (Stan-Lotter & Bragg, 1986a,b). Ultimately one would like to correlate these different reactivities with different points in catalysis.

The ϵ subunit strongly inhibited product release in the unisite experiments. We interpret this result to reflect inhibition by ϵ of a conformational change that is required for product release. It is tempting to suggest that ϵ may exert this effect directly at the β subunit which has the occupied catalytic site. However, if this were the case, one might expect to see an effect on the equilibrium of substrate to products at the tight catalytic site. For example, Duncan and Senior (1985) have found that F_1 from *uncD* mutants, which have altered β subunits, has very different ratios of ATP to ADP·P_i at the catalytic sites. The lack of such an effect indicates that ϵ did not seriously perturb the site. Furthermore, if ϵ had any effect on the rate constants for ATP hydrolysis or its reversal, the effect must have been the same on both of these constants. Thus it seems likely that reduction of the rate of the conformational change required for product release was the only significant effect of ϵ on unisite catalysis. As this conformational change probably affects the entire enzyme, ϵ could inhibit without binding near the occupied catalytic site.

As unisite experiments are performed at high enzyme concentrations, where nearly all ϵ will remain bound, it is not surprising that the product dissociation rate constant reported by Duncan and Senior (1985) for wild-type *E. coli* F_1-ATPase (1×10^{-3} s^{-1}) is close to the value we observed for the ϵ-replete enzyme. Small differences between their results and ours are found in the ratio of bound ATP to bound P_i at the active site (Duncan and Senior reported a ratio of 1 whereas we found a ratio between 2 and 3) and in our higher rate of ATP release. Grubmeyer and co-workers (Grubmeyer et al., (1982) reported a ratio of ATP to ADP·P_i of 2 for mitochondrial F_1-ATPase.

The multisite catalysis studies show that each form of F_1-ATPase has kinetic components with K_m values in the vicinity of 20 μM and 0.2 mM, suggesting again that ϵ does not greatly alter the catalytic or promotional sites. Also, the major kinetic component detected in membrane-bound F_1-ATPase, which contains ϵ subunit, had an apparent K_m of 0.13 mM (Dunn & Tozer, 1987). It should be remembered that according to the alternating sites theory the observed components do not arise from independent sites that operate in an additive manner. Rather, as the lower affinity component becomes saturated the hydrolysis mode with promotion from two sites displaces that with promotion from a single site. The true velocity of the reaction with promotion at two sites is thus the sum of the apparent velocities of the two components. Our results are surprising in indicating that ϵ inhibits both unpromoted unisite hydrolysis and multisite hydrolysis promoted at two sites, but not multisite hydrolysis promoted at just one site. One possible explanation is that while ϵ slows the rate

of the conformational change required for product release regardless of promotion, the subunit may also tighten the coupling between the catalytic and promotional sites, so that a single promotional site is more effective. This would explain the overall trend toward higher affinity expressed in the presence of ϵ and would allow the mechanism of ϵ inhibition to be the same in unisite and multisite hydrolysis. A second possible explanation is that the two promotional sites are basically dissimilar and promote by different mechanisms. Occupation of the higher affinity site would promote by a mechanism that overcomes the effect of ϵ on unisite release, while further promotion from the second site would still be inhibited. Slater and co-workers (Slater et al., 1986) have recently outlined a model with dissimilar promotional sites, one of them being essentially regulatory.

The aurovertin results show that ϵ has an effect on the conformation of at least one of the β subunits. The ϵ-replete enzyme had high affinity for aurovertin only if adenine nucleotides were present. In contrast, the ϵ-depleted form showed high affinity regardless of the presence of nucleotides. Thus, regarding the single β subunit where binding aurovertin produces high fluorescence (Issartel et al., 1983), the ϵ subunit favors a conformation different from that favored by aurovertin or nucleotides. This effect of ϵ may be responsible for the general observation that aurovertin enhances the binding of nucleotides by F_1-ATPase (Wise et al., 1981). The finding that the ϵ subunit is required for modulation of aurovertin binding and fluorescence by nucleotides suggests that studies on these effects may have little or no applicability to the ϵ-depleted form which normally predominates in multisite hydrolysis assays.

Issartel et al. (1983) found that *E. coli* F_1-ATPase exhibited high-affinity binding of 0.3 mol of [^{14}C]aurovertin/mol of enzyme in buffer lacking Mg^{2+} and adenine nucleotides. Under similar conditions, we also detected, by fluorescence, some high-affinity binding in our standard preparation of F_1-ATPase, but with a very low apparent molar binding ratio. The elimination of this high-affinity component by added ϵ subunit implies that it was due to enzyme molecules that had lost ϵ due to dissociation either during purification or during the experiment. Inclusion of excess ϵ is therefore essential for maintaining *E. coli* F_1-ATPase in an ϵ-replete form, even when working at high enzyme concentrations.

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