Catalytic Site Nucleotide and Inorganic Phosphate Dependence of the Conformation of the ϵ Subunit in *Escherichia coli* Adenosinetriphosphatase†

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ABSTRACT: The rate of trypsin cleavage of the ϵ subunit of *Escherichia coli* F_1 (ECF_1) has been found to be ligand-dependent, as measured indirectly by the activation of the enzyme that occurs on protease digestion, or when followed directly by monitoring the cleavage of this subunit using monoclonal antibodies. The cleavage of the ϵ subunit was fast in the presence of ADP alone, ADP + Mg^{2+}, ATP + EDTA, or AMP-PNP, but slow when P_i was added along with ADP + Mg^{2+} or when ATP + Mg^{2+} was added to generate ADP + P_i (+Mg^{2+}) in the catalytic site(s). The half-maximal concentration of P_i required in the presence of ADP + Mg^{2+} to protect the ϵ subunit from cleavage by trypsin was 50 μM, which is in the range measured for the high-affinity binding of P_i to F_1. The ligand-dependent conformational changes in the ϵ subunit were also examined in cross-linking experiments using the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). In the presence of ATP + Mg^{2+} or ADP + Mg^{2+} + P_i, the ϵ subunit cross-linked to β in high yield. With ATP + EDTA or ADP + Mg^{2+} (no P_i), the yield of the β–ϵ cross-linked product was much reduced. We conclude that the ϵ subunit undergoes a conformational change dependent on the presence of P_i. It has been found previously that binding of the ϵ subunit to ECF_1 inhibits ATPase activity by decreasing the off rate of P_i [Dunn, S. D., Zadorozny, V. D., Tozer, R. G., & Orr, L. E. (1987) *Biochemistry 26*, 4488–4493]. This reciprocal relationship between P_i binding and ϵ-subunit conformation has important implications for energy transduction by the *E. coli* ATP synthase.

An F_1F_0-type ATP synthase, found in the plasma membrane of bacteria, the thylakoid membrane of chloroplasts, and the inner membrane of mitochondria, is responsible for the synthesis of ATP during oxidative or photophosphorylation. This multicomponent complex can also function as an ATP-driven proton pump, with the pH gradient so generated used in active transport of metabolites.

The simplest F_1F_0-type ATP synthases are those in bacteria. The F_1 moiety of the enzyme of *Escherichia coli* (ECF_1)[1] contains five subunits, α, β, γ, δ, and ϵ, in the stoichiometry of 3:3:1:1:1; the F_0 moiety (ECF_0) is made up of three subunits, a, b, and c, in the ratio 1:2:10–12 [for recent reviews, see Cross (1988), Senior (1988), and Futai et al. (1989)]. The functions of the subunits of the F_1 moiety are partly known. There are three catalytic sites per F_1 located on β subunits, possibly at the interface between α and β subunits. The α subunits probably contain so-called nonexchangeable nucleotide binding sites (Cross, 1988; Senior, 1988). The δ subunit is important in linking the F_1 to the F_0 part (Sternweis, 1980) while the γ and ϵ subunits appear to regulate enzymic activity (Nalin & McCarty, 1984; Dunn et al., 1987); the ϵ subunit acts as an inhibitor of ATP hydrolysis in isolated F_1 (Sternweis & Smith, 1977).

Both ATP hydrolysis and ATP synthesis catalyzed by the F_1F_0-type ATP synthases are cooperative processes, now thought to involve ligand-induced and energy-dependent conformational changes which modulate the affinity of catalytic sites for substrate and products (Rosen et al., 1979; Cross et al., 1982). Approaches which have been used to monitor conformational changes in F_1 and in F_1F_0 preparations include hydrogen exchange (Ryrie & Jagendorf, 1972), chemical labeling with general protein-modifying reagents (Stan-Lotter & Bragg, 1986), binding changes of inhibitors (Issartel et al., 1983), and protease digestion experiments (Bragg & Hou, 1987). These conformational changes appear to be large and involve most of the subunits of the enzyme.

We have recently used trypsin cleavage of ECF_1 and ECF_1F_0 to probe the topology of the enzyme complex (Gavilanes et al., 1988). We found that all five subunits of ECF_1 were cleaved by the protease, with the α subunit clipped from the N-terminus, the β subunit cleaved at a position close to the C-terminus, the γ subunit separated into two parts by cleavage at around residue 200, and both the δ and ϵ subunits cleaved into several fragments. We now describe the kinetics of the cleavage of the subunits of ECF_1 as a function of nucleotides present in the catalytic sites, and with respect to the presence or absence of Mg^{2+} and P_i on the enzyme. These protease digestion studies, together with cross-linking experiments, and studies using cryoelectron microscopy (E. P. Gogol, E. Johnston, R. Aggeler, and R. A. Capaldi, unpublished results), provide evidence that the conformation of the ϵ subunit and the binding of this subunit to the core ECF_1 complex are controlled by the presence of P_i.

Experimental Procedures

Preparation of ATPase. ECF_1 was purified from *E. coli* strain AN 1460 by a modified method of Senior et al. (1979) and Wise et al. (1981), and stored in liquid nitrogen in 50 mM

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[1] Abbreviations: ECF_1, water-soluble, membrane-extrinsic ATPase sector of the F_1F_0 complex of *E. coli*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; DTT, DL-dithiothreitol; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PVDF, poly(vinylidene difluoride); $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; P_i, inorganic phosphate; AMP-PNP, 5′-adenylyl imidodiphosphate; TPCK, tosylphenylalanine chloromethyl ketone; ϵACA, ϵ-aminohexanoic acid.

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Tris-HCl (pH 7.5), containing 1 mM ATP, 2 mM EDTA, 40 mM ϵACA, 1 mM DTT, and 20% glycerol. Samples of F_1 containing 2 mol of nucleotide/mol of ECF (1 ATP:1 ADP) were prepared at 25 °C by passing the stored F_1 (1–4 mg/mL) through two consecutive centrifuge columns (Penefsky, 1977) (Sephadex G-50, fine, 0.5 × 5.5 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.5 mM EDTA, as described (Hanada et al., 1989). Additions were made to equivalent aliquots to produce ECF_1 samples of equal volume and protein concentration (1–4 mg/mL) containing 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and (a) ±5 mM $MgSO_4$, (b) 5 mM ADP ± 5 mM $MgSO_4$, (c) 5 mM ATP ± 5 mM $MgSO_4$, and (d) 5 mM ADP with 5 mM NaH_2PO_4 ± 5 mM $MgSO_4$. Samples were allowed to equilibrate for 30 min at room temperature before further treatment. (In EDC cross-linking experiments, 50 mM MOPS, pH 7.0, was substituted for 50 mM Tris-HCl pH 8.0.)

Trypsin Digestion Studies. ECF_1 samples equilibrated in the presence or absence of various ligands were incubated at a concentration of 1–4 mg/mL with TPCK-treated trypsin in a ratio of 1:50 or 1:100 (w/w) at room temperature. At times indicated, aliquots were withdrawn, inhibited by 1 mM PMSF and soybean trypsin inhibitor in a ratio to trypsin of 10:1 (w/w), and immediately assayed for ATPase activity.

EDC Cross-Linking. ECF_1 samples (1 mg/mL) equilibrated in the presence or absence of various ligands were incubated with 5 mM EDC for 3 h at room temperature according to Lotscher et al. (1984). The cross-linking reaction was stopped by addition of 4 M Tris to a final concentration of 100 mM.

ATPase Activity Assay. The ATPase activity was assayed in 1 mL of solution containing 25 mM Tris, pH 7.5, 25 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 5 mM KCN, 2 mM phosphoenolpyruvate, 0.5 mM NADH, 30 units of L-lactic acid dehydrogenase, and 30 units of pyruvate kinase at 37 °C. The absorbance change at 340 nm was followed in a Beckman DU7 spectrophotometer. The specific activity is expressed as units per milligram; 1 unit corresponds to 1 μmol of ATP hydrolyzed/min.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Samples for $NaDodSO_4$–polyacrylamide gel electrophoresis were dissolved with 0.25 volume of 10% $NaDodSO_4$, 0.25 M DTT, 25% glycerol, and 0.625 M Tris, pH 6.5. Slab gels (1.5 mm thick) were run as described by Laemmli (1970), with a 3% acrylamide stacking gel and a 10–18% separating gel in a linear gradient.

When separation of α and β subunits along with higher molecular weight cross-linked products was required, a 7–10% linear gradient of acrylamide was used in the separating gel. Staining with Coomassie brilliant blue R and destaining were carried out according to the method of Downer et al. (1976).

Immunodetection of Tryptic Fragments and Cross-Linked Products. $NaDodSO_4$–polyacrylamide gels used for electroblotting were duplicates of those stained with Coomassie brilliant blue but containing only 10-μg aliquots of protein applied per lane.

Immunoblotting was conducted according to the procedure of Towbin et al. (1979) using a Hoefer TE22 transblot apparatus and transferring at 400 mA for 3 h, using a buffer containing 10 mM CAPS, pH 11.0, and 10% methanol. Additional protein sites on the nitrocellulose blot were saturated with 5% nonfat dry milk (Carnation). Alkaline phosphatase conjugated goat anti-mouse antibodies were used for color development (Hawkes et al., 1982) in the presence of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. All saline buffers used during antibody incubations contained 0.095% Tween 20. Monoclonal antibodies against the subunits of ECF_1, supplied by Dr. R. Aggeler, were isolated and characterized as described (Gogol et al., 1989).

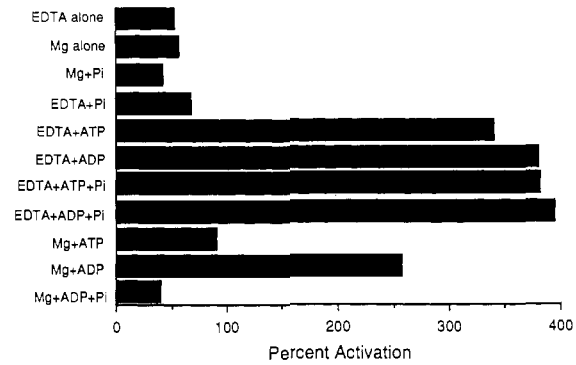

FIGURE 1: Effect of trypsin cleavage on the ATPase activity of ECF_1 in various ligand-induced conformations. Trypsin cleavage was conducted as described under Experimental Procedures. After 4 min, a time determined for maximal activation, each sample was inhibited and assayed for ATPase activity. ATP, ADP, $MgSO_4$, and NaH_2PO_4 were added to 5 mM and EDTA to 0.5 mM as indicated prior to proteolysis.

Electroblotting onto a PVDF Membrane. Protein samples resolved by polyacrylamide gel electrophoresis were electrophoretically transferred onto a poly(vinylidene difluoride) (PVDF) membrane (Immobilon, Millipore) following the procedure of Matsudaira (1987). After being stained with Coomassie brilliant blue R and destained for a minimum amount of time, bands were excised with a razor blade and stored at −20 °C until sequence analysis was performed.

Sequence analysis was performed by a gas-phase protein sequencer (Applied Biosystems Model 470A) equipped with an on-line PTH analyzer (Applied Biosystems Model 120A).

Other Methods. Protein concentrations were determined according to the method of Lowry et al. (1951) as modified by Markwell et al. (1978).

RESULTS

The ECF_1 preparation used in this study was made under conditions in which the ϵ subunit remains bound to and inhibits the ATPase activity of the enzyme complex. The activities of these preparations measured under standard assay conditions with 5 μg/mL or more of enzyme were in the range 6–8 μmol of ATP hydrolyzed min^{-1} (mg of enzyme)$^{-1}$ compared with 80–90 units/min for ϵ-free enzyme. Lower concentrations of ECF_1 were used in some experiments, leading to higher initial rates of activity because of partial dissociation of the ϵ subunit [see Dunn et al. (1987) and Gavilanes-Ruiz et al. (1988)].

Effect of Protease Digestion on the Activity of ECF_1 under Different Nucleotide Conditions. Enzyme to be used for trypsin cleavage studies was centrifuged twice through a 1-mL Sephadex G50 column (Penefsky, 1977) in 50 mM Tris-HCl, pH 8.0, to remove free nucleotides (except where stated). This gave preparations containing 2.1 ± 0.2 mol of tightly bound nucleotide (ATP + ADP) per mole of enzyme in confirmation of the report by Hanada et al. (1989). Samples were incubated with ATP or ADP under a variety of conditions and then subjected to trypsin treatment using a ratio of protease to enzyme (w/w) of 1:50. A time course of the protease digestion was obtained by stopping the reaction at selected times with the combination of trypsin inhibitor (10-fold excess) and PMSF (1 mM final concentration), and the effect of proteolysis on both ATPase activity and on subunit composition of the enzyme was monitored. Figure 1 shows the results of a

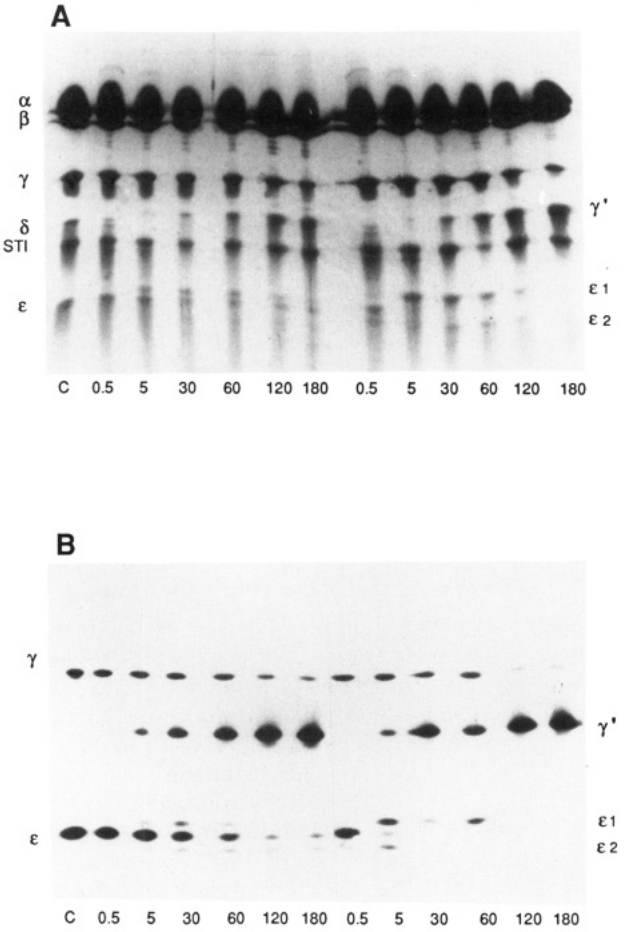

FIGURE 2: Time course of trypsin cleavage of ECF_1. ECF_1 was incubated with trypsin (1:50 w/w) in the presence of ATP + Mg^{2+} (left part, i.e., lanes 2–7) or ATP + EDTA (right part, i.e., lanes 8–13). At the times (min) indicated, proteolysis was inhibited by addition of trypsin inhibitor. (A) Samples were electrophoresed on a 10–18% $NaDodSO_4$–polyacrylamide gel. (B) Immunoblot of same samples, with antibodies against the γ and ϵ subunits.

typical experiment in which the effect of trypsin treatment on the ATPase activity of ECF_1 was measured under a variety of ligand conditions. In the presence of EDTA, Mg^{2+} alone, EDTA + P_i, or Mg^{2+} + P_i, there was little or no change in ATPase activity as a result of trypsin treatment. Addition of ADP or ATP (but not AMP), along with EDTA, increased proteolysis and activated ECF_1 dramatically. Addition of P_i along with nucleotide in the presence of EDTA did not alter this activation. In the presence of Mg^{2+}, the activation obtained with ADP was much less, and when ATP + Mg^{2+} were present, there was no activation of the enzyme due to protease digestion. The effect of ATP + Mg^{2+}, which is converted to ADP + Mg^{2+} + P_i on the enzyme, was reproduced when ADP + Mg^{2+} + P_i were added directly rather than being generated by enzyme turnover.

Rate of Subunit Cleavage under Different Nucleotide Conditions. For the various experimental conditions above, the cleavage of subunits of ECF_1 by trypsin (1:50 w/w) was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. Time courses of cleavage in the presence of ATP + EDTA (representative of conditions in which the enzyme was activated) and in the presence of ATP + Mg^{2+} (representative of low activity conditions) are shown in Figure 2. Trypsin cleaved all five subunits of ECF_1 under all conditions [see also Bragg & Hou (1987) and Gavilanes-Ruiz et al. (1988)]. The rates of cleavage of the α and β subunits followed on 10% polyacrylamide gels and of the δ subunit (monitored at a 1:1000 trypsin:protein ratio) were not significantly altered by different ligand binding conditions (results not shown). In Figure 2B, the rates of cleavage of the γ and ϵ subunits in two different ligand binding conditions were followed by monoclonal antibody binding using Western blotting. The rate of cleavage of the ϵ subunit but not the γ subunit was sensitive to the ligands present during proteolysis, with the rate of cleavage of the ϵ subunit in all cases proportional to the extent of activation of the enzyme. Thus, the ϵ subunit was only slowly cleaved in the presence of EDTA, Mg^{2+}, EDTA + P_i, and Mg^{2+} + P_i and when ATP + Mg^{2+} or ADP + Mg^{2+} + P_i were added prior to proteolysis. In contrast, the cleavage of the ϵ subunit was fast when ATP + EDTA or ADP + EDTA were present. In the presence of ADP + Mg^{2+}, an intermediate rate of proteolysis of the ϵ subunit was observed, which also followed the time course of the rate of activation of the enzyme.

The rate of cleavage of the ϵ subunit was also monitored in the presence of the nonhydrolyzable ATP analogue AMP–PNP (5 mM), both in the presence and in the absence of P_i. The ϵ subunit was cleaved rapidly in the presence of AMP–PNP, whether Mg^{2+} was present or not (result not shown), the rates being comparable to when ATP + EDTA were in the catalytic sites. Addition of P_i to the (AMP–PNP + Mg^{2+})-reacted enzyme failed to slow the cleavage of the ϵ subunit.

Some experiments were conducted using ECF_1 that had been passed only once through a Penefsky column, conditions insufficient to remove nucleotides from the catalytic sites; i.e., the enzyme contained 5 mol of ADP + ATP/mol of enzyme [see also Hanada et al. (1989)]. With residual nucleotide present in the catalytic sites, Mg^{2+} alone, and EDTA alone, gave rapid activation and similarly rapid cleavage of the ϵ subunit. The combination of Mg^{2+} + P_i in contrast, by reacting with the bound ADP, caused slow activation of the enzyme and slow cleavage of the ϵ subunit.

Phosphate Dependence of the Protection of the ϵ Subunit from Trypsin Cleavage When Nucleotides Are Bound. The important role of P_i in converting ECF_1 from a rapidly activated to slowly activated form when ADP is in the catalytic sites was monitored more extensively. ECF_1 was reacted with trypsin (1:50 w/w) for 4 min in the presence of ADP + Mg^{2+} over a range of [P_i] from 0 to 1200 μM, with care being taken to exclude adventitious P_i from enzyme and buffers (see figure legend). The half-maximal effect of P_i was observed at around 50 μM (Figure 3). This activation effect was directly related to the extent of cleavage of the ϵ subunit under the different conditions. The effect of added P_i was not seen with enzyme in the presence of ADP + EDTA, Mg^{2+} alone, or ATP + EDTA (result not shown).

Trypsin Cleavage Sites in the ϵ Subunit. The immunoblot analyses (in Figure 2, part B) identify the fragments of ϵ generated by the protease treatment and show that cleavage of this subunit occurs in at least three steps. The first product formed migrates more slowly than intact ϵ, the second product runs faster than the position of intact ϵ, and in the third cleavage step, small fragments are generated which are lost from the gel in staining and destaining, and/or the epitope for the mAb is destroyed.

The two visible cleavage products, ϵ^1 and ϵ^2 in Figure 2B, were isolated by electroblotting onto Immobilon after protein bands were stained with Coomassie brilliant blue. The N-terminal sequence of both fragments, AMTYHLDVV, is identical with the sequence of the intact ϵ (Figure 4), indicating that cleavage had occurred from the C-terminus. The intact

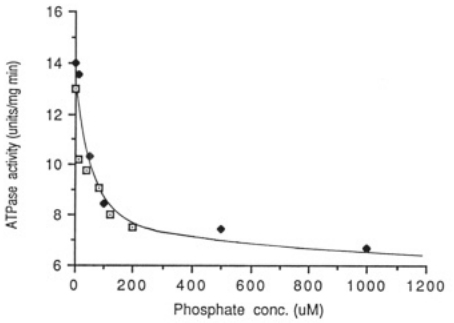

FIGURE 3: Effect of P_i on trypsin activation of the ATPase activity of ECF_1. ECF_1 samples were centrifuged twice through Penefsky columns as described under Experimental Procedures using new plastic syringes and gel material, each rinsed several times in deionized water. All buffers were made in plasticware washed many times in deionized water, which was also used to make all buffer solutions. Enzyme containing 5 mM $MgSO_4$, 5 mM ADP, and various concentrations of NaH_2PO_4 was reacted with trypsin (1:100 w/w) for 4 min, under conditions described under Experimental Procedures. Following trypsin inhibition, the ATPase activity of each sample was measured. The different symbols are typical results from experiments with different preparations of ECF_1.

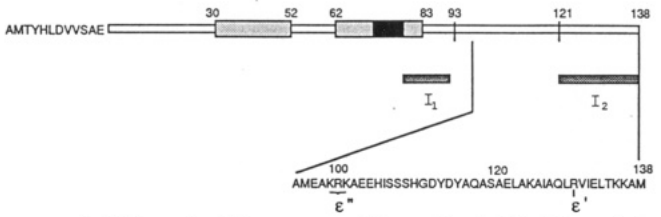

FIGURE 4: Schematic of the sequence of the ϵ subunit. The N-terminal sequence obtained in our sequencing studies is shown. Putative sites of cleavage of the ϵ subunit are indicated as are other features of the subunit. The gray boxed areas are highly conserved regions of this subunit among bacteria and chloroplasts. The dark boxed region is required for binding ϵ to ECF_1 in the experiments of Kuki et al. (1988). The region I_1 is claimed to be required for the inhibitory effect of ϵ (Kuki et al., 1988) while region I_2 is a segment with sequence homology to the inhibitor protein of F_1 in mitochondria (Frangione et al., 1981).

ϵ subunit migrates anomalously in $NaDodSO_4$–polyacrylamide gel electrophoresis to give an apparent molecular weight of 10 500 [cf. with a true molecular weight based on sequence data of 15 051 (Walker et al., 1984)]. The generation of a product with an apparent molecular weight greater than the untreated subunit may be a consequence of removing the highly charged C-terminus. Figure 4 shows the likely sites of trypsin cleavage of the ϵ subunit based on size considerations, along with features of this subunit deduced from genetic studies (Kuki et al., 1988).

The sizes of the various fragments of the ϵ subunit generated under different ligand binding conditions were all the same (see Figure 2); only the rates of cleavage were different.

EDC Cross-Linking of ECF_1 under Different Nucleotide Conditions. We have previously shown that the ϵ subunit can be cross-linked to a β subunit by the water-soluble carbodiimide EDC (Lotscher et al., 1984). The amount of this cross-linked product was reduced in ECF_1 which had been activated by the detergent LDAO, suggesting that the yield of the β–ϵ product could be used to monitor the conformation and/or affinity of the ϵ subunit for the core ECF_1 complex. Figure 5 shows a typical experiment in which the reaction of ECF_1 with EDC was examined in the presence of ATP + EDTA and ADP + Mg^{2+} + P_i, respectively. The Coomassie brilliant blue stained gels of the cross-linked enzyme and their corresponding Western blots using the anti-ϵ mAb are also shown for the two conditions. Cross-linking of ϵ to a β subunit was found in greatest yield when ADP, Mg^{2+}, and P_i were present. Under these conditions, there was essentially no free ϵ. In the presence of ATP + EDTA, ADP + EDTA, or ADP + Mg^{2+}, the amount of cross-linking of ϵ to β was much lower, i.e., 20–30% of that when P_i was present (three different experiments).

EDC also catalyzed internal cross-linking of the ϵ subunit. As seen in Figure 5, the positions both of the β–ϵ cross-link and of ϵ by itself are shifted on the gels in enzyme reacted with EDC in the presence of ATP + EDTA when compared to enzyme reacted in the presence of ATP + Mg^{2+}. This proved to depend on the presence or absence of Mg^{2+}. The internal cross-link was only obtained when Mg^{2+} was absent, i.e., with EDTA added. Magnesium ions protected the ϵ subunit from internal cross-linking in the absence of added nucleotide, indicating that the effect is related to the well-characterized divalent metal ion shielding of carboxyls from reaction with carbodiimides [see Lotscher et al. (1984)]. Note then that the cross-linking between β–ϵ was greater in the presence of Mg·ATP than in EDTA·ATP.

Discussion

Energy coupling in F_1F_0-type ATPases has been studied for more than 20 years [reviewed in Cross (1988) and Senior (1988)]. It is now generally assumed that events at the catalytic sites of the F_1 moiety are coupled to proton translocation through the F_0 moiety by conformational changes in the protein complex [Rosen et al., 1979; Cross et al., 1982; reviewed in Senior (1988) and Cross (1988)]. However, the subunits involved and the nature of these ligand-induced and energy-dependent conformational changes are poorly defined.

Approaches that monitor conformational changes in F_1 are aurovertin binding and associated fluorescence changes (Chang & Penefsky, 1974; Wise et al., 1981; Matsuno-Yagi et al., 1985) and protease digestion experiments (Bragg & Hou, 1987; this study). Aurovertin has been found to bind in the C-terminal part of the β subunit of F_1 (Satre et al., 1980; Lee et al., 1989). This inhibitor of ATPase activity fluoresces weakly when bound to nucleotide-depleted MF_1 or ECF_1 in the presence of EDTA (Wise et al., 1981; Bragg & Hou, 1987). Addition of ADP or ATP causes a marked increase in fluorescence which is reduced by addition of Mg^{2+} and P_i (Wise et al., 1981; Bragg & Hou, 1987; Languin et al., 1980). Our studies and the work of Bragg and Hou (1987) indicate that the protease digestion pattern of ECF_1 is also sensitive to ligand binding. Bragg and Hou (1987) found rapid cleavage of the β, γ, and ϵ subunits upon trypsin cleavage of the enzyme in the absence of additions (i.e., in EDTA), rapid cleavage of the same subunits in Mg^{2+} alone or ATP + EDTA, but slow cleavage of these subunits with Mg^{2+} + ADP or Mg^{2+} + ATP present. These workers did not control for nucleotide binding or for the presence of P_i in their experiments, and this explains some of the differences in results between our study and their work.

We have followed the effects of trypsin digestion of ECF_1 under a wide range of ligand binding conditions by monitoring activity changes and subunit conformational changes. We find that cleavage of the ϵ subunit is sensitive to ligand binding conditions. This is not true of the γ subunit which is only cleaved after digestion of the ϵ subunit. The resolution of uncleaved and cleaved β subunit on our gels was not good enough to monitor unequivocally whether the rate of cleavage of this subunit was ligand-dependent. However, cleavage of the β subunit was slower than that of the ϵ subunit under all conditions, suggesting the changes in the ϵ subunit are paramount.

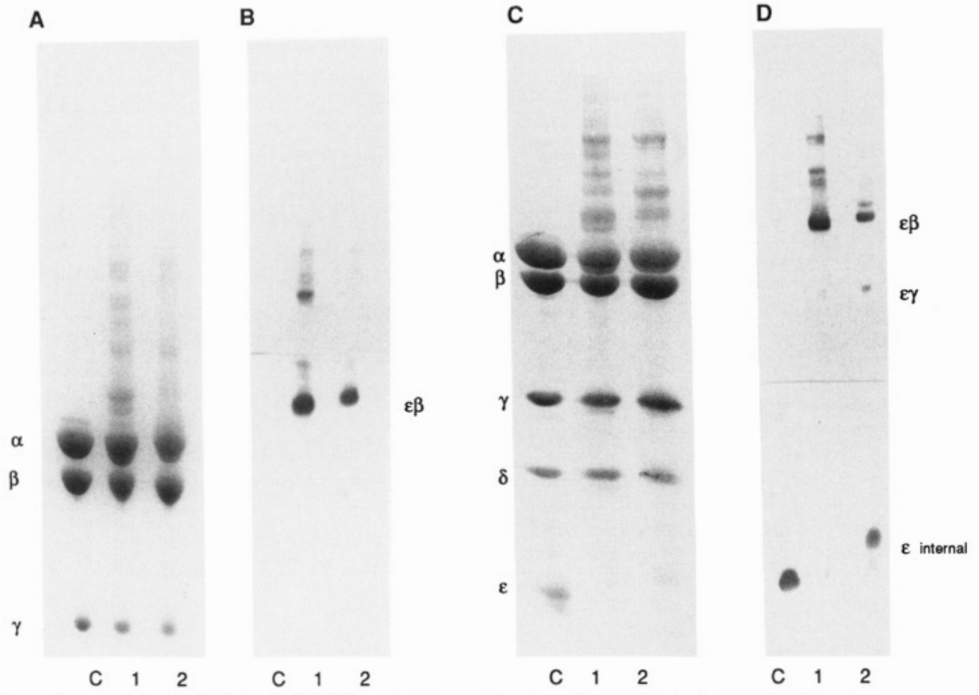

FIGURE 5: Formation of cross-links by EDC. ECF_1 (1 mg/mL) was incubated with (1 mg/mL) EDC in the presence of 5 mM ATP and 5 mM $MgSO_4$ (lane 1) or 5 mM ATP and 0.5 mM EDTA (lane 2), as described under Experimental Procedures. After incubation for 3 h at room temp, the cross-linking reaction was stopped by addition of 0.25 volume of 4 M Tris. The samples were then dissolved in $NaDodSO_4$ denaturing buffer and electrophoresed in duplicate on two different gradient slab gels, as described under Experimental Procedures. Half of each gel (A and C) was stained with Coomassie blue, while the other half was electrophoretically transferred to nitrocellulose paper and incubated with monoclonal antibody against the ϵ subunit (B and D). (A) and (B) represent separation of cross-linked products on a 7–10% polyacrylamide separating gel. (C) and (D) are the stained gel and immunoblot, respectively, of the same samples, run on a 10–18% separating gel. Lane C, ECF_1, control (no EDC present).

We find that the rate of cleavage of the ϵ subunit parallels the increase in ATPase activity caused by trypsin treatment, under all ligand binding conditions. These results support previous work indicating that the ϵ subunit is an inhibitor of ATPase activity in isolated ECF_1 (discussed later).

The enzyme preparation used in most of our studies contained 2 mol of nucleotide, and these were in noncatalytic sites. Trypsin digestion of this preparation in EDTA or Mg^{2+} alone gave only slow activation and slow cleavage of the ϵ subunit. Trypsin cleavage of enzyme passed through a Penefsky centrifugation column only once, conditions used by Bragg and Hou (1987), gave rapid activation and rapid cleavage of the ϵ subunit. This can be explained by the presence of nucleotide in catalytic sites which had not been removed by a single column procedure [such enzyme preparations contain 5 mol of nucleotide (ADP + ATP) per mole of enzyme; see also Hanada et al. (1989)]. Addition of ADP or ATP to nucleotide-depleted enzyme converted the ϵ subunit from a protease-resistant to protease-sensitive structure if Mg^{2+} was absent. These are conditions which convert aurovertin-modified enzyme from a low-fluorescence form to a high-fluorescence form, suggesting that changes in the β subunit (seen by aurovertin) and in the ϵ subunit (from protease digestion) are part of the same conformational switch.

Addition of Mg^{2+} along with ATP and consequent enzyme turnover to generate ADP + P_i + Mg^{2+}, or addition of ADP + Mg^{2+} + P_i directly, converted the ϵ subunit from a protease-sensitive to a protease-insensitive form. Addition of Mg^{2+} to nucleotide-replete ECF_1 also causes a change in aurovertin fluorescence as does the presence of P_i along with ADP + Mg^{2+}. Thus, our results provide evidence for two conformational changes in ECF_1, one induced by adding ATP or ADP to nucleotide-depleted enzyme and the second a conformational difference between ECF_1 loaded with Mg^{2+} and ADP and enzyme containing Mg^{2+} + ADP + P_i.

The role of bound nucleotide in maintaining a stable F_1 structure is well documented by cold inactivation and reconstitution studies (Dunn & Futai, 1980; Miwa & Yoshida, 1989; Rao et al., 1988). Our results and previous aurovertin studies indicate that a significant conformational change occurs as nucleotides bind, which involves at least β and ϵ subunits. This conformational change is unlikely to be a part of the enzyme mechanism as nucleotides would be bound in F_1 at all times in vivo. More important from a functional standpoint is the P_i-dependent conformational change of ECF_1.

Previous studies have established that the ϵ subunit acts to inhibit ECF_1 by decreasing the off rate of P_i from the catalytic site as much as 15-fold (Tozer et al., 1987). When the ϵ subunit is bound, ^{18}O exchange into P_i is extensive on ECF_1, while in ϵ-depleted enzyme, the exchange is negligible (Wood et al., 1987). Thus, the presence of the ϵ subunit clearly alters the binding of the P_i in catalytic sites on the enzyme. The results presented here suggest that there is a reciprocal relationship between P_i binding and the ϵ-subunit conformation.

We found a half-maximal effect of P_i on the conformation of the ϵ subunit at 50 μM. This is the range of the K_d values obtained in direct binding experiments for the single high-affinity binding site for P_i and F_1 (Penefsky, 1977; Kasahara & Penefsky, 1978; Languin et al., 1983; Koslov & Vulfson, 1985). Koslov and Vulfson (1985) have argued that the high-affinity site for P_i is a catalytic site containing Mg ADP. The K_d for P_i has been calculated from kinetic data based on the measured rates for all of the individual reactions in ATP hydrolysis except for the on rate of P_i in the presence of ADP + Mg^{2+} [K_{-3} in the scheme of Grubmeyer et al. (1982)]. The calculated K_d for P_i binding to F_1 from this approach is in the range 0.8–10 M (Grubmeyer et al., 1982; Cunningham & Cross, 1988; Al-Shawi et al., 1990). It remains possible, therefore, that the P_i effect we observe is due to binding in a noncatalytic site. We can only rule this out by kinetic studies

in which the rate of the conformational change in ε is related to the rate of cleavage of ATP to ADP + P_i or release of P_i during unisite ATP hydrolysis. We have recently modified the ε subunit by site-directed mutagenesis by converting selected Ser to Cys residues. These Cys residues will be reacted with fluorescent probes with the aim of monitoring the kinetics of the conformational change in this subunit.

The idea that binding of P_i changes the affinity of the core $\alpha_3\beta_3\gamma$ complex for the ε subunit, suggested by our cross-linking studies, is supported by recent cryoelectron microscopy studies. In the presence of ADP + Mg^{2+} or ATP + EDTA, we can visualize the ε subunit (as tagged by an Fab derived from a subunit-specific mAb) randomly distributed with respect to β subunits. When P_i is present along with ADP + Mg^{2+}, the ε subunit relocates predominantly to one β subunit (E. Gogol, E. Johnston, R. Aggeler, and R. A. Capaldi, unpublished results). A relocation of the ε subunit from one β subunit to another could occur as the first β subunit releases P_i and the ε now has equal affinity for all three β subunits. ATPase activity would still be inhibited if the ε subunit is being serially released and rebound, because enzymatic activity will be limited by P_i release from each catalytic site in turn.

An interesting finding of the present study relates to the segments of the ε subunit important for the inhibitory effect of this subunit on ECF_1. Kuki et al. (1988), using genetic approaches, have reported that a truncated form of the ε subunit including residues 1–93 confers inhibition but that a shorter ε fragment (1–80) shows no inhibition of ATPase activity. We find that the activation of ATPase activity by protease digestion occurs with cleavage of the ε subunit from the C-terminus but the activation is maximal when fragment ϵ_{II} (in Figure 2) is still present in a large amount. Our results, therefore, imply that the site important for inhibition is the C-terminal 30 amino acids that includes residues homologous to the inhibitor protein of mitochondrial F_1-type ATPases.

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Registry No. ATPase, 9000-83-3; P_i, 14265-44-2; ATP-Mg^{2+}, 1476-84-2; ADP-Mg^{2+}, 7384-99-8.

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Kinetic Analysis of Lactose Exchange in Proteoliposomes Reconstituted with Purified *lac* Permease

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ABSTRACT: Lactose exchange catalyzed by purified *lac* permease reconstituted into proteoliposomes was analyzed with unequal concentrations of lactose on either side of the membrane and at low pH so as to prevent equilibration of the two pools. Exchange with external concentrations below 1.0 mM is a single-exponential process, and the apparent affinity constants for external and internal substrate are close to the apparent K_Ms reported for active transport and efflux, respectively [Viitanen, P. V., Garcia, M. L., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1629]. At external lactose concentrations above 1.0 mM, a second kinetic pathway becomes evident with an apparent affinity constant of about 6 mM which is similar to the apparent K_M for facilitated influx. A second pathway is not observed with respect to internal lactose even when the concentration is increased up to 80 mM. Furthermore, high internal or external lactose concentrations do not inhibit the exchange reaction. Biphasic kinetics with respect to external lactose are retained in a mutant permease that catalyzes exchange but is defective in H^+-coupled lactose transport. It is suggested that *lac* permease has more than one binding site and that this may be the underlying reason for the biphasic kinetics observed for both exchange and H^+-coupled lactose transport.

The β-galactoside transport system of *Escherichia coli* catalyzes cotransport (i.e., symport) of lactose and H^+ across the cytoplasmic membrane with a stoichiometry of unity [for reviews, see Kaback (1983, 1989, 1990)]:

$$\text{lactose}_{out} + H^+_{out} \Leftrightarrow \text{lactose}_{in} + H^+_{in} \quad (1)$$

lac permease, the product of the *lac Y* gene, is the only gene product required, and the protein has been solubilized from the membrane, purified to homogeneity, and reconstituted into proteoliposomes in a functional state (Newman et al., 1981; Foster et al., 1982). The reconstituted system is ideal for studying the kinetic properties of *lac* permease due mainly to the passive impermeability of the proteoliposomes to ions and lactose (Garcia et al., 1983). Furthermore, both the turnover number of the permease and also its apparent K_M for lactose are similar in proteoliposomes and membrane vesicles with respect to membrane potential driven lactose accumulation, counterflow, facilitated diffusion (i.e., lactose influx under nonenergized conditions), and efflux (Viitanen et al., 1984).

The permease is a hydrophobic polytopic plasma membrane protein, and based on circular dichroic measurements and hydropathy analysis of the primary sequence (Foster et al., 1983), a secondary structure model was proposed in which the protein consists of 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion, connected by hydrophilic segments with the N- and C-termini on the cytoplasmic surface (Kaback, 1983, 1989, 1990). The general features of the model are consistent with other spectroscopic measurements (Vogel et al., 1985),[1] chemical modification (Page & Rosenbusch, 1988), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1984, 1986; Herzlinger et al., 1984). Moreover, recent studies on a large number of *lacY-phoA* (*lac* permease–alkaline phosphatase) fusions have provided strong exclusive support for the 12-helix model (Calamia & Manoil, 1990).

Mechanistic studies with right-side-out membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) and reconstituted proteoliposomes (Garcia et al., 1983; Viitanen et al., 1983) have led to a kinetic model for efflux, equilibrium exchange, and counterflow. The model is consistent with the effects of D_2O (Viitanen et al., 1983), the behavior of a monoclonal antibody that uncouples lactose from H^+ translocation (Carrasco et al., 1984), and the properties of various uncoupled mutants (Herzlinger et al., 1985; Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987). Accordingly, efflux down a concentration gradient consists of a minimum of five steps (Figure 1): (1) binding of substrate and H^+ on the inner surface of the membrane (order unspecified); (2)

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[1] In addition to circular dichroic and laser Raman spectroscopy, Fourier-transform infrared studies also show that purified *lac* permease is largely helical (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished work).

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