Communication

ATP Hydrolysis-linked Structural Changes in the N-terminal Part of the γ Subunit of Escherichia coli F₁-ATPase Examined by Cross-linking Studies*

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Robert Aggeler and Roderick A. Capaldi*

From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

A mutant of Escherichia coli F₁-ATPase (ECF₁) in which the serine residue in position 8 of the γ subunit has been replaced by a cysteine residue (γS8C) has been used to study nucleotide-dependent cross-linking of the γ subunit to a β subunit. When examined in the presence of ADP + Mg²⁺, either supplied directly or as produced during catalytic turnover of ATP + Mg²⁺, the main cross-linked product generated using the heterobifunctional, photoactivatable, cross-linker tetrafluorophenylazide maleimide-6 had a Mr(app) of 108,000. When ATP hydrolysis was inhibited, either by cold or by reaction with sodium azide, or when ATP hydrolysis was prevented by the use of adenyl-5'-yl P₄-y-imidodiphosphate, the main cross-linked products were species with Mr(app) of 102,000 and 84,000. The nucleotide-dependent switching from one cross-linking pattern to another could only be observed when the ε subunit was bound to ECF₁; it was not seen in ECF₁*, an enzyme preparation missing δ and ε subunits, but was observed in preparations selectively depleted of the δ subunit. We conclude that the changes detected in these cross-linking experiments are occurring during the hydrolysis of ATP when the β-γ phosphate bond is cleaved and that they are related to the coupling of ATP hydrolysis to proton translocation.

An F₂,F₀-type ATP synthase, found in the bacterial plasma membrane, the mitochondrial inner membrane, and the chloroplast thylakoid membrane, catalyzes ATP synthesis in response to a transmembrane proton gradient. This enzyme can also generate a proton gradient by using the energy released by ATP hydrolysis (reviews in Refs. 1–3). The best characterized F₁,F₀-type ATPase, the Escherichia coli enzyme, ECF₁,F₀, is composed of eight different subunits, five of which, α, β, γ, δ, and ε, are present in the membrane extrinsic F₁ part in the stoichiometry 3:3:1:1:1 (1, 2). We are studying ECF₁ and ECF₁,F₀ by a variety of biophysical and biochemical methods in order to understand how catalytic sites of the F₁ are coupled conformatively to the proton channel in the membrane intercalated F₀ part of the complex. Our general approach is to create mutants by site-directed mutagenesis to include a reactive cysteine in a subunit of choice, a site that can then be selectively modified with reporter groups such as fluorescent reagents, spin labels, and bifunctional cross-linking reagents (4–6). Recently we described the generation of mutants of the γ subunit including the mutant γS8C (6). Reaction of this mutant with the cross-linker TFPAM-6 led to covalent linkage of the γ to a β subunit. Preliminary experiments indicated that this cross-linking depended on which nucleotides were present during the photolysis reaction (6). We have now explored the nucleotide dependence of the cross-linking from the cysteine residue at position 8 of the γ subunit to a β subunit in more detail and here provide evidence that this cross-linking is tied to ATP hydrolysis in catalytic sites in a manner that is dependent on the tight binding of the ε subunit to the core of the enzyme complex.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—E. coli ATPase from the mutant γS8C, containing the plasmid pRA12 in the unc⁻ strain AN489 (6), was isolated and purified by a modified method of Senior et al. (7) and Wise et al. (8). ECF₁,F₀, an ECF₁ preparation depleted of δ and ε subunits, was isolated essentially as described by Tuttas-Dorschug and Hanstein (9), followed by two passages through a monoclonal anti-ε antibody affinity column according to Dunn (10). The ε antibody used (ε4 in Ref. 10) was a kind gift from Dr. Stanley Dunn (University of Western Ontario). ATPase depleted only of the δ subunit, was prepared by the procedure of Lottescher et al. (11).

Cross-linking of E. coli F₁; ATPase—Cross-linking experiments were carried out with TFPAM-6 essentially as described in Aggeler et al. (4). ATPase was partially depleted of nucleotides by the use of centrifuge columns, and nucleotides were added at final concentrations of 5 mM, either in the presence or in the absence of Mg²⁺. The photolysis was carried out either at room temperature for 2 h with a 6-watt 365-nm lamp or in ice water for 15 min with a 100-watt 365-nm lamp. Cross-link products were analyzed by electrophoresis on 6–12% polyacrylamide gels containing 20% mol tetrafluorophenylazide maleimide. Excess label was removed by two consecutive passages through centrifuge columns, and nucleotides were added at final concentrations of 5 mM, either in the absence or in the presence of 5 mM Mg²⁺.

RESULTS

Nucleotide Dependence of Cross-linking between γCys-8 and the β Subunit—For the experiments reported here, ECF₁ isolated from the mutant γS8C was freed of loosely bound nucleotide by two centrifugation columns in EDTA-containing buffer, thereby generating an enzyme retaining only 2 mol of tightly bound nucleotides in (two) non-catalytic sites (15). This enzyme preparation was reacted with TFPAM-6 via the maleimide and then cross-linked by photolysis after first adding different combinations of Mg²⁺ and/or nucleotides. Fig. 1 shows the results of cross-linking from Cys-8 of the γ subunit by TFPAM-6 under various nucleotide conditions. Three different major cross-linked products were seen in Coomasie Brilliant Blue stained gels, and each contained the β and γ subunits as determined by Western blotting using subunit-specific monoclonal antibodies as before (6).
ATP in a catalytic site during the photolysis reaction. As shown while that was not the case at room temperature. This difference could not be attributed to denaturation of ATPase in the control conditions. In EDTA alone (lane 3), 0.5 mM EDTA + 5 mM MgCl₂ (lane 3); 0.5 mM EDTA + 5 mM ADP (lane 4); 0.5 mM EDTA + 5 mM ATP (lane 5). A control was kept in the dark (lane 1). 70 μg of protein was applied on each lane, and electrophoresis was carried out on a SDS-containing 6-12% gradient polyacrylamide gel. The doublet of cross-linked products migrating below the 84-kDa band is α-δ and β-δ, respectively.

The key data are shown in lanes 6-9. With ADP + Mg²⁺ (lane 6), when ADP + Mg²⁺ + P, were generated in catalytic sites by turnover of ATP + Mg²⁺ on the enzyme (lane 7) and in the presence of AMP-PNP + Mg²⁺ (lane 8), the predominant cross-linked product was the 108-kDa species, with a small amount of a 102-kDa species also produced. In contrast, with the non-hydrolyzable ATP analogue AMP-PNP + Mg²⁺ present (lane 9), cross-linked products of 102 and 84 kDa were generated with little or no 108-kDa species produced.

Fig. 1, lanes 1-5, shows cross-linking under several different control conditions. In EDTA alone (lane 2) or Mg²⁺ alone (lane 3) the 102- and 84-kDa species were formed, but in low yield. With ADP + EDTA (lane 4) or ATP + EDTA (lane 5) the same two products were generated, but in higher amounts.

The difference in cross-linked products with AMP-PNP + Mg²⁺ versus AMP-PNP + Mg⁺⁹, or ADP + Mg²⁺ suggests that a conformational change occurs around Cys-8 of the γ subunit related to the cleavage of the β-γ bond of ATP by the enzyme. To explore this possibility more fully, cross-linking was conducted in ATP + Mg²⁺ at 0 °C to slow down ATP hydrolysis and retain ATP in a catalytic site during the photolysis reaction. As shown in Fig. 2A, the presence of uncleaved ATP along with Mg²⁺ increased the amount of the 102- and 84-kDa species with a concomitant reduction of the 108-kDa species (cf. lane 3 with lane 2, where there had been complete hydrolysis of ATP to ADP + P). In the cold, therefore, the cross-linking patterns of the Mg²⁺ + ATP and Mg²⁺ + AMP-PNP (lane 4) were identical, while that was not the case at room temperature. This difference could not be attributed to denaturation of ATPase in the cold. Preincubation in the cold followed by photolysis at room temperature, as well as preincubation at room temperature followed by photolysis in the cold, showed the 108-kDa cross-linked species as the main product (data not shown). A further indication of a native structure of ECF1 in the cold is provided by the observation that the cross-linking pattern in the presence of Mg²⁺ + ADP was identical on ice (lane 5) and at room temperature (Fig. 1, lane 6).

Cross-linking was also conducted in the presence of azide, a potent non-competitive inhibitor of F₁-ATPase activity, that abolishes cooperative interactions between catalytic sites (16, 17). Fig. 2B shows the concentration dependence of the effect of sodium azide. With an increasing concentration of the inhibitor, the amount of the 108-kDa species was reduced while the amount of the 102- and 84-kDa species increased.

Nucleotide-dependent Differences in Cross-linking from γCys-8 to the β Subunit Requires the Binding of the ε Subunit —ECF₁⁺⁺ is a preparation of ECF₁, missing both the δ and ε subunits. This preparation has a high ATPase activity due to release of the inhibitory ε subunit (i.e. 90 μmol of ATP hydrolyzed per min per mg versus 7-12 μmol per mg for the five-subunit enzyme) (18). Fig. 3 shows cross-linking studies similar to those presented in Fig. 1 but performed on ECF₁⁺⁺. As with the five-subunit enzyme, cross-linking in the presence of ATP + EDTA gave the 102- and 84-kDa species (lane 4). However, with ECF₁⁺⁺ the 102- and 84-kDa species were also obtained when ADP + Mg²⁺ were bound in catalytic sites either directly (lane 2), with ADP + Mg²⁺ + P, added (lane 3), or by enzyme turnover of ATP + Mg²⁺ (lane 5). The 108-kDa species was not generated in significant amounts under any nucleotide conditions tested with ECF₁⁺⁺. This difference in cross-linking patterns between ECF₁ and ECF₁⁺⁺ must be related to removal of the ε subunit. When ECF₁ selectively depleted of the δ subunit was examined (Fig. 4), similar results to those obtained with the five-subunit ECF₁ were obtained.

![FIG. 1. Nucleotide dependence of TFPAM-6 cross-linking of the γS8C mutant.](image)

![FIG. 2. Cross-linking of cold-treated and azide inhibited γS8C ECF₁. A, photolabeling at 0 °C. The cysteines of ECF₁ (4 mg/ml) were modified in 300 μl of labeling buffer with 200 μM TFPAM-6 as described in Fig. 1. Photolysis was carried out for 15 min with a 100-watt Blak-Ray lamp, either at room temperature after preincubating for 15 min with 0.5 mM EDTA, 5.5 mM MgCl₂ + 5 mM ATP (lane 2) or at 0 °C in 0.5 mM EDTA + 5.5 mM MgCl₂ + 5 mM ATP (lane 3). 0.5 mM EDTA + 5.5 mM MgCl₂ + 5 mM ATP-PNP (lane 4). A control sample was kept in the dark (lane 1). 100 μg of protein was loaded on each lane.](image)

![FIG. 3. Cross-linking of γS8C ECF₁⁺⁺, depleted of δ and ε subunit. ECF₁⁺⁺ was labeled at 3 mg/ml with TFPAM-6. Photolysis was carried out at 0.5 mg/ml for 2 h at room temperature in the presence of 0.5 mM EDTA + 5.5 mM MgCl₂ + 5 mM ADP (lane 2), 0.5 mM EDTA + 5.5 mM MgCl₂ + 5 mM ATP (lane 4) or 0.5 mM EDTA + 5.5 mM MgCl₂ + 5 mM ATP-PNP (lane 5). A control sample was kept in the dark (lane 1). 35 μg of protein was loaded on each lane of a 6-12% gradient polyacrylamide gel.](image)
The gradient was run at 40,000 rpm in a VTi50 rotor (Beckman) for 20 h at 4 °C. The ATPase-containing fractions were pooled and applied onto a Sephacryl S300 column (1 x 40 cm) in 50 mM Tris-HCl, pH 7.4, 20% glycerol, 2 mM EDTA, 1 mM MgCl₂, and 10 mM 6-aminohexanoic acid for removal of LDAO. The 6-free ATPase eluted from the column was precipitated with n-hexanoic acid for removal of LDAO. The 6-free ATPase eluted from the column was precipitated with n-hexanoic acid for removal of LDAO. The 6-free ATPase eluted from the column was precipitated with n-hexanoic acid for removal of LDAO.

DISCUSSION

There are several interesting aspects to the cross-linking data presented here. There is a clear difference in cross-linking from the Cys at position 8 of the y subunit to a subunit when the photolysis is performed in ATP + Mg²⁺ as opposed to ADP + Mg²⁺. Thus, when uncleaved ATP was present along with Mg²⁺ during photolysis, whether the rate of hydrolysis of the substrate has been slowed by cold (0 °C), or azide inhibition (17), or prevented by using the non-cleavable analogue AMP-PNP (26), the main products of cross-linking had Mᵣₑₐₓ of 102 and 84 kDa. This cross-linking pattern with ATP + Mg²⁺ bound is the same one seen when nucleotides ADP or ATP were bound without Mg²⁺ present. In the presence of ADP + Mg²⁺, the main cross-linked product was a 108-kDa species with the 102-kDa product much reduced in amount and the 84-kDa species absent. The temperature effect and the azide sensitivity of the observed changes in cross-linking pattern make it unlikely that these result from nucleotide binding changes in the non-catalytic sites. Instead, we conclude that the structural changes around Cys-8 of γ are related to ATP hydrolysis in catalytic sites.

Supporting evidence for the conclusion comes from fluorescence studies. We have labeled Cys-8 of the γ subunit in the mutant ySCC with the fluorescent reagent coumarin maleimide and followed changes in the fluorescence spectrum from this site as a function of ATP hydrolysis. There is a quenching of the fluorescence spectrum on addition of ATP + Mg²⁺ that follows the cleavage of the β-P bond of ATP. This quenching is not seen when ATP + EDTA, ADP + Mg²⁺, or AMP-PNP + Mg²⁺ are added, and it is blocked by prior treatment of the enzyme with the inhibitor azide.

The difference in cross-linking of the γ to β subunit in ATP + Mg²⁺ compared with ADP + Mg²⁺ was found to depend on the presence of the ε subunit. In ECF₂⁺, which is missing the ε and subunits, the 102- and 84-kDa cross-linked species were obtained in both nucleotide conditions. This must be a consequence of the loss of the ε subunit because enzyme selectively depleted of the ε subunit behaves identically to intact ECF₁. (The fluorescence changes detectable with coumarin maleimide bound at Cys-8 of the γ subunit are also lost on removal of the ε subunit.)

ECF₂⁺ is a much more active ATPase than intact ECF₁ because of the removal of the inhibitory ε subunit (18). The interaction of the ε subunit with the core ECF₁ complex has been shown to involve both the γ and β subunits (4, 20–23). In the intact ATP synthase (where the ε subunit is tightly bound), we propose that changes in the γ subunit couple catalytic site events with proton pumping in the F₀ part of the complex.

The generation of the 108-kDa species obtained when ADP is bound to intact ECF₁ is Mg²⁺-dependent. In the absence of Mg²⁺ (i.e. when ADP + EDTA are present), the 108-kDa species does not form, and instead, the 102- and 84-kDa species are obtained. There is good evidence that the catalytic sites on the β subunits of F₁ are similar to the GTP binding site of Ras, where it participates in binding the β- and γ-subunits of ATP and in liganding the Mg²⁺ ion (28, 29). Movements of the phosphate binding loop along with rearrangements of other close by segments of the Ras protein occur on GTP hydrolysis, and these changes alter the binding of Ras with other proteins (29, 28). Similar changes in and around the catalytic site of F₁ could trigger changes in the structure of the γ subunit under coupled conditions, i.e. in the presence of the ε subunit. We are in the process of mapping the sites of cross-linking of Cys-8 of γ with the β subunit. In the 108-kDa cross-linked product, this site has been localized to the peptide fragment (residues 145–155), which includes the phosphate loop region.

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