Rotation of a γ-ε Subunit Domain in the Escherichia coli F₁F₀-ATP Synthase Complex

THE γ-ε SUBUNITS ARE ESSENTIALLY RANDOMLY DISTRIBUTED RELATIVE TO THE αβδ DOMAIN IN THE INTACT COMPLEX*

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A triple mutant of Escherichia coli F₁F₀-ATP synthase, αQ2C/εS411C/εS108C, has been generated for studying movements of the γ and ε subunits during functioning of the enzyme. It includes mutations that allow disulfide bond formation between the Cys at α411 and both Cys-87 of γ and Cys-108 of ε, two covalent cross-links that block enzyme function (Aggeler, R., and Capaldi, R. A. (1996) J. Biol. Chem. 271, 13888–13891). A cross-link is also generated between the Cys at α2 and Cys-140 of the δ subunit, which has no effect on functioning (Ogilvie, I., Aggeler, R., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 16652–16656). CuCl₂ treatment of the mutant αQ2C/εS411C/εS108C generated five major cross-linked products. These are α-γ-δ, α-γ-ε-δ, α-ε, and α-ε. The ratio of α-γ-δ to the α-γ product was close to 1:2, i.e. in one-third of the ECF₁F₀ molecules the γ subunit was attached to the α subunit at which the δ subunit is bound. Also, 20% of the ε subunit was present as a α-δ-ε product. With regard to the δ subunit, 30% was in the α-γ-δ, 20% in the α-δ-ε, and 50% in the α-δ products when the cross-linking was done after incubation in ATP + MgCl₂. The amounts of these three products were 40, 22, and 38%, respectively, in experiments where Cu²⁺ was added after pre-incubation in ATP + Mg²⁺ + azide. The δ subunit is fixed to, and therefore identifies, one specific α subunit (α3). A distribution of the γ and ε subunits, which is essentially random with respect to the α subunits, can only be explained by rotation of γ-ε relative to the αβδ domain in ECF₁F₀.

F₁F₀-type ATPases are found in the plasma membrane of bacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts. These enzymes can both use a proton gradient to synthesize ATP and in the reverse direction hydrolyze ATP to establish a proton gradient for subsequent substrate and ion transport processes (1–3). The F₁ part of the enzyme from Escherichia coli, ECF₁F₀, is composed of α, β, γ, δ, and ε subunits in the stoichiometry 3:3:1:1:1. This part is linked by a narrow stalk to the F₀ part that is composed of α, b, and ε subunits where it interacts with the δ subunit distributed at all three β subunits was found to contain product, and the third is closing to trap the substrate.

An important tenet of the binding change mechanism is that catalytic sites are sequentially linked to the proton channel for energy coupling by a rotation of the small subunits. Early evidence for such rotation came from cryoelectron microscopy studies on ECF₁ that showed the γ subunit distributed at all three β subunits rather than fixed at one site (19, 20). Consistent with the idea of rotation of γ within the αβδ domain, cross-linking of this subunit to α or β subunits was found to fully inhibit the functioning of ECF₁ (16, 21).

Additional evidence for rotation of the γ subunit relative to the αβδ domain has been provided more recently by Duncan et al. (22). These authors isolated a complex containing a (unlabeled) β and γ subunit, stably linked by a disulfide bond between Cys-87 of γ and a Cys introduced at position 380 of β. They mixed this β-γ complex with a [35S]-labeled β subunit along with α, δ, and ε subunits to regenerate a functional F₁. This reconstituted enzyme was then shown to undergo subunit switching when the disulfide bond was broken and MgCl₂ + ATP was added, i.e. the γ moved from unlabeled to labeled β subunits. Sabbert et al. (23) have also provided evidence of

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rotation of the \( \gamma \) subunit in chloroplast \( F_1 \). Finally, rotation of the \( \gamma \) subunit in the \( \alpha_3\beta_2\gamma \) subcomplex of \( TF_1 \) has been visualized directly in recent elegant single molecule studies (24).

However, all of the above studies have focused on the movements of the \( \gamma \) subunit in \( F_1 \), and in terms of functional relevance it remains important to show that rotation of the \( \gamma \) subunit in conjunction with other subunits (e.g., the \( \epsilon \) subunit) occurs in the intact \( F_1F_0 \). Also, for an understanding of the coupling mechanisms, it is necessary to establish which subunits are moving with the \( \gamma \) subunit and which are fixed with respect to the \( \alpha_3\beta_2 \) subdomain. One attempt to examine rotation in \( ECF_1F_0 \) has been reported recently by Cross and colleagues (25) using the same cross-linking and reconstitution approach they used before for \( F_1 \). They showed an ATP-driven and dicyclohexylcarbodiimide-sensitive scrambling of \( \gamma \) relative to \( \beta \) subunits, although the level of this scrambling was lower than would be expected if all enzyme molecules were active.

We have previously observed that disulfide bonds can be formed from an \( \alpha \) subunit via a \( \gamma \) cyst to position 2 to the \( \delta \) subunit (in the mutant \( \alpha Q2C \) (26)) and via a \( \gamma \) cyst at residue 411 to the \( \gamma \) and \( \epsilon \) subunits (in the mutant \( \alpha S411C/eS108C \) (21)). Here, we report cross-linking studies with \( ECF_1F_0 \) from the mutant \( \alpha Q2C/eS411C/eS108C \) where cross-linking from the \( \alpha \) subunit to all three small subunits can be obtained at the same time. The combinations of cross-linked products obtained provide information about which subunits have to be moving in the ATP synthase and which do not.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants and Isolation of Enzymes**—The triple mutant \( pRA170 \) (\( \alpha Q2C/eS411C/eS108C \)) was obtained by ligating the 5.8-kilobase \( XhoI/NsiI \) fragment of \( pRA140 \) (21), which contains the mutations \( eS411C \) and \( eS108C \), to the 6.8-kilobase \( XhoI/NsiI \) fragment of \( pIO1 \) (26), which contains the mutation \( \alpha Q2C \). Mutant \( pRA141 \) (\( \alpha S411C \)) was created by ligation of the 2.9-kilobase \( NsiI/NsiI \) fragment of \( pRA100 \) (27) with the 9.7-kilobase \( SstI \) fragment of \( pRA140 \) (21), which contains the mutation \( \alpha S411C \). The triple mutant, \( pRA141 \), and the isolate of \( \alpha Q2C/eS411C/eS108C \) from \( E. coli \) were prepared as described by Gogol et al. (31) and Aggeler et al. (32), respectively.

**Other Methods**—\( ECF_1F_0 \) was reconstituted in egg lecithin on a Sephadex G-50 column (medium, 1.5 × 60 cm) in 50 mM Tris, pH 7.5, 2 mM MgCl\(_2\), 2 mM DTT\(^1\) and 10% glycerol, and cross-linking of the enzyme was carried out with CuCl\(_2\) in 50 mM MOPS, pH 7.0, 2 mM MgCl\(_2\), 2 mM ATP, 10% glycerol as described by Aggeler et al. (16). Cross-linked products were separated by electrophoresis on SDS-containing polyacrylamide gels according to Lammli (33). Two-dimensional SDS-PAGE of polyacrylamide gel electrophoresis was carried out by resolving cross-linked products in a first dimension without prior treatment with reducing agents on an 8% polyacrylamide gel. A portion of a lane was cut out and exposed to 50 mM DTT in dissociation buffer for 2 h at room temperature. The gel piece was rotated 90° and positioned with agarose on a stacking gel of a 10–18% polyacrylamide gel for the second dimension. Protein concentration was determined with the BCA protein assay from Pierce. Gels were stained with Coomassie Brilliant Blue R (34). Cross-linked products were identified with Western blotting, using monoclonal antibodies against \( F_1 \) subunits (35).

**RESULTS**

The experiments described here utilize the mutant \( \alpha Q2C/eS411C/eS108C \). The ATP hydrolysis rates of this mutant were in the range of 25–30 \( \mu \text{mol} \) of ATP hydrolyzed per min per mg, which is the same as for wild-type enzyme. Also, the \( ECF_1F_0 \)

\(^1\) The abbreviations used are: DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.
catalysis (23, 24). A priori, this rotational movement could be an artifact of a freedom of the \( \gamma \) subunit that is allowed only when the \( F_1 \) is dissociated from the \( F_0 \). However, the results of Zhou et al. (25) and the data presented in this study are evidence that this is not the case. The key observations here are that in ECF, \( F_0 \) from the mutant \( \alpha Q2C/\alpha S411C/\epsilon S108C \) there is cross-linking of \( \gamma \) and \( \epsilon \) subunits separate to the same \( \alpha \) subunit that binds the \( \delta \) subunit (Fig. 3). However, the \( \gamma \) and \( \epsilon \) subunits are never bound to the same \( \alpha \) subunit. The significance of these results is clear when the activity effects of cross-linking of the \( \gamma \), \( \delta \), or \( \epsilon \) subunits to \( \alpha \) subunits are considered. Covalent cross-linking of the \( \delta \) to an \( \alpha \) subunit has been found to have little or no effect on either cooperative ATP hydrolysis or on the proton pumping function of ECF, \( F_0 \) (26). This is in contrast to the cross-linking of \( \gamma \) or \( \epsilon \) to \( \alpha \) subunits, which completely blocks functioning (21). The conclusion from these activity data is that movements of \( \gamma \) and \( \epsilon \) but not \( \delta \) are an essential part of the functioning of the enzyme. It follows that the \( \delta \) subunit must be fixed with its interaction thereby identifying one of the three \( \alpha \) subunits (\( \alpha_a \) that can be visited by both \( \gamma \) and \( \epsilon \) since both \( \alpha-\delta-\gamma \) and \( \alpha-\delta-\epsilon \) cross-linked products were obtained.

The distribution of cross-linked products observed is understandable when it is considered that the experiments reported here involve a population of ECF, \( F_0 \) molecules that are not synchronized. Thus, at any time during enzyme turnover after ATP hydrolysis has stopped, around one-third of any rotating subunits should be at each of the three \( \alpha-\beta \) pairs. This is approximately the observed distribution of both \( \gamma \) and \( \epsilon \) subunits in our experiments whether enzyme activity was ended by substrate depletion or by addition of azide. In previous studies, it was shown that cross-linking of \( \epsilon \) to the \( \gamma \) subunit did not block activity (7, 27), indicating that these two subunits move together as a mobile domain. In electron microscopy studies (19), we have seen movements of the \( \gamma \) subunits relative to \( \epsilon \), but this is because the antibody fragments to \( \alpha \) and \( \epsilon \) were used to tag specific subunits release \( \epsilon \) from \( \gamma \) so that it is fixed at one \( \alpha-\beta \) subunit pair.

In summary, the scrambling of \( \gamma \) and \( \epsilon \) subunits with respect to the three \( \alpha \) subunits, one of which is clearly distinguished by interaction of the \( \delta \) subunit, is evidence for rotational movements of the main stalk forming subunits in ECF, \( F_0 \). The only other explanation of the data, that ECF, \( F_0 \) assembles with a fixed random distribution of the small subunits, does not seem feasible. The \( \gamma \) and \( \epsilon \) subunits appear to move as one domain, although there may be small movements of the two relative to one another as part of the coupling between catalytic sites and proton channel functioning (6).

For rotational movements of the \( \gamma \) and \( \epsilon \) subunits to occur within ECF, \( F_0 \), the \( \alpha-\beta \) domain must be fixed relative to the \( F_0 \) part by a stator. Recent evidence suggests that this stator is contributed by the \( \delta \) with the \( b \) subunits (9, 26, 36). For cou-

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**Fig. 2. Resolution and quantitation of subunits involved in CuCl₄-induced cross-link of ECF₁F₀ from \( \alpha Q2C/\alpha S411C/\epsilon S108C \) by two-dimensional SDS-polyacrylamide gel electrophoresis.**

ECF₁F₀ in egg lecithin vesicles in 50 mM MOPS, 2 mM MgCl₂, 2 mM ATP, and 10% glycerol was cross-linked at a concentration of 0.46 mg/mL with 100 \( \mu \)M CuCl₂ for 1 h at room temperature. The reaction was stopped with 7 mM EDTA, and 20 mM N-ethylmaleimide was added before the dissociation buffer without reducing agent. For the first dimension, 70 \( \mu \)g of protein was applied on a 8% polyacrylamide gel. After electrophoresis the top 12.5 cm of the 18-cm long resolving gel of a lane was cut out, soaked in 12 ml of dissociation buffer with 50 mM DTT for 2 h, and placed on a 10–18% polyacrylamide gel for the second dimension. A, the bands were visualized with Coomassie Brilliant Blue. B, relative intensities were determined by scanning the gel with Adobe Photoshop and NIH Image. The cross-linked products giving rise to the peaks are indicated.

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**Fig. 3. Rotation of \( \gamma \) and \( \epsilon \) in ECF₁F₀.** The \( \gamma \) and \( \epsilon \) subunits (shaded) rotate relative to \( \delta \) subunit. Cysteines involved in cross-links were either introduced by site-directed mutagenesis at positions 2 and 411 of the \( \alpha \) subunit and 108 of the \( \epsilon \) subunit or were endogenous in positions 87 of \( \gamma \) and 140 of \( \delta \).
pling, ATP hydrolysis-driven movements of the γ-ε domain must be linked to proton translocation. It has been established that both the γ and ε (Refs. 7 and 14, respectively) interact directly with the c subunit oligomer of the Fₐ subunit. The covalent cross-linking of γ or ε to the c subunit ring does not block ATP hydrolysis (7, 14), which implies that the rotatory element in ECF₁F₀ is a γ-ε-c oligomer domain moving relative to the α₃β₃δ-a₁b₂ complex.

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