The Second Stalk Composed of the b- and δ-subunits Connects F₀ to F₁ via an α-Subunit in the Escherichia coli ATP Synthase*

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The b- and δ-subunits of the Escherichia coli ATP synthase are critical for binding ECF₁ to the F₀ part, and appear to constitute the stator necessary for holding the α₃β₃ hexamer as the c-e-γ domain rotates during catalysis. Recent studies have determined that the b-subunits are dimeric for a large part of their length, and interact with the F₁ part through the δ-subunit (Rodgers, A. J. W., Wilkens, S., Aggeler, R., Morris, M. B., Howitt, S. M., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 31058–31064). To further study b-subunit interactions, three mutants were constructed in which Ser-84, Ala-144, and Leu-156, respectively, were replaced by Cys. Treatment of purified ECF₁F₀ from all three mutants with CuCl₂ induced disulfide formation resulting in b-subunit dimer cross-link products. In addition, the mutant bL156C formed a cross-link from a b-subunit to an α-subunit via aCys90. Neither b-b nor b-α cross-linking had significant effect on ATPase activities in any of the mutants. Proton pumping activities were measured in inner membranes from the three mutants. Dimerization of the b-subunit did not effect proton pumping in mutants bS84C or bA144C. In the mutant bL156C, CuCl₂ treatment reduced proton pumping markedly, probably because of uncoupling caused by the b-α cross-link formation. The results show that the α-subunit forms part of the binding site on ECF₁, for the b-δ domain and that the b-subunit extends all the way from the membrane to the top of the F₁ structure. Some conformational flexibility in the connection between the second stalk and F₁ appears to be required for coupled catalysis.

An F₁F₀ type ATPase is located in mitochondrial, chloroplast, and bacterial membranes where it catalyzes the terminal step in oxidative- and photo-phosphorylation. In Escherichia coli, the enzyme contains five different subunits in the F₁ part, α, β, γ, δ, and ε, in the stoichiometry 3:3:1:1:1, and three different subunits in the F₀ part, a, b and c, in the ratio 1:2:9–12. The F₁ part contains three catalytic sites on β-subunits and is an ATPase when released from the F₀, whereas the F₀ part forms a proton pore (1, 2).

The F₁ and F₀ parts of the E. coli enzyme are joined by a central stalk 40–45 Å in length (3) that is constituted by the γ- and ε-subunits (4), both of which make contact with the c-subunit ring (5, 6). Two other subunits, δ of the F₁ part and the two copies of the b-subunit of the F₀ part, are also involved in binding F₁ to F₀ (7–10). Recent studies indicate that the b-δ domain forms a second connection, a stator that fixes the α₃β₃ subdomain to the α-subunit to allow rotation of a γ-ε-δ subunit subdomain during energy coupling within the complex (9, 10). This stator function is consistent with the observed binding of the b-subunits to F₁ as a dimer and via the δ-subunit (10) and with experiments which show that, in the mutant αG2C, a disulfide cross-link can be formed between α (at Cys-2) and δ (at Cys-140), which is without effect on ATPase, ATP-dependent proton pumping, or ATP synthesis activities (11). The second stalk has recently been visualized by electron microscopy of single particles (12).

There are four Cys residues intrinsic to the wild-type α-subunit. Of these, Cys-90 readily forms a disulfide bond with δ in isolated ECF₁ (13). Based on the crystal structure of bovine mitochondrial F₁-ATPase (14), αCys47 and αCys90 are in close proximity, whereas the two remaining Cys residues are buried in the protein. Interestingly, only one αCys90 residue per F₁ complex is reactive to labeling with N-ethylmaleimide (15), demonstrating an inherent asymmetry of F₁.

Previous studies have shown that the b-subunit is dimeric for at least a portion of its length proximal to the C terminus (10, 16, 17) and also in the N-terminal membrane-spanning region (18). It has not been determined whether dimerization extends to the very C terminus of the protein. Interaction of the b-subunit with F₁ has been shown to involve the δ-subunit, specifically its C terminus (10), although it is not clear whether the b-subunit interacts with α or β as well.

To examine interactions involving the b-subunit more fully, three new mutants were constructed in which Cys replaced Ser-84, Ala-144, and Leu-156 (the C-terminal residue), respectively, and disulfide cross-link formation in ECF₁F₀ was studied. Upon treatment with CuCl₂, b-subunit dimers were formed in the mutant bL156C in competition with cross-linking to an α-subunit. This interaction of the very C terminus of b is shown to involve αCys90. Dimers of subunit b were also formed via Cys residues introduced at positions 84 and 144. These results show that the α-subunit forms part of the binding site on F₁, for the second stalk and that the b-subunit extends from the membrane to the N-terminal domain of the α-subunit.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Containing Mutations in the uncF Gene—Site-directed mutagenesis was carried out on an M13 mp18 clone containing the unc genes B, E, F, and H on a 2356-bp base HindIII/EcoRI fragment, according to the method of Kunkel et al. (19). Residues bSer84, bAla144, and bLeu156 were replaced by Cys using the following oligonucleotides (sequence changes underlined): AAACGCCGCTGCCAGCTGAC (bSer84), GGATGAAGCTTGTAACAGCGAC (bAla144C), and GGAGTGAAATCTGACATCGTG (bLeu156). The 909-base pair BsrGI/BstHHII fragments carrying the mutated uncF genes were excised from the M13 mp18 replicative form and ligated into the vector pRA100 (described in Aggeler et al. (20)). These plasmids were used to transform the unc⁻ E. coli strain AN888 (21). Strains XL1-Blue (Stratagene) and C3236 (New England Biolabs) were used in cloning and mutagenesis procedures.

CuCl₂-induced Cross-linking of ECF₁F₀—ECF₁F₀ was isolated and

* This work was supported by National Institutes of Health Grant HL58671 and by a grant from the Human Frontiers Science Program (both ro R. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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reconstituted into egg-lecithin vesicles as described in Aggeler et al. (22). Final resuspension was in 50 mM MOPS, pH 7.0, 2 mM MgCl₂, and 10% glycerol, at a protein concentration of 1 mg/ml. Cross-linking was carried out at 22 °C for 2 h using concentrations of between 5 μM and 200 μM CuCl₂. All cross-linking reactions were stopped by the addition of 5 mM EDTA, and ATPase activities (23) were measured with and without prior incubation of the samples with 20 mM DTT for 2 h at 22 °C.

Cross-linking in inner membranes was carried out at 22 °C for 2 h using 400 μM CuCl₂. Cross-linking reactions were stopped by the addition of 5 mM EDTA, and ATPase and atebrin fluorescence quenching activities were measured with and without prior incubation of the samples with 50 mM DTT for 2 h at 22 °C.

Preparation of Stripped Vesicles and Reconstitution with F₁-ATPase—ECF₁F₀ was prepared as described above and resuspended in 50 mM MOPS, pH 7.0, 2 mM MgCl₂, and 10% glycerol, at a protein concentration of 1 mg/ml. Stripped ECF₁F₀ was prepared by adding KSCN to a final concentration of 1 mM and pelleting the enzyme at 175,000 × g for 30 min at 4 °C in a Beckman TLA100.2 rotor. The pellets were washed twice by successive resuspension and centrifugation steps in 50 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM DTT, 40 mM 6-amino-n-hexanoic acid, and 20% glycerol. Stripped vesicles and F₁-ATPase dissolved in this buffer were mixed to give final protein concentrations of 0.5 mg/ml and 3.0 mg/ml, respectively, and incubated at 22 °C for 4 h. The enzyme was pelleted at 175,000 × g for 30 min at 4 °C in a Beckman TLA100.2 rotor and washed twice by successive resuspension and centrifugation steps in 50 mM MOPS, pH 7.0, 2 mM MgCl₂, and 10% glycerol.

Other Methods—Atebrin fluorescence quenching activities were assayed as described by Hatch et al. (24). Protein concentrations were determined using the BCA protein assay from Pierce, with bovine serum albumin as standard. F₁-ATPase was prepared from membranes of strain AN1460 (25) as described by Wise et al. (26) and modified by Gogol et al. (27). Strains used for the preparation of inner membranes and of F₁F₀-ATPase were grown in minimal medium with supplements (28).

RESULTS

Studies reported here involve three new mutants in which residues Ser-84, Ala-144, and Leu-156 are each replaced by Cys. Previous studies had established that a Cys at position 146 can form cross-links with both the corresponding residue of its b-subunit pair and the a-subunit (10). The implication is that the C-terminal region of the b-subunit is involved in binding to the F₁. It was of interest therefore to examine a mutant with a Cys at the very C terminus of the b-subunit. Residue 144 lies on the opposite face of a predicted α-helix which is continuous with that formed by a hydrophobic patch (residues 124 to 131). This patch is known to be important for dimer formation (16). Residue 84 is within a putative β-turn (residues 82–85) that is sensitive to tryptic digestion (10) and is one of the few regions not predicted to be α-helical.

A Cys at Position 156 Forms b-Subunit Dimers and Cross-links to an α-Subunit at Cys-90.—The CuCl₂ dependence of cross-linking of reconstituted ECF₁F₀ from the mutant bL156C is shown in Fig. 1a. Monomeric subunit b disappeared after incubation with 50 μM CuCl₂ into two competing products with approximate Mr 70,000 and 38,000, respectively. Analysis by Western blotting showed that both of these products contained b-subunit and that the Mr 70,000 band also contained α-subunit (see Fig. 1b). Additional evidence that the Mr 70,000 band consists of b cross-linked to α was obtained by excising the cross-link product from the gel, incubating the band in loading buffer containing DTT, and then identifying the components by SDS-PAGE in a second dimension (see Fig. 1c).

Previous studies have established that only one of the four Cys residues in the α-subunit can be labeled with N-ethylmaleimide, and this is αCys90 (11). The other three Cys residues are buried within the protein and are not expected to be avail-

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1 The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
able for interaction with the b-subunit. The α-β cross-link, therefore, almost certainly forms between αCYs90 and bCys156. The M₉ 38,000 band is a dimer of the b-subunit based on antibody binding as well as on molecular weight considerations. Table I lists the effect of cross-linking from the Cys at position 156 on the ATPase activity of the purified enzyme. Essentially full cross-linking of b-subunits into dimers or b-α caused no more than 15% inhibition of activity (Table I).

To establish more directly that Cys-156 of b is interacting with Cys-90 of α, a reconstitution experiment was conducted using F₁ from the mutant bL156C along with ECF₁ isolated from the mutant αC90A. Rebinding of wild-type or mutant F₁ to membranes containing F₀ from the bL156C mutant reconstituted a functional ECF₁F₀. Cross-linking of these reassembled enzyme preparations by CuCl₂ treatment is shown in Fig. 2. Rebinding of wild-type ECF₁ led to cross-links between α and b as well as generation of the b-dimer (Fig. 2, lane 2) as in the experiments in Fig. 1. An α-β cross-link occurs, migrating just above the α-b product, which is most likely produced in that fraction of ECF₁ improperly bound back to the F₀-containing membranes (it is not seen in intact ECF₁F₀; see Ref. 11). No α-b cross-linking was observed in ECF₁F₀ reconstituted to contain the Cys at b156 but with the Cys at α90 absent (Fig. 2, lane 4). However b-dimer was obtained (note that b-dimer is present even in the absence of Cu²⁺, being formed during the reconstitution procedure). In the reconstituted mutant αC90A/bL156C enzyme, there is internal cross-linking within the b-subunit, as seen previously, Ref. 11.

**CuCl₂ Treatment of Mutants bS84C and bA144C Yields b-Subunit Dimers**—Treatment of ECF₁F₀ from the mutant bA144C with CuCl₂ at a concentration as low as 25 μM caused essentially full cross-linking of b-subunit into dimers (see Fig. 3). In the mutant bS84C, a concentration of 150 μM also gave high yields of cross-linking into dimers (result not shown). The essentially complete linkage of b-subunit into dimers at positions 84 or 144 had no major effect on ATPase activity (Table I).

**Cross-linking of Mutant E. coli Inner Membranes and Effect on Enzyme Functioning**—Cross-linking in the mutants bA144C and bL156C was examined in E. coli inner membranes so that effects on proton pumping activity could be measured more readily. Dimers of b-subunits were produced in both mutants, which could be detected with the monoclonal antibody to this subunit. Based on the disappearance of monomeric b-subunit, the yield of cross-link in membranes from the mutant bL156C was around 70% in the experiment shown in Fig. 4. However, the intensity of the cross-link products on the blots was not as high as expected for such a yield. This may be because the cross-linking affects the accessibility of the antibody for its epitope. Cross-linking of the mutant bA144C in E. coli inner membranes also gave a greater disappearance of monomeric b-subunit than appearance of dimeric cross-link products (result not shown).

The effect of Cu²⁺-induced cross-linking on ATP-dependent proton pumping by the various mutants is shown in Fig. 5. With the mutants bS84C and bA144C, the effect on proton pumping was minimal (see Figs. 5, a and b). Proton pumping was reduced in bl156C, consistent with the lower growth rate of this strain on succinate (see Table I). Either the mutation itself or a small amount of cross-linking between α and b in the absence of added Cu²⁺ makes the membranes leaky to protons. Cross-linking by addition of Cu²⁺ further diminished the function of the mutant bL156C (Fig. 5c). DTT treatment reversed
The cross-linking-induced inhibition, confirming that it was the disulfide bond formation that was affecting function and not the effect of Cu2+ on other membrane components. Proton-translocating activity was not recovered completely on addition of DTT, possibly because of poor accessibility of the reductant to the disulfide bond in the bL156C mutant. We have seen similar variations in accessibility of disulfide bridges to DTT in our previous cross-linking studies.

**DISCUSSION**

An important new result of the present study is that the b-subunit with a Cys at position 156 forms a cross-link with an a-subunit or of the b-subunit to an a-subunit (as reported before) does not block cooperative, multisite ATPase activity. This is in contrast to a to b, a to e, b to e, a to γ, and b to γ cross-links, all of which block this activity essentially fully (see Ref. 11). The lack of effect on ATP hydrolysis would be anticipated if the two b-subunits and the b-subunit together form the second stalk, a stator connecting F1 to the F0.

Cross-links through Cys residues introduced at any position up to residue 146 of the b-subunit were found to have little effect on proton pumping. In contrast, cross-linking in the mutant bL156C inhibited proton pumping markedly, an effect reversed by breaking the disulfide bond with DTT. As described already, this mutant forms a cross-link with the a-subunit. It may, therefore, be the b-α cross-link rather than the b-β-cross-link that uncouples ATP hydrolysis from proton movements through the F0. The torque generated by rotation of the γ-ε domain within the α3βε hexamer could require compensation by way of conformational flexibility in the connection between the second stalk and F1. Based on the results here, such flexibility would be in interactions at the very C terminus of the b-subunit only, e.g., in its interactions with the a-subunit. Using genetic approaches, Takeyama et al. (31) also found a critical role for the very C terminus of the b-subunit. Removal of this single residue caused significant reduction of both F1 binding and 146 adopts a structure other than α-helix. Higher resolution structural data on the b-subunit than are available now will be needed to decide between these possibilities.

Cross-linking of the two b-subunits to one another, of a b-subunit to an a-subunit or of the b-subunit to an a-subunit (as reported before) does not block cooperative, multisite ATPase activity. This is in contrast to a to b, a to e, b to e, a to γ, b to δ, and b to γ cross-links, all of which block this activity essentially fully (see Ref. 11). The lack of effect on ATP hydrolysis would be anticipated if the two b-subunits and the b-subunit together form the second stalk, a stator connecting F1 to the F0.

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In summary, we add to the evidence that the b-subunits, along with the δ-subunit, form a second stalk connecting the F1 and F0 parts of the ATP synthase. Cross-linking has been observed between α and b which does not affect ATPase activity but inhibits proton pumping, presumably by disrupting the F1 subunit b interface.

Acknowledgment—The expert technical assistance of Kathy Chicas-Cruz is gratefully acknowledged.
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