The γɛ-c Subunit Interface in the ATP Synthase of Escherichia coli

CROSS-LINKING OF THE ε SUBUNIT TO THE c SUBUNIT RING DOES NOT IMPAIR ENZYME FUNCTION, THAT OF γ TO c SUBUNITS LEADS TO UNCOUPLING*

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Mutants with a cysteine residue in the γ subunit at position 207 and the ε subunit at position 31 were expressed in combination with a c-dimer construct, which contains a single cysteine at position 42 of the second c subunit. These mutants are called γY207C/εε’Q42C and εE31Cc/εQ42C, respectively. Cross-linking of ε to the c subunit ring was obtained almost to completion without significant effect on any enzyme function, i.e. ATP hydrolysis, ATP synthesis, and ATP hydrolysis-driven proton translocation were all close to that of wild type. The γ subunit could also be linked to the c subunit ring in more than 90% yield, but this affected coupling. Thus, ATP hydrolysis was increased 2.5-fold, ATP synthase was dramatically decreased, and ATP hydrolysis-driven proton translocation was abolished, as measured by the 9-amino-6-chloro-2-methoxyacridine quenching method. These results for εE31Cc/εQ42C indicate that the c subunit ring rotates with the central stalk element. That the γ-ɛ cross-linked enzyme retains ATPase activity also argues for a γɛ-c subunit rotor. However, the uncoupling induced by cross-linking of γ to the c subunit ring points to important conformational changes taking place in the γɛ-c subunit interface during this. Blocking these structural changes by cross-linking leads to a proton leak within the F0.

A proton-translocating F0,F1-type ATP synthase can be found in the periplasmic membrane of bacteria, the thylakoid membrane of chloroplasts, and the cristae membranes of mitochondria. This enzyme can use a proton gradient to synthesize ATP, a process that is reversible in bacteria, where the hydrolysis of ATP is used to generate a proton motive force for substrate and ion transport (1, 2). The best-characterized F1F0-type ATP synthase, that from Escherichia coli, is composed of two parts: a membrane-embedded F0 part containing three different subunits (a, b2, c12) (3–5) and a water-soluble ECF1 part with five different subunits (α3, β3, γ, δ, ε, e). The structures of the α and β subunits are known in detail from x-ray crystallography (6, 7). The F1 portion contains the three catalytic sites, each predominantly on a β subunit, but with the contribution of residues of the α subunits. Recent electron microscopy studies show that the F1 and F0 parts are connected by two stalks (8, 9). The more central stalk has been shown to include the γ and ε subunits (10, 11), whereas the outer stalk is made by the b and δ subunits (12, 13). The available evidence indicates that the central stalk is rotating inside the α-β subunits, with the more peripheral stalk acting to hold the α-β hexagon in position relative to the a subunit (14–16).

Various models of the catalytic mechanism have been proposed where the ring of c subunits is a part of the rotor coupling proton translocation across the membrane to conformational changes that lead to the formation of ATP (17, 18). However, the direct evidence that the c subunit ring rotates with the γ-ε subunits is lacking. One approach to establishing that γ, ε, and c subunits work together as the rotor would be to show that cross-linking of these subunits does not block energy-coupling within the complex. A positive result would be strong evidence that the linked subunits move in unison. A negative result would not rule out co-rotation but would require a molecular explanation, which might give important insight into the coupling mechanism.

The sites of interaction between γ and ε with the c subunit have been partly mapped by genetic and chemical cross-linking studies. The γ subunit interacts with the polar loop of c subunits via a region including residues Tyr-205, Tyr-207, and Glu-208 (19, 20). The interaction of the ε subunit with the polar loop of c subunit is via a loop provided by residues Glu-31 to His-38 (21). Although cross-linking of both γ and ε to the c subunit ring have been obtained already, the effects of these cross-links on function have been hard to assess. Introducing Cys residues into each of the polar loop regions of c causes dimer formation of this subunit, and the effects of these are difficult to differentiate from γ-ε or ε-ε interactions. To avoid such problems, we have now generated mutants in which Cys residues are present only in the polar loop of every second c subunit. This was accomplished by generating subunit c fusions genetically, where the linkage is from the C terminus of the first to the N terminus of the second copy of c. The mutant is constructed so that a Cys is present only in the second c subunit of each pair. As a result, there is very little cross-linking between c subunits, allowing the functional effects of cross-linking of γ or ε to the c subunit ring to be assessed unambiguously.

MATERIALS AND METHODS

Strains and Plasmids—E. coli strains used were CJ236 (New England Biolabs) for site-directed mutagenesis according to Kunkel et al. (22), XL1-Blue (Stratagene) for cloning (23, 24), and the unc-RAI (11) to express the mutant ATP synthases.

The c subunit dimer was created using a two-stage polymerase chain reaction strategy (26). The oligonucleotide 5’-CGTGAATTCGCTGCGCGGTACCATTGGTTAGGTCCTTATTATTAAGAGCC’ incorporates consecutive in-frame KpnI (bold) and SpeI (bold, underlined) restriction enzyme sites at the 3’ end of the uncE subunit (c) gene before the stop codon. This oligonucleotide was used in a polymerase chain reaction in conjunction with an antisense primer (CTGGCGGCAAAGGGCGC), which anneals downstream of the uncE region of the wild-type template pRA100 (described in Agyger et al. (27)). The purified “megaprimer” product of this reaction was used in a second polymerase chain reaction with a sense primer (CTGTCGGAATGGACGA),

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which anneals upstream of the uncE region of the template plasmid pRA100. The product, which encodes subunit c with a C-terminal 4-residue GTTS extension, was excised and integrated into the plasmid pRA100 with BssHII/BorGI, creating the plasmid pJM1.

In a separate polymerase chain reaction, sense and antisense oligonucleotides were employed to amplify the uncE region. The sense primer, 5’-CGGTAGACGTCTAGACCCGCGAGTACGCCGT-GAAAAACCTGAAT-3’, incorporates a 5’ KpnI restriction site (bold) preceding a 27-base region (italic, underlined) encoding the sequence SASNGASSA, a random sequence predicted to be flexible. The corresponding antisense primer, 5’-TAACTCATGCCGACAGGCAATC-3’, incorporates a 3’ SpeI restriction site (bold, underlined). Using the plasmid pBS106, which was created by inserting a 7.3-kilobase BorGI/NsiI fragment of pYZ215 into pRA100, as template DNA, the amplification product resulted in an uncE region with the mutation Q42C. This amplification product is inserted into pJM1 using restriction sites KpnI and SpeI, creating pRA197, which encodes two c subunits linked by the 11-residue loop GTSSAGGASSA with a cysteine residue in position 42 and threonine-serine at the C terminus of the second c subunit only (cc’Q42C). The double mutant pRA198, containing the c-dimer and a cisteine in position 31 of the c subunit (cc’Q42C/e31C), was generated by ligation the 7.2-kilobase Xhol/NsiI fragment of pRA197 with the 5.8-kilobase fragment of pBS106.

Mutant pRA230 containing a cysteine in position 207 of the γ subunit (γ207C) was created by site-directed mutagenesis, using the oligonucleotide 5’-GGGATTACCTGTCGAAACCCTACCC-3’, and subcloning of the mutated gene into pRA100 as described in Aggeler and Capaldi (28). pRA214 with the c-dimer and a cysteine in position 207 of the γ subunit (cc’Q42C/γ207C) was obtained from pRA197 and pRA230.

Other Methods—Inner membranes were isolated from the strain RA1 (11), which was transformed with mutant plasmid as described by Foster and Fillingame (29). ATP synthase was purified according to Foster and Fillingame (29), modified by Aggeler et al. (30). Reconstitution of F1,F0 in egg lecithin vesicles and CuCl2-induced cross-linking at 0.5 mg of protein/ml in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, and 10% glycerol was carried out as described by Aggeler et al. (31). ATP hydrolysis activity was measured with a regenerating system (32). ATP synthase was determined with a luciferin/luciferase detection system (Sigmas). NADH and ATP-dependent proton translocation to catalytic site activity was further evaluated by ATP synthesis measurements, studies not done by Jones and Fillingame (4).

RESULTS

Characterization of the Genetically Fused c Subunit Mutant cc’Q42C—It has recently been demonstrated that c subunits of the F0 part of ECF,F0 can be linked from the C terminus of one copy to the N terminus of a second copy to form fused dimers (4). Furthermore, the enzyme containing these genetically fused c subunits was shown to retain function. By introducing a Cys residue in the polar loop of only one of the two copies of such a c subunit dimer, we reasoned that it might be possible to prevent the cross-linking between polar loops of near neighbor c subunits, which has been problematic in previous studies that examined the effects of cross-linking γ or ϵ subunits to the c subunit ring. To this end, the mutant shown in Fig. 1 was constructed.

This new mutant contained the c subunit as a dimer connected by a loop of 11 amino acids from the C terminus of the first to the N terminus of the second copy of the subunit. The second c subunit of each pair also had two additional amino acids at its C terminus, as a result of the cloning strategy used. Finally, the polar loop region of the second, but not the first c subunit of the construct, had a Cys replacing the Gln at position 42. If the c subunits were arranged as a ring of 12 (33), there would have been 6 Cys residues at alternating positions in the ring in ECF,F0 from this mutant.

As shown in Fig. 2A, the ATPase activity of the inner membranes from the cc’Q42C mutant was approximately one-half that of wild-type cell membranes. ECF,F0 isolated from the mutant had an ATPase activity of 16.5 units/mg compared with around 24 units/mg for wild type, a reduction of around 30%. The lower activity appeared to be in part due to lower levels of assembled enzyme, but as shown by data for the purified enzyme, the mutant also had a lower turnover rate. Enzyme in inner membranes from the mutant cc’Q42C retained a level of DCCD sensitivity of the ATPase activity close to that for wild-type, 70% compared with 80% using 20 μM DCCD. Proton pumping was only marginally affected (compare plot C against plot B of wild type in Fig. 2). In this respect, the dimer construct prepared in this study was different from that described recently by Jones and Fillingame (4), which had a much reduced proton-pumping function. The efficient coupling of proton translocation to catalytic site activity was further evaluated by ATP synthesis measurements, studies not done by Jones and Fillingame (4).

As hoped, there was no significant cross-linking between Cys residues in the cc’ subunit ring at levels of Cu2+, which generated high yields of ϵcc’ or eϵ products. At higher Cu2+ concentrations, tetramer formation was observed without concomitant loss of activity.

Covalent Linkage of ϵ to the c Subunit Ring in the Mutant ϵ31C/cc’Q42C—To examine the effects of cross-linking of the ϵ subunit to the c subunit ring, the mutant ϵ31C/cc’Q42C was constructed. As shown in Fig. 3, there was ready disulfide bond formation between ϵ and the cc’ in this mutant. At 150–200 μM Cu2+, the yield of eϵ cross-linking was 80–90% based on the disappearance of the ϵ subunit in the Coomassie Blue-stained gels when purified ECF,F0 from the mutant was reacted with Cu2+. A similar yield of cross-linking was calculated in inner membranes after treatment with the same Cu2+ concentration,
The effects of cross-linking of ε to cc’ on function of ECF1F0 are summarized in Fig. 4. High cross-linking yields had no significant effect on ATP hydrolysis rates or on proton pumping function when measured in the ACMA quenching assay (Fig. 4B). The rate of ATP synthesis was only reduced 30% when 200 μM Cu²⁺ was used for cross-linking. However, a similar level of inhibition was obtained when wild-type enzyme was treated in the same way. Therefore, this effect could be due to cross-linking of other enzymes in the membrane that lead to some proton leakage or an effect on ECF1F0 other than cross-linking of ε to cc’.

Covalent Linkage of γ to the c Subunit Ring in the Mutant γY207C/cc’Q42C—Our previous studies have shown that a Cys at positions 205 or 207 (19) can be cross-linked to the polar loop of the ε subunit in high yield. The mutant γY205C/cc’Q42C was examined in detail previously (19). Here, the mutant γY207C/cc’Q42C was studied because it grew like wild type on succinate, whereas the γY205C/cc’Q42C mutant did not.

As shown in Fig. 5A, there were significant levels of cross-linking of γ to cc’ in ECF1F0 purified from the mutant at 200 μM Cu²⁺, but maximal yields required levels up to 1 mM. In membranes, lower levels of Cu²⁺ gave higher levels of γ/cc’ product than in the isolated membrane, based on monoclonal antibody binding in Western blots (Fig. 5C), but cross-linking was still incomplete at 200 μM. Fig. 5B shows the effects of the cross-linking on ATPase activity. At 200 μM Cu²⁺, with an approximate 50% yield of cross-link, there was a 170% increase in enzyme activity. When the enzyme was treated with 1 mM Cu²⁺, there was an increase of 300%. The maximum yield of cross-link was obtained at 5 mM Cu²⁺, with a 50% increase in activity. At 10 mM Cu²⁺, there was a 70% increase in activity. These results suggest that the cross-linking of γ to cc’ leads to an increase in enzyme activity, possibly by stabilizing the enzyme structure.

DISCUSSION

The cross-linking of γY207C or εE31C to cc’Q42C has been studied before (19). However, functional studies were complicated by the fact that the Cys introduced into the c subunit led to the formation of c dimers in addition to the γ/ε or ε/ε product being examined. In our previous work (19), this dimer formation caused an inhibition of the enzyme function, making interpretation of the effects of cross-linking of the central stalk subunits to the ε subunit ring difficult. To avoid this problem, we followed an approach developed by Jones and Fillingame (4)
Polyacrylamide gels were run after the addition of 7 mM EDTA, 50 mM dithiothreitol (±), and created a dimer in which the two copies were connected by a flexible loop at the periplasmic side. We then introduced 1 cysteine/dimer in the second subunit in the polar loop part at position 42. The effect was to place Cys at alternate c subunits around the ring on the F1-facing side. This proved to eliminate significant cross-linking between adjacent polar loops of the c subunits. Tetramers, i.e. cross-links of two cc' subunits, were obtained at higher Cu²⁺ concentrations. As reported by Jones and Fillingame (4), E. coli with ECF₁F₀ in which the c subunit is a dimer instead of monomer, grew well on succinate. With the linkage between the two monomers on the periplasmic side, ECF₁F₀ retained ATP-coupled proton translocation close to that of wild-type enzyme.

In the mutant e31C/cc'Q42C, there was ready cross-linking of the c subunit ring, an observation first made by Zhang and Fillingame (38) using a mutant incorporating a Cys at position 31 in e but with a Cys for Gln-42 in all 12 c subunits. In their studies, they compared enzyme function in membranes of their mutant that had been prepared with and without reducing conditions. This was possible because cross-linking between the Cys at position 31 in e and a Cys in the polar loop of the c subunit blocks alteration of their mutant which had been prepared with and without reducing conditions. As reported by Jones and Fillingame (4), E. coli with ECF₁F₀ in which the c subunit is a dimer instead of monomer, grew well on succinate. With the linkage between the two monomers on the periplasmic side, ECF₁F₀ retained ATP-coupled proton translocation close to that of wild-type enzyme.

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Here, we examined the effects of cross-linking of a Cys in ε at position 31 and in the subunit at position 42 in both purified ECF₃F₀ from the mutant E31CεεεQ42C and in inner membranes from this mutant. Cross-linking had essentially no effect on ATPase activity in purified enzyme, where the yield of ε-ε₂ cross-linking was very high, and yet generation of cross-linking between polar loops of εεε subunits was essentially zero. Cross-linking of ε to the εεε subunit ring also had no significant effect on ATPase activity in inner membranes, and there was little or no effect on proton translocation or ATP synthesis because of conformational interactions of the two subunits. These results are consistent with the ε subunit ring and the ε subunit rotating together during coupling of catalytic site events and proton channeling in both ATP hydrolysis and ATP synthesis.

It is interesting to note that the yield of cross-link of the ε to ε₂ was close to 100% (and that of γ to the εεε subunits greater than 80%), although only 50% of the 12 polar loops of the εεε subunit ring contain a Cys. The most likely explanation is that the polar loop regions of ε are deformable enough that the polar ε₂ can reach Cys-31 of ε or Cys-207 of γ. This is supported by the fact that some tetramer formation was observed, requiring significant flexibility of the loop.

There is now considerable data to indicate that both γ and ε subunits rotate during functioning of F₁F₀-type ATPases (14–16). The observation that the two can be cross-linked together without effect on ATP hydrolysis or ATP synthesis is strong evidence that the two subunits move together during coupling (25, 27). It was surprising, then, to find that cross-linking of γ to the ε subunit ring induced an uncoupling of the F₁ from the F₀ part such that ATPase activity was increased by release from constraints of the F₀, and that the F₀ part became leaky to protons. This leak through the F₀ not only affected ATP-driven proton translocation but also NADH-driven proton pumping (see Fig. 6). Accepting that γ, ε, and ε subunits all rotate in unison, the data for the mutant γεεεεεεQ42C are best interpreted as indicating conformational changes of the γ subunit and/or ε subunit ring during the rotation that are important for functioning. It is important to note that the mutant functions like wild type in the absence of cross-link formation. Only after disulfide bond formation, which would prohibit the conformational change, was there loss of coupling. It is interesting that the recent model of the ε subunit ring provided by Fillingame and co-workers (33) has a key residue in the proton-translocating path, Asp-61, pointing away from the surface of the α subunit thought to complete the proton channel. These authors propose that there must be rearrangement of the ε subunits as each is brought into interaction with the α subunit during the rotation process. It could be this reorientation of all or a part of the ε subunits that is being blocked by the cross-linking described here. Clearly, the γεεεεεεQ42C mutant will be interesting to study by physical methods such as video microscopy to explore the linkage of rotation of the γεεεεεε rotor to proton translocation in more detail.

In summary, we provide the first strong evidence that the ε subunit ring rotates along with the γεεεεεε rotor, based on results with the mutant εεεεεεεQ42C. It appears that the rotor is not a rigid entity but undergoes conformational rearrangements transmitted from γεεεεεε to the ε subunits during coupling steps based on studies of the γεεεεεεεεQ42C mutant. Such conformational flexibility of the γεεεεεε to the ε subunit ring interface was already apparent in previous cross-linking studies in that a Cys at position 31 of ε readily cross links at several sites on the polar loop of ε and that a Cys at 205 of γ can link to several positions on this loop of the ε subunit.

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