Interactions between the F₁ and F₀ Parts in the *Escherichia coli* ATP Synthase

ASSOCIATIONS INVOLVING THE LOOP REGION OF C SUBUNITS

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The N-ethylmaleimide reactivity of c subunits in *Escherichia coli* F₁F₀ ATP synthase (ECF₁F₀) isolated from five mutants, each with a cysteine at a different position in the polar loop region (positions 39, 40, 42, 43, and 44), has been investigated. The maleimide was found to react with Cys placed at positions 42, 43, and 44 but not at 39 or 40. All copies of the c subunit reacted similarly when the Cys was at position 43 or 44. In contrast, the Cys in the mutant cQ42C reacted as two classes, with 60% reacting relatively rapidly and 40% reacting at a rate 40-fold slower. After removing F₀, all copies of the c subunit in this mutant reacted equally fast. Therefore, the slow class in the cQ42C mutant represents c subunits shielded by, and probably involved directly in, the interaction of the F₀ with γ and ε subunits of the F₁ part. Based on the estimated stoichiometry of c subunits in the ECF₁F₀ complex, 4 or 5 c subunits are involved in this F₁ interaction. N-Ethylmaleimide modification of all of the c subunits reduced ATPase activity by only 30% in ECF₁F₀ from mutant cQ42C. Modification of the more rapidly reacting class had little effect on ATP hydrolysis-driven proton translocation, and did not alter the DCCD inhibition of ATPase activity. However, as those c subunits involved in the F₁ interaction became modified, DCCD inhibition was progressively lost, as was coupling between ATP hydrolysis and proton translocation.

F₁F₀ ATP synthases are found in the plasma membrane of bacteria, the thylakoid membrane of chloroplasts, and the inner membrane of mitochondria, where they use the energy of a proton electrochemical gradient to drive ATP synthesis. These large multi-subunit complexes are composed of two domains, as the name implies: an F₁ part, which is extrinsic to the membrane and contains the catalytic sites, and an F₀ part, which spans the bilayer and contains a proton pore. The F₁ part of the *Escherichia coli* enzyme is composed of five subunits, α, β, γ, δ, and ε, in the stoichiometry 3:3:1:1:1. The F₀ part contains three subunits, a, b, and c, in the molar ratios 1:2.9–12 (1–4).

The recent high resolution structure determination of bovine mitochondrial F₁ shows the α and β subunits arranged as a hexamer surrounding a central cavity in which the γ subunit is located. This γ subunit extends from the αβδε barrel into the narrow stalk region that joins the F₁ and F₀ parts (4, 5). Recent evidence indicated that the γ subunit binds directly to the c subunits of the F₀ part (6, 7). Also present in the stalk is the ε subunit (8), which has direct interaction with c subunits (9). The δ subunit has also been considered a component of the stalk (10, 11). However, recent studies indicate that this subunit is bound close to the top of the F₁, at an α-β interface, where it binds to the F₀ part by interaction with the b subunit (12, 13).

The c subunit oligomer is arranged in F₀ as a ring that is 70 Å in diameter based on atomic force microscopy (14). Each c molecule is folded as a helical hairpin (15–17), with two α helices traversing the membrane. The N and C termini are in the periplasmic space, and the polar loop region is in the cytosol (18, 19). A key residue of the c subunit is Asp⁵¹. DCCD reacts with this residue, blocking proton translocation and inhibiting F₁ catalytic function (10, 20). How many of the c subunits are involved in the interaction between the F₀ and γ and ε subunits is not known. To examine this question, we have reacted Cys residues introduced into this polar loop region with [¹⁴C]NEM. Our results show two types of c subunits within the ring: a group of 4–5 c subunits that are shielded from reaction with the maleimide by interaction of F₁ subunits, and a second group of 6–7 c subunits that are more readily accessible for modification. NEM reaction of these two groups affects functioning differently, and this has significance for the mechanism of energy coupling within ECF₁F₀.

EXPERIMENTAL PROCEDURES

**Mutant Purification and Reconstitution—**Mutants ca39C, ca40C, cQ42C, cp43C, and cd44C have been described previously (6, 9). Purification of ECF₁F₀ from these mutants was according to Aggeler et al. (21). Reconstitution of ECF₁F₀ into egg lecithin vesicles was as described previously (7) for the vesicles used in ACMA fluorescence quenching assays. ECF₁F₀ was prepared by KSCN extraction of purified F₁F₀ after reconstitution into egg lecithin vesicles (22). The F₀ was resuspended in Buffer A (50 mM MOPS, pH 7.0, 10% glycerol, 2 mM MgCl₂) by washing twice by centrifugation at 150,000 × g for 30 min at 4 °C. [¹⁴C]NEM Reaction with ECF₁F₀ and F₀ Preparations—[¹⁴C]NEM was added to ECF₁F₀ and ECF₀ at 1 and 0.3 mg/ml of protein, respectively, in Buffer A. For time course experiments, an aliquot was removed immediately, 2% SDS was added to act as a control for full incorporation, and this was incubated for 2 h at 22 °C. At the times indicated, aliquots were removed and the NEM reaction stopped by incubating at 22 °C for 30 min with 50 mM DTT. The data were analyzed using a curve fit that assumed two independent rates for Cys residues introduced into this polar loop region with [¹⁴C]NEM.

The abbreviations used are: DCCD, dicyclohexylcarbodiimide; ECF₁, soluble portion of the F₁ part of the E. coli F₁F₀ ATP synthase; ECF₀, membrane-bound portion of the E. coli F₁F₀ ATP synthase; ECF₁F₀, E. coli F₁F₀ ATP synthase; ACMA, 9-amino-6-chloro-2-methoxyacridine; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; NEM, N-ethylmaleimide.
group-NEM adduct, respectively; $A = B + C = 100$; $k_A$ and $k_B$ are second-order rate constants for group$_1$ and group$_2$, respectively; $N =$ initial NEM concentration; $D =$ protein concentration; and $t =$ time.

Quantitation of $[^{14}C]$NEM Incorporation—The quantitation of the incorporation of $[^{14}C]$NEM into the $c$ subunits was as described previously (22, 23). The percentage of the Cys in all c subunits that reacted with $[^{14}C]$NEM after different treatments was determined on the basis of the total incorporation of the maleimide into the appropriate detergent-solubilized control sample incubated with 800 $\mu$M $[^{14}C]$NEM for 2 hr at 22 °C to ensure full labeling. The reaction was stopped by the addition of 50 mM DTT.

**DCCD Inhibition and NEM Titration—**ECF$_F_0$ reconstituted in egg lecithin vesicles suspended in Buffer A (0.5 mg/ml protein) was reacted with 50 $\mu$M DCCD for 1 hr at 22 °C. Aliquots were then mixed with equal volumes of various concentrations of NEM dissolved in DMSO, and samples were incubated for 1 h at 22 °C. The reaction was stopped by addition of 600 $\mu$M DTT with incubation at 22 °C for 30 min. Samples were assayed for ATPase activity in 5 mM ATP and 2 mM MgCl$_2$ as described previously (24).

**ACMA Fluorescence Quenching and NEM Titration—**Samples treated with NEM were reacted with 600 $\mu$M DTT for 30 min to quench the maleimide reaction, and then 20 $\mu$g of protein from each sample was assayed for ACMA fluorescence quenching as described previously (22) at 1 mM ATP and 2 mM MgCl$_2$.

Other Methods—Samples for SDS-PAGE were mixed with a one-half volume of dissociation buffer (10% SDS, 6.8, 30% glycerol) and 50 mM DTT and incubated for 1 h at room temperature. Polypeptides were separated using a 10–22% linear gradient (25), and protein bands were visualized by staining with Coomassie Brilliant Blue R according to the method of Downer et al. (26). Protein concentrations were determined by the BCA assay from Pierce. $[^{14}C]$NEM was obtained from DuPont NEN (40 mCi/mmol).

**RESULTS**

Five different mutants (cA39C, cA40C, cQ42C, cP43C, and cD44C) were used in this study. ECF$_F_0$ isolated from each of these mutants had activities in the range of 25–33 $\mu$mol ATP hydrolyzed per min/mg, and each mutant was DCCD sensitive (85% or more inhibition on addition of 50 $\mu$M DCCD). Enzyme from each of the mutants was reconstituted into proteoliposomes at a ratio of protein to lipid of 1:2 (w/w) and the reactivity of the Cys introduced at different positions in the c subunit loop, native ECF$_F_0$ from each of the mutants was reacted with 100 $\mu$M $[^{14}C]$NEM for 1 hr at room temperature. Fig. 1 summarizes the results obtained. The labeling of the Cys at positions 39 and 40 was negligible. In contrast, Cys at position 42 or 44 was labeled completely under the reaction conditions chosen. With the mutant cQ42C, only around 70% of the c subunits were labeled, implying that in this mutant, there are two populations of c subunits, one class that reacts readily and a second class that is protected from labeling. In Fig. 1, the shaded bars show the labeling of $F_0$ preparations from the various Cys mutants. After the removal of the $F_1$ part, the Cys at 39 and 40 remained unreactive, indicating that these residues are not buried by contact with $F_1$ subunits but, instead, either by protein-protein contacts within the $F_0$ or by lipid-protein interactions. After removal of $F_1$ from the mutant cQ42C, all of the c subunits became reactive to NEM.

The Interaction of the F$_1$ Part Differentiates a Subclass of c Subunits—The reactivity of the c subunit in ECF$_F_0$ from the mutant cQ42C was examined in more detail by both concentration dependences and time courses of $[^{14}C]$NEM labeling. Fig. 2A shows the reaction of the c subunit in this mutant with 0–200 $\mu$M NEM. Modification of all of the c subunits occurred at significantly lower NEM concentrations in the isolated $F_0$ than in the intact $F_1F_0$ complex. A time course of reaction of c subunits in ECF$_F_0$ with 100 $\mu$M $[^{14}C]$NEM is presented in Fig. 2B. Two phases of the reaction were observed. A fraction of the c subunits reacted less readily than the remainder. The best fit to these kinetic data was obtained with 60% of the c subunits reacting at the same rate of 62 s$^{-1}$ m$^{-1}$, and 40% reacting with a second rate of 1.5 s$^{-1}$ m$^{-1}$. When c subunits were reacted with NEM under identical conditions, but in isolated $F_0$, all reacted rapidly, with a rate of 94 s$^{-1}$ m$^{-1}$. For comparison, 100% of c subunits of ECF$_F_0$ from the mutant cD44C reacted with $[^{14}C]$NEM at one rate of 100 s$^{-1}$ m$^{-1}$.

**Effect on Functioning of NEM Modification of the Cys in the Mutant cQ42C—**The activity effects of NEM modification of the Cys at position 42 of the c subunit was examined as a function of the percentage of c subunits reacted. The extent of the modification was established by altering the concentrations of NEM used (as in Fig. 2A). Reaction of all copies of Cys$^{42}$ labeling in SDS for that mutant.

**Relative Exposure of Cys at Different Positions in the c Subunit Loop—**To compare the relative reactivity of Cys at different positions in the c subunit loop, native ECF$_F_0$ from each of the mutants was reacted with 100 $\mu$M $[^{14}C]$NEM for 1 hr at room temperature. Fig. 1 summarizes the results obtained. The labeling of the Cys at positions 39 and 40 was negligible. In contrast, Cys at position 42 or 44 was labeled completely under the reaction conditions chosen. With the mutant cQ42C, only around 70% of the c subunits were labeled, implying that in this mutant, there are two populations of c subunits, one class that reacts readily and a second class that is protected from labeling. In Fig. 1, the shaded bars show the labeling of $F_0$ preparations from the various Cys mutants. After the removal of the $F_1$ part, the Cys at 39 and 40 remained unreactive, indicating that these residues are not buried by contact with $F_1$ subunits but, instead, either by protein-protein contacts within the $F_0$ or by lipid-protein interactions. After removal of $F_1$ from the mutant cQ42C, all of the c subunits became reactive to NEM.

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NEM in the native and denatured forms of ECF1F0 profoundly destroys function, so this specific site was not studied here. Position 41 is a highly conserved Arg, and its replacement Cys at residue position 39, 40, 42, 43, or 44 in this loop (7–9). The effect of NEM modification on ATP hydrolysis-coupled proton translocation by ECF1F0 from mutant cQ42C is presented in Fig. 4. Modification of the more reactive c subunits (curve 2) had very little effect on ATP-driven proton translocation as measured by the ACMA fluorescence quenching assay. However, when the modification of c subunits exceeded 60%, coupled proton translocation was essentially lost (curves 3–5).

**DISCUSSION**

We have been using mutants of ECF1F0 in which Cys residues have been introduced into various subunits to probe structure-function relationships, using cross-linkers (e.g. Ref. 27), binding fluorescent probes (e.g. Ref. 28), or, as in this study, by monitoring the reactivity of the Cys and the effect of the modification on functioning (see also Ref. 29). Here, we have examined the arrangement and role of the polar loop region of the c subunits by taking advantage of various mutants that have introduced residues into the F0 subunits by taking advantage of various mutants that have introduced Cys residues into the periplasmic space. The polar loop, which is predicted to include residues 39–44, is in the cytosol pointing toward the F1 part (18). Our results show that residues 39 and 40 are buried, not by interaction of the F1 part, but either by lipid-protein interaction or protein-protein contacts within the F0 part. In the latter case, these must be interactions between c subunits given the low copy number of a and b subunits.

The number of c subunits per ECF1F0 remains unclear; values of 10–12 have been reported (18, 19). The labeling data here for enzyme denatured in SDS are no more definitive for technical reasons. The value obtained was different for each mutant, but in the range of 9.4–12.2.

The key result of the present study is that the c subunits in ECF1F0 are not all equivalent, but fall into two classes based on the NEM reactivity of the Cys at position 42. On labeling of ECF1F0 from the mutant cQ42C, 60% of the c subunits reacted essentially with N- and C-terminal regions in the periplasmic space. The polar loop, which is predicted to include residues 39–44, is in the cytosol pointing toward the F1 part (18). Our results show that residues 39 and 40 are buried, not by interaction of the F1 part, but either by lipid-protein interaction or protein-protein contacts within the F0 part. In the latter case, these must be interactions between c subunits given the low copy number of a and b subunits.

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The key result of the present study is that the c subunits in ECF1F0 are not all equivalent, but fall into two classes based on the NEM reactivity of the Cys at position 42. On labeling of ECF1F0 from the mutant cQ42C, 60% of the c subunits reacted relatively rapidly with maleimide, all at a similar rate of around 62 s⁻¹ M⁻¹. The other 40% reacted much more slowly (rates of 1.5 s⁻¹ M⁻¹). These more slowly reacting c subunits are involved in F1 binding because after removal of the F1, all of the c subunits were equally accessible.
No subset of shielded c subunits was observed on labeling ECF, F, from mutants cP43C or cD44C with [14C]NEM, suggesting that the interaction of the F₁ part with c subunits is relatively limited and involves mainly Arg²¹ and Glu₄².

The effect of NEM incorporation at position 42 on DCCD inhibition of ATP hydrolysis supports the idea that only a few of the c subunits interact with the F₁ part. Modification of the faster reacting 60% of c subunits has little or no effect on the DCCD sensitivity of ATP hydrolysis in the mutant. However, with reaction of the last 40%, there was a progressive loss of the inhibition caused by the covalently bound carbodiimide. The implication is that NEM reaction with the more shielded Cys disrupts the F₁F₀ interaction and causes loss of DCCD sensitivity. Genetic studies have shown that Glu₄² can be replaced by Val, Ala, or Cys but not by more bulky side chains without disrupting the F₁F₀ interface (30). Presumably the size difference between Cys and the Cys-NEM adduct is sufficient to alter binding of the γ and ε subunits with the c subunit oligomer. Reaction of the Cys at position 42 was also found to affect ATP-driven proton translocation in the mutant cP44C. As the number of c subunits modified by NEM was increased, the coupling of ATP hydrolysis to proton translocation as measured by ACMA fluorescence quenching was decreased. This assay is not quantitative, but it is clear that modification of more than 60% of the c subunits is required before proton pumping is greatly reduced. The uncoupling of ATP hydrolysis from proton translocation is explained if NEM modification of the Cys at position 42 disrupts the F₁ and F₀ interface.

The question of how many c subunits are involved in binding of F₁ to F₀ is not answered absolutely by the present studies because of uncertainties about the stoichiometry of the c subunit in the complex. However, if there are 10 copies, 4 are involved, whereas if there are 12, then 5 are involved. The organization of c subunits in the F₀ part remains to be defined precisely. However, the recent single-particle, atomic force microscopy studies show the c subunit oligomer as a ring within which is a pit (14). This would appear to preclude the idea that the c subunits are a tightly packed bundle with an inner core set that binds the F₁ part.

If, as proposed, the c subunits are arranged in a single ring, then the fact that two classes of c subunits can be detected in activity assays has important functional implications. The emerging model of the coupling of catalytic site events with proton translocation includes the rotation of the stalk subunits γ (31–33) and ε (32, 34). This rotation of γ and ε could be relative to a fixed c subunit ring, or there could be concerted movements of a domain that includes both γ and ε subunits and the c oligomer. We have previously found that the cross linking of γ (via Cys at 205) to the c subunit ring does not significantly affect ATP hydrolysis rates. We interpreted these results in favor of a model in which the γ and ε subunits oligomers, along with the c subunit oligomer, move together (7). The results presented here are consistent with this idea. If the γ and ε subunits were moving relative to the c subunit ring, all of the c subunits should become equivalent during enzyme turnover and modification of any group of the c subunits by NEM should have the same overall effect. As a consequence, NEM incorporation into the faster-reacting class of c subunits would be expected to disrupt the F₁F₀ interface as the γ-ε domain moves around to interact with these. This is not the case, as both the DCCD inhibition of ATPase activity and the coupling of ATP hydrolysis to proton translocation are not lost until more than half of the c subunits have been modified.

In summary, evidence is presented that 4 or 5 c subunits are involved in the binding of the γ and ε subunits to the F₀. Furthermore, it appears that this interaction is fixed such that the γ and ε subunits do not switch between c subunits during functioning. Our working model is that rotational catalysis involves movements of a complex of the γ, ε, and c subunits and is relative to other parts of the F₁ and F₀ that are linked by a stator constituted by the δ and b subunits (see Ref. 13).

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