Subunits Coupling H\(^+\) Transport and ATP Synthesis in the Escherichia coli ATP Synthase

Cys-Cys CROSS-LINKING OF F\(_1\) SUBUNIT \(\epsilon\) TO THE POLAR LOOP OF F\(_0\) SUBUNIT \(\epsilon\)

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Second site suppressor mutations at position 31 of F\(_1\) subunit \(\epsilon\) recouple ATP-driven H\(^+\) translocation in the uncoupled Q42E mutant of subunit \(\epsilon\) of the Escherichia coli F\(_1\)F\(_0\) ATP synthase (Zhang, Y., Oldenburg, M., and Fillingame, R. H. (1994) J. Biol. Chem. 269, 10221–10224). This finding suggests a functional interaction between subunit \(\epsilon\) and subunit \(\epsilon\) during the coupling of H\(^+\) transport through F\(_0\) to ATP synthesis of F\(_1\). However, the physical proximity of the two subunits remained to be defined. In this study, Cys residues were introduced into residues in the polar loop region of subunit \(\epsilon\) surrounding Gin42 and at position 31 of subunit \(\epsilon\) to see whether the subunits could be cross-linked. Disulfide bridge formation between subunit \(\epsilon\) and subunit \(\epsilon\) was observed in membranes of three double mutants, i.e. ca40C/e31C, cQ42C/e31C, and cP43C/e31C, but not in wild type membranes or in membranes of the ca39C/e31C double mutant. These results indicate that the polar loop of subunit \(\epsilon\) and the region around residue 31 of subunit \(\epsilon\) are physically close to each other in the F\(_1\)F\(_0\) complex and support the hypothesis that these two subunits interact directly in the coupling of H\(^+\) transport to ATP synthesis. Disulfide cross-linking of the Q42C subunit \(\epsilon\) and E31C subunit \(\epsilon\) leads to inhibition of ATPase coupled H\(^+\) transport, as might be expected in a model where the catalytic sites of the F\(_1\) ATPase alternate during H\(^+\) transport-coupled ATP hydrolysis/synthesis. However, a quantitative relationship between the extent of inhibition of transport and the extent of cross-linking could not be established by the methods used here, and the possibility remains that the \(\epsilon\)-C cross-linked F\(_1\)F\(_0\) complex retains residual H\(^+\) transporting activity.

The H\(^+\)-transporting, F\(_1\)F\(_0\) ATP synthase of Escherichia coli utilizes an H\(^+\) electrochemical gradient to drive ATP synthesis during oxidative phosphorylation (Senior, 1988). Similar enzymes are found in mitochondria, chloroplasts, and other bacteria. The enzymes are composed of two sectors, termed F\(_1\) and F\(_0\). The F\(_1\) sector contains the catalytic sites for ATP synthesis, and when released from membrane, it shows ATPase activity. The F\(_0\) sector traverses the membrane and functions as the H\(^+\) transporter. When F\(_1\) is bound to F\(_0\), the complex acts as a transport-coupled ATP hydrolysis/synthesis.

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FIG. 1. Plasmids used in polymerase chain reaction mutagenesis and in construction of mutated unc operon plasmids. Key restriction digest sites are shown. The unc fragments in the plasmids are shaded.

TABLE I

| Mutations | Oligonucleotidea | unc# at 3’ |
|-----------|------------------|------------|
| A39C | 5`-G ACG CGC ACA GCC TTC CAG GAA TTT AC-3’ | 1983 |
| A40C | 5’-C AGC ATG TGG AGC GCA TGC GCC TTC-3’ | 1994 |
| Q42C | 5’-C AGC ATG AGC GCA ACC GCG TGC-3’ | 2000 |
| P43C | 5’- G AAT CAG ATC AGG CCC TTC-3’ | 2007 |
| D44C | 5’- CAG AAT CAG GGG AGG TGC-3’ | 2007 |
| E31C | 5’-A GAT CCC CAC ACA GCC TTC-3’ | 7187 |

a Oligonucleotides are complementary to the sense strand. Nucleotides that have been changed are underlined.

Complementation of Chromosomal unc Deletion Mutant—Strain MO204 (AN346, unc (B-C, Ilv+) (Fraga et al., 1994) was transformed with pMO142 and its mutant derivatives. Transformant colonies were transferred to minimal medium 63 agar plates (Miller, 1972), containing 22 μM succinate as carbon source and incubated at 37°C with scoring for growth after 1-5 days.

Membrane Preparations, Assays, and Buffers—Cells were grown on M63 minimal medium containing 1% glucose, 2 mg/l thiamine, 0.2 mM l-arginine, 0.2 mM uracil, 0.02 mM dihydroxybenzoinic acid, and supplemented with 10% LB (Miller, 1972). Ampicillin was added to a final concentration of 100 μg/ml. Membranes were prepared in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 10% glycerol) by rupture of cells in a French press (Mosher et al., 1979). Immunostaining was carried out using the “Enhanced Chemiluminescence System” (Amersham Corp.). The rabbit antiserum to subunit c used was that described by Girvin et al. (1989). Antibodies that nonspecifically cross-reacted with E. coli membrane proteins were removed by preabsorption with membranes prepared from a mutant strain with a deleted unc operon (Girvin et al., 1989). The mouse monoclonal antibody to subunit ε (13-A7, e ε; Agger et al., 1990) was a gift from Dr. R. Capaldi (University of Oregon, Eugene, OR). Subunit c was purified as described by Hermein and Fillingame (1989). Purified subunit ε was a gift from Dr. S. Dunn (University of Western Ontario, Canada).

Cross-linking and Purification by Immunoprecipitation—Membrane vesicles at 10 mg/ml in TMG buffer were treated with 1.5 mM Cu(II)-2-hydroxy-1,1-bis(hydroxymethyl)ethyl glycine. After a 1-h incubation at room temperature, EDTA was added to a final concentration of 15 mM, and the sample was incubated for 20 min. F₀ was isolated from 5 mg of these oxidized membrane vesicles by the method of Schneider and Altendorf (1984), following stripping of F₀ from the membranes, except that dithiothreitol was omitted in all buffers. The isolated F₀ was resuspended in 50 μl of radioummune precipitation buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholate). Immunoprecipitation was performed according to the method described by Sambrook et al. (1989) with some modifications. The solubilized F₀ mixtures were first diluted to 0.5 ml with IPP150 buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Nonidet P-40, 0.02% NaN₃) and incubated for 4 h at 4°C with mouse monoclonal antibody to subunit ε which had been preabsorbed to protein A-Sepharose beads. The immunoprecipitates were centrifuged and washed twice with 1 ml of radioummune precipitation buffer, three times with 1 ml of IPP150 buffer, and finally once with 1 ml of 10 mM Tris-HCl, pH 7.5. The beads were suspended in 40 μl of SDS sample buffer in the absence of reducing agent and incubated 30 min at 50°C. Following centrifugation for 20 s in a microcentrifuge, the supernatant solution was collected and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting; half of the sample was made 10% in β-mercaptoethanol to reduce disulfide cross-links prior to electrophoresis.

RESULTS

Substitutions and Their Effect on Function—Cys residues were introduced at positions 39, 40, 42, 43, and 44 in the polar loop of subunit c or/and position 31 of subunit ε. Plasmids coding the whole unc operon were transformed into strain MO204, which carries a deletion of the chromosomal unc operon. The growth of transformants was tested using a succinate carbon source, where growth depends upon a functional oxidative phosphorylation system. As shown in Table II, each of the single mutant plasmids and most of the double mutant plasmids promoted growth as well as the wild type plasmid. The p43C/e31C plasmid transformant grew less well.

The ATPase and ATP-driven ACMA quenching activities of membrane vesicles from these transformant strains are shown in Table III. Membrane vesicles were prepared under nonreducing conditions in buffer lacking dithiothreitol. The e31C substitution by itself led to a 2-fold elevation in ATPase activity,

TABLE II

| Plasmid | Substitutions | Growth on succinate |
|---------|---------------|---------------------|
| pMO142 | Wild type | 1.2 |
| pYZ203 | cA39C | 1.2 |
| pYZ204 | cA40C | 1.2 |
| pYZ207 | eA39C | 1.2 |
| pYZ208 | eA40C | 1.2 |
| pYZ209 | cA39C | 1.2 |
| pYZ210 | cA40C | 1.2 |
| pYZ211 | eA39C | 1.2 |
| pYZ212 | eA40C | 1.2 |

* Colony size measured after incubation at 37°C for 48 h.

The abbreviations used are: ACMA, 9-amino-6-chloro-2-methoxy-acridine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

The plasmids were constructed by ligating NcoI-digested plasmids containing single Cys substitutions in the polar loop of subunit c and in construction of mutagenized plasmids used in this study. mML -arginine, 0.2 mM uracil, 0.02 mM dihydroxybenzoic acid, and 24610 unit plasmids containing single Cys substitutions in the polar loop of subunit c and in construction of mutagenized plasmids used in this study.

The abbreviations used are: ACMA, 9-amino-6-chloro-2-methoxy-acridine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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### Table III

| Mutation | ATPase activity* | ATP-driven ACMA Quenching* |
|----------|------------------|----------------------------|
| None     | 0.46             | 88                         |
| εE31C    | 0.83             | 87                         |
| cA39C    | 0.38             | 86                         |
| cA40C    | 0.43             | 84                         |
| cQ42C    | 0.31             | 82                         |
| cP43C    | 0.39             | 46                         |
| cA39C/cE31C | 0.83    | 83                         |
| cA40C/cE31C | 0.89    | 46                         |
| cQ42C/cE31C | 0.22    | 61                         |
| cP43C/cE31C | 0.43    | 35                         |

*ATPase activity was measured in 50 mM Tris-HCl, pH 7.8, 0.2 mM MgSO₄, 0.4 mM [γ-32P]ATP. Activities shown are the average of two sets of assays (done in triplicate with S.D. < 10%) on a single membrane preparation. The percent of ACMA fluorescence quenched after addition of 0.94 mM ATP to 0.25 mg/ml membrane vesicles in HMK buffer, pH 7.5. Percentages given are the average of duplicate or triplicate trials on a single membrane preparation.

whereas the polar loop substitutions by themselves had little effect on ATPase activity. The combined effect of mutations on ATPase activity was variable. The cP43C mutation by itself caused a significant reduction in the ATP-driven ACMA quenching response. Three of the four polar loop mutations, when combined with the eE31C mutation, also led to significant reductions in the ACMA quenching response. The significance of these changes in activity is considered in greater detail below.

Spontaneous Sulphydryl Cross-link Formation in the Membrane Vesicles—Membrane vesicles prepared under nonreducing conditions (TMG buffer lacking dithiothreitol) were solubilized with SDS and analyzed by electrophoresis and immunoblotting. A single band corresponding to subunit ε was detected with monoclonal antibody to subunit ε in each of the single Cys mutant membranes and in wild type membranes (Fig. 2). A slower moving protein band with a molecular mass around 25 kDa was detected in three of the double Cys mutants, cA40C/eE31C, cQ42C/eE31C, and cP43C/eE31C, but not in the cA39C/eE31C double Cys mutant. The apparent molecular mass of this band (25 kDa) corresponds to the size expected for a heterodimer of subunit c and ε (i.e. 24 kDa). When the gel was analyzed by immunoblotting using antibodies to subunit c, a 25-kDa protein band of apparently corresponding mobility was observed in the same three double Cys mutants, although the patterns were more complicated due to subunit c oligomer formation (Fig. 2). A heavy protein band with a mobility around 15 kDa was also detected with subunit c antisera in all the subunit c Cys mutant membranes, but not in wild type or the eE31C single mutant membranes. This protein band likely results from cross-linking of the polar loop Cys of subunit c. The 25-kDa band was not detected in any of the other F0 preparations. A 15-kDa protein band, corresponding in size to a subunit c monomer, was detected by subunit c antisera in the F0 preparations of all the subunit c Cys mutants.

Following dissociation of F0 subunits with detergent, the putative c-ε cross-linked product was precipitated by antibody to subunit ε. The immunoprecipitates were analyzed by immunoblotting (Fig. 4). In the three double Cys mutants shown, the 25-kDa protein band was detected both with antiserum against subunit c and antibody to subunit ε. A corresponding band was not detected in equivalent preparations from wild type or eE31C single Cys mutant membranes (Fig. 4A). The 25-kDa protein band was not observed if the sample was treated with β-mercaptoethanol before electrophoresis. Instead, two protein bands were observed, and they migrated to the positions corresponding to subunit c and subunit ε monomers (Fig. 4B). From these results we conclude that a disulfide bridge is indeed formed between polar loop residues of subunit c and position 31 of subunit ε, after introduction of Cys groups at these positions, and that these two regions lie physically close to each other at the surface of the membrane.

Effect of Cross-linking on Function of the Enzyme—To examine the effect of disulfide cross-link formation on the function of the enzyme, membrane vesicles from one of the double mutants, cQ42C/eE31C, were prepared under either nonreducing or reducing conditions, i.e. in the presence or absence of 5 mM dithiothreitol. The proportions of ε subunit in the 25-kDa product versus monomeric ε subunit were compared after solubilization of membranes in SDS sample buffer lacking β-mercap-
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In the membrane prepared under reducing conditions, antibody staining of the 25-kDa band was nearly undetectable; >95% of the stain was estimated to track with monomeric subunit e (Fig. 5B). In membranes prepared under nonreducing conditions the yield of cross-linked product was estimated by comparing the staining density of the two bands and found to vary in the range of 63–78% with different gels and different sample loadings.

The ATP-driven ACMA quenching response of the cQ42C/eE31C vesicles, prepared under reducing and nonreducing conditions, was compared to assess the effect of cross-linking on ATPase-coupled H⁺ transport function.4 As shown in Fig. 5A, the ATP-driven ACMA quenching response of cQ42C/eE31C membranes prepared under nonreducing conditions was approximately 70% of that given by membranes prepared under reducing conditions (curve 1 versus curve 2). A nearly complete quenching response was restored when the oxidized preparation was assayed in HMK assay buffer with 10 mM dithiothreitol (Fig. 5A, curve 3). The final experiment shown in Fig. 5A (curve 4) is the quenching response of cQ42C membrane vesicles prepared under nonreducing conditions. These vesicles show a normal quenching response. This control suggests that the oxidation-induced inhibition of quenching with cQ42C/eE31C vesicles can be attributed to formation of the c–e cross-link rather than c–c cross-links.

Unfortunately, the ACMA quenching response does not decrease linearly with decreases in ATPase activity (Miller et al., 1990). Using the calibration curves previously described by Miller et al. (1990), we estimate that a 30% reduction in quenching response would occur under conditions where activity was reduced by 50–90%. The reduction in quenching response is thus approximately that expected from the extent of cross-link formation, if the c–e cross-linked F₁F₀ is inactive in proton pumping. The results therefore do indicate that c–e cross-link formation markedly reduces enzyme function, but do not rule out the possibility that the c–c cross-linked enzyme retains residual proton pumping activity.

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prepared under nonreducing conditions was also measured. As shown in Table III, the quenching response by the cA39C and cA40C single mutant membranes approached that of wild type membranes, even though subunit c homodimer formation was observed in both preparations (Fig. 2). The quenching responses by the double mutant membranes, i.e. cA40C/eE31C and cP43C/eE31C, were decreased compared with that of their subunit c single mutant membrane counterparts, whereas the quenching response by the cA39C/eE31C mutant membranes was not significantly different from that of cA39C single mutant membranes. The cA39C/eE31C mutant was the only double mutant not showing formation of a cross-link between subunit c and subunit e. These results provide further support to the above conclusion that cross-linking between subunit c and e inhibits the function of the enzyme.

**DISCUSSION**

In a previous study (Zhang et al., 1994), we demonstrated that the uncoupled phenotype of the cQ42E mutation could be suppressed by second site mutations in Glu31 of F1 subunit e. This discovery suggested a possible functional interaction between the polar loop of subunit c and subunit e in the coupling process. To test whether the loop region of subunit c and residue 31 of subunit e are actually physically close to each other in the F1-F0 complex, Cys residues were introduced into both regions and cross-linking attempted by oxidation. Heterodimeric cross-linked products were formed in the membrane vesicles of three of the double Cys mutants, i.e. cA40C/eE31C, cQ42C/ eE31C, and cP43C/eE31C, but not in the membrane vesicles of the cA39C/eE31C mutant. These experiments provide the first evidence of a contacting interface between the polar loop of subunit c and subunit e and support the hypothesis that these two subunits may interact directly. Our conclusions are consistent with the previous cross-linking experiments of Suss (1986), who concluded that subunit c and e of chloroplast F1-F0 could be cross-linked by bifunctional imidoesters.

The cA39C/eE31C mutant was the only double mutant not forming a cross-link between subunit c and subunit e. The Cys of the A39C subunit c does appear to be reactive in forming subunit c dimers (see Figs. 2 and 3). The residue also appears to be accessible to Cu(II)-(1,10-phenanthroline)3 oxidation since c-c dimerization was increased by this reagent. On the other hand, the A39C sulphydryl reacted less readily with N-[H]methylmaleimide than the other polar loop Cys residues (data not shown). The Cys31 sulphydryl may be buried in the F1 complex in a less reactive, more hydrophobic environment, relative to the other polar loop substitutions.

A direct interaction between the polar loop of subunit c and subunit e has important implications in the mechanism of coupling H+ transport to ATP synthesis. The conformation of the polar loop of subunit c is hypothesized to change with the ionization state of Asp61 in the middle of the membrane (Fillingame, 1990; Fillingame et al., 1992). The conformation of subunit e and its position within F1 also changes with the occupancy of catalytic sites (Capaldi et al., 1992). Binding of Mg-ADP-Pi at catalytic sites results in a simultaneous association of γ and e with β (Gogol et al., 1990), an association that is manifested by increased N-ethyl-N’-dimethylaminopropylcarbodiimide catalyzed cross-link formation between Ser108 of subunit e and Glu381 in the conserved DELSEED sequence of subunit β (Dallman et al., 1992; Mendel-Hartvig and Capaldi, 1991). According to the recently published, atomic resolution crystal structure of the αβγδ domain of beef heart mitochondrial F1 ATPase (Abraham et al., 1994), the DELSEED sequence lies below the catalytic site at the bottom of subunit β. When ATP is bound in the catalytic site of subunit β, the DELSEED region of the same subunit directly contacts several references

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Kuki et al. (1988) have shown that mutant F1-F0 with truncated versions of subunit e, terminating after residue 78, are still active in oxidative phosphorylation and ATP-driven proton transport.

The ATPase-coupled H+ transport function of three double Cys mutants membranes, i.e. cA40C/eE31C, cQ42C/eE31C and cP43C/eE31C, was decreased under conditions where cross-link formation between subunits c and e was observed. The inhibition of H+ transport appears to relate to c-e heterodimer formation rather than c-c dimer formation. In the cA39C, cA40C, and cQ42C single mutants, c-c dimers were formed but normal activity observed. Normal activity was also observed in the cA39C/eE31C mutant where c-c dimers, but not c-e dimers, were formed. Formation of c-e dimers might be expected to inhibit activity by fixing the conformation of the two regions and preventing further coupling at alternating catalytic sites (Boyer, 1993).

We were not able to proportionally relate the extent of inhibition of ATPase-coupled H+ transport function to the extent of c-e cross-link formation by the methods used here. The major problem stems from the nonlinear decrease in ACMA quenching response with ATPase function (Miller et al., 1990). The cross-linked F1-F0 studied most thoroughly in cQ42C/eE31C membranes was generated by spontaneous oxidation during membrane preparation, and the extent of e incorporation into the c-e dimer was approximately 70%. It might be possible to relate e-c cross-link formation to inhibition more easily under conditions where the cross-linking of subunits e to c approached 100%. Although further cross-linking could be achieved by mild treatment with Cu(II)-(1,10-phenanthroline)3 and activity further reduced, the inhibition following this treatment was not effectively reversed by dithiothreitol treatment. In conclusion, cross-link formation between subunit e and a single subunit c might be expected to totally inhibit ATPase-coupled proton transport by preventing alternation between catalytic sites in F1. The inhibition observed here is suggestive of such a phenomenon, but we cannot rule out residual activity in an c-e complex. The relatively high ATPase activity of the cross-linked F1-F0 complexes may reflect mutational induction of uncoupling of F1 from F0.

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