Organization of the F_0 Sector of *Escherichia coli* H^+-ATPase: The Polar Loop Region of Subunit c Extends from the Cytoplasmic Face of the Membrane†

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Received November 4, 1988; Revised Manuscript Received January 9, 1989

ABSTRACT: The membrane-spanning F_0 sector of the *Escherichia coli* H^+-transporting ATP synthase (EC 3.6.1.34) contains multiple copies of subunit c, a 79 amino acid residue protein that is thought to insert in the membrane like a hairpin with two membrane traversing α-helices. The center of the protein is much more polar than the putative transmembrane α-helices and has been postulated to play a crucial role in coupling H^+ translocation through F_0 to ATP synthesis in the membrane extrinsic, F_1 sector of the complex. However, the direction of insertion of subunit c in the membrane has not been established. We show here that the "polar loop" lies on the F_1 binding side of the membrane. A peptide corresponding to $Lys_{34} \rightarrow Ile_{46}$ of the polar loop was synthesized. Antisera were generated to the $Lys_{34} \rightarrow Ile_{46}$ cognate peptide, and the polyclonal antipeptide IgG was shown to bind to a crude F_0 fraction by using enzyme-linked immunosorbent assays. The antipeptide serum did not bind tightly enough to F_0 to disrupt function. However, a polyclonal antiserum made to purified, whole subunit c was shown to block the binding of F_1 to the F_0 exposed in F_1-stripped membranes. Incubation of the antisubunit c serum with the peptide reduced the inhibitory effect of the antiserum on the binding of F_1 to F_0. The reversal of inhibition by the peptide was specific to the antisubunit c serum in that the peptide had no effect on inhibition of F_1 binding to F_0 by antiserum to subunit a of F_0. We conclude that the antisubunit c serum blocks F_1 binding to the cytoplasmic side of the inner membrane by recognizing epitope(s) in the $Lys_{34} \rightarrow Ile_{46}$ sequence.

A reversible, H^+-translocating ATPase catalyzes the synthesis of ATP during oxidative phosphorylation. The enzyme is composed of two functionally distinct sectors termed F_1 and F_0. The F_1 sector catalyzes ATP synthesis or hydrolysis and is bound to F_0 at the surface of the membrane. The F_0 sector traverses the membrane and promotes H^+ translocation. When the two sectors of the complex are coupled together, the enzyme functions as an H^+-translocating ATP synthase, the driving force for ATP synthesis being the H^+ electrochemical potential generated by electron transport (Senior, 1988). In *Escherichia coli*, the F_0 sector is made up of three types of subunits, each of which seem to have counterparts in both mitochondria and chloroplasts (Senior, 1988). These subunits are found in an unusual stoichiometric ratio of $a_1b_2c_{10}$ (Foster & Fillingame, 1982). Subunit c is a small, hydrophobic protein that is thought to be a component of the H^+-translocating apparatus of F_0 (Hoppe & Sebald, 1984). Dicyclohexylcarbodiimide (DCCD)[1] reacts quite specifically with a single carboxyl group in the protein (Asp_{61} of *E. coli* subunit c) to block H^+ translocation. The "DCCD binding proteins" from the F_0 of a variety of species have similar structures (Hoppe & Sebald, 1984). The N-terminal and C-terminal segments of the protein are hydrophobic and are proposed to traverse the membrane as α-helices. The DCCD-reactive carboxyl residue would be expected to lie toward the center of the C-terminal membrane traversing helix. The middle or loop region of the protein is much more polar and is the region predicted to be most antigenic (Hopp & Woods, 1981), but there is no direct information establishing the direction of insertion. Loo and Bragg (1982) prepared antiserum to purified subunit c and demonstrated that it blocked binding of F_1 to the F_0 exposed in F_1-stripped membranes, but the epitopes recognized by the antiserum were not defined. We have proposed that the evolutionarily invariant Arg_{41}-Gln_{42}-Pro_{43} sequence of the polar loop plays a critical role in coupling H^+ translocation through F_0 to ATP synthesis in F_1, based upon the properties of mutants in this sequence (Mosher et al., 1985; Miller et al., 1989). In this study we have addressed the question of whether the polar loop sequence extends from the F_1 binding inner surface of the *E. coli* inner membrane and conclude that it does.

Table I: Amino Acid Analysis of Synthetic Peptide

amino acid	molar ratio found[a]	molar ratio predicted	amino acid	molar ratio found[a]	molar ratio predicted
Asp	0.99	1	Leu	2.00	2
Glu	2.02	2	Phe	0.98	1
Pro	1.36	1	Lys	0.92	1
Gly	0.86	1	NH_3	0.99	1
Ala	2.05	2	Arg	0.98	1
Ile	1.05	1			

[a] The peptide was hydrolyzed in 6 N HCl in vacuo at 110 °C for 24 h. Moles found are normalized to 2.00 mol of Leu.

Experimental Procedures

Peptide Synthesis. The $Lys_{34} \rightarrow Ile_{46}$ cognate peptide Lys-Phe-Leu-Glu-Gly-Ala-Ala-Arg-Gln-Pro-Asp-Leu-Ile was synthesized on a Beckman Model 990 automatic peptide synthesizer by using solid-phase peptide synthesis procedures

†Supported by U.S. Public Health Service Research Grant GM-23105 from the National Institutes of Health to R.H.F. M.E.G. was partially supported by U.S. Public Health Service Fellowship Award F32-GM11096.
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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/89/0428-4340$01.50/0

(Barany & Merrifield, 1980). The details are provided as supplementary material (see paragraph at end of paper regarding supplementary material). The amino acid composition of the purified peptide is shown in Table I.

Coupling of Peptide to Carrier Protein. Peptide (5–7 mg) was coupled to porcine thyroglobulin or bovine serum albumin (10–15 mg) by using either glutaraldehyde (Sigma, St. Louis, MO) as described by Briand et al. (1985) or EDC (Pierce, Rockford, IL) in a method similar to that described by Goodfriend et al. (1964).

Purification of Subunit c. Subunit c was purified from whole cells of *E. coli* by extraction into chloroform–methanol (2:1) and ether precipitation according to Beechey et al. (1979) and by CM-cellulose chromatography as described (Graf & Sebald, 1978; Hermolin & Fillingame, 1989), applying ≤1 mg of ether-precipitated proteolipid/mL of CM-cellulose. The protein eluting from the CM-cellulose column with chloroform–methanol–H_2O (5:5:1) was judged to be pure on the basis of SDS–polyacrylamide electrophoresis and the amino acid composition (Fillingame, 1976).

Preparation of Antisera. Polyclonal antibodies were generated by immunizing young (6–8 lb) New Zealand White rabbits subcutaneously with 2 mL of either whole subunit c diluted to 0.3 mg/mL or EDC-coupled thyroglobulin–peptide diluted to 0.125 mg/mL. Initial immunizations were done with antigen diluted in Freund's complete adjuvant while subsequent boostings were done at 3–4-week intervals with antigen diluted in Freund's incomplete adjuvant. The presence of antibodies was detected with ELISA assays. For subunit c, high titers were obtained 4–5 months after the initial immunization (using subunit c as test antigen). Significant antipeptide activity (against the peptide–albumin conjugate) was observed 4 months after the first injection. The maximal titer against a crude deoxycholate-solubilized F_0 complex required 8 months of immunization. Antiserum to subunit a was a gift from Dr. K. Altendorf [Universität Osnabrück, Federal Republic of Germany; see Deckers-Hebestreit and Altendorf (1986)].

Antisera were further treated by incubation overnight at 4 °C with 8 mg/mL mutant strain LW125 membranes in order to absorb nonspecific antibodies. LW125 membranes lack all subunits of the F_1F_0 complex due to genetic deletion of the appropriate genes (Fillingame et al., 1986). After the incubation, the membranes were removed by centrifugation, and the supernatant was stored at −70 °C. Antisubunit c serum was tested on immunoblots of SDS–acrylamide gels of wild-type membranes and found to react with a single band that corresponded in migration to subunit c. Serum against the $Lys_{34} \rightarrow Ile_{46}$ peptide was also found to react with protein c on immunoblots. However, much higher concentrations of antipeptide serum were required to detect a positive reaction (i.e., 1×10^{-1} dilution versus 5×10^{-3} dilution).

ELISA Assays. An ELISA procedure was used to test for the presence of specific antibodies in the processed serum. The protein used as a test antigen varied, depending upon the purpose of the experiment. Test antigens included the glutaraldehyde peptide–albumin conjugate, purified subunit c (dissolved in SDS), and a detergent-solubilized F_0 fraction. The F_0 was solubilized from induced MM598 stripped membranes (at 20 mg/mL) with 0.6% Na^+ deoxycholate and 1.5% Na^+ cholate in the presence of 1 M KCl for 10 min at 0 °C, according to Fillingame et al. (1983), but was not precipitated with ammonium sulfate.

The test antigen was bound to the wells of a microtiter plate (Flow Laboratories, McLean, VA) by incubating 100 μL of antigen solution for 2–3 h at 20 °C and then overnight at 4 °C. The antigen was diluted in binding buffer, which contained 150 mM NaCl, 1 mM $MgCl_2$, 20 μM $ZnCl_2$, 0.25% gelatin, 0.03% NaN_3, and 50 mM Tris-HCl, pH 7.4. After the antigen was bound, the wells were rinsed with washing buffer (binding buffer without the gelatin). Dilutions of serum in binding buffer (100 μL per well) were added, and the plate was incubated overnight at 4 °C. After the wells were washed again, a solution containing alkaline phosphatase coupled to goat anti-rabbit IgG was added (100 μL per well) and the plate incubated for 2 h at 20 °C. The alkaline phosphatase solution contained 9 mL of binding buffer, 1 mL of calf serum, and 2.5 μg (50 μL) of Kirkegaard and Perry (Gaithersburg, MD) alkaline phosphatase conjugate. After a final wash, enzyme substrate (200 μL per well) 4-methylumbelliferyl phosphate (Research Organics, Cleveland, OH) was added. The 4-methylumbelliferyl phosphate was dissolved at 0.2 mg/mL in 25 μM $ZnCl_2$, 1.25 mM $MgCl_2$, and 1 M 2-amino-2-methyl-1-propanol at pH 10.3, and the fluorescence of the hydrolyzed product was read at several time intervals.

Indirect Assay for F_1 Binding to F_0 via Reconstitution of ATP-Driven Quenching of Quinacrine Fluorescence. F_1-stripped membranes were isolated from the F_1F_0 overproducing strain MM598 as previously described (Hermolin et al., 1983). The F_1 used in the assay was purified from strain ML308-225 membranes by releasing F_1 from membranes into TEDG buffer [1 mM Tris-HCl, pH 8.0, 0.5 mM Na_2EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol], followed by DEAE-Sepharose CL-6B column chromatography in 50 mM Tris-HCl, pH 8.0, 2 mM Na_2EDTA, 1 mM ATP, 1 mM DTT, and 10% glycerol buffer with a 0–0.5 M NaCl gradient and precipitation with 65% ammonium sulfate. Stripped membrane vesicles were reconstituted with F_1 by incubating 250 μg of membrane in 1 mL of HMK buffer (10 mM HEPES–NaOH, pH 7.5, 5 mM $MgCl_2$, 0.3 M KCl) with varying amounts of F_1 for 30 min at room temperature. The function was assayed by measuring ATP-induced quenching of quinacrine fluorescence as described (Hermolin et al., 1983).

In order to test the effect of antisubunit c serum on binding of F_1 to stripped membranes, 10 mg/mL stripped membranes were mixed in HMG buffer (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 10% glycerol) with an equal volume of a 1/10 dilution of antiserum, incubated overnight at 4 °C, and then reconstituted with F_1 as described above. To test for competition between F_0 and the peptide for antiserum binding, the $Lys_{34} \rightarrow Ile_{46}$ peptide was included at 1 mg/mL in the incubation with antiserum.

Results

We had initially hoped to test whether epitopes from the polar loop were present on the cytoplasmic side of the inner membrane using antisera generated to the $Lys_{34} \rightarrow Ile_{46}$ peptide. The antipeptide serum did bind to purified subunit c or native F_0, as judged by ELISA assays, but the binding was rather weak. Further, in competitive ELISA assays, we had difficulty in reproducibly demonstrating a convincing difference in competition by wild-type inside-out membranes ($\pm F_1$), mutant inside-out membranes lacking F_0, and right-side-out membranes. We therefore turned to an alternative approach based upon function.

Loo and Bragg (1982) had previously shown that an antisubunit c serum prevented binding of F_1 to F_0. We were not able to confirm this effect by a direct binding assay using our antisubunit c serum, but ultimately did so by the following procedure. Stripped MM598 membranes, which lack ATP-driven quenching of quinacrine fluorescence, were titrated with varying amounts of F_1. F_1 binding resulted in restoration of

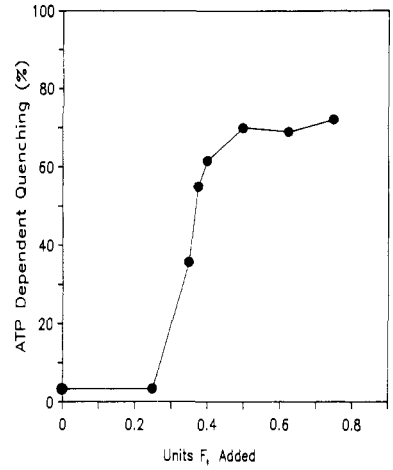

FIGURE 1: Titration of stripped MM598 membranes with purified F_1 as monitored by ATP-dependent quenching of quinacrine fluorescence. Stripped membranes (0.25 mg in 1.0 mL of HMK buffer) were incubated with the amount of F_1 shown for 30 min at room temperature (1 unit of activity = 1 μmol/min). Quinacrine was added to 0.75 μg/mL and the initial fluorescence recorded. ATP was then added to 1 mM with a resulting quenching of fluorescence. ATP-dependent quenching is expressed as the percentage of initial fluorescence that was quenched.

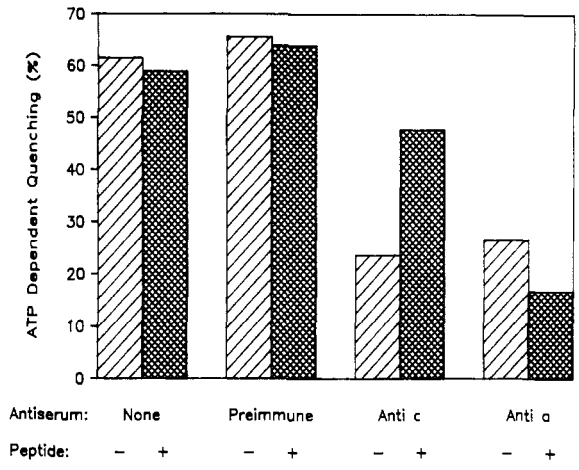

FIGURE 2: The $Lys_{34} \rightarrow Ile_{46}$ peptide competes for binding of antisubunit c serum to F_0. Stripped MM598 membrane, suspended at 5 mg/mL in HMG buffer, was incubated with the antiserum shown, ±1 mg/mL peptide, overnight at 4 °C. A 250-μg sample of the preincubated membrane was then diluted to 1 mL in HMK buffer and reconstituted with 0.4 unit of F_1 as described in Figure 1. After 30 min, quinacrine was added and the ATP-dependent quenching response recorded as described in Figure 1. The preincubations were with 5×10^{-2} dilutions of preimmune serum, antisubunit c serum, or antisubunit a serum, all of which were preabsorbed with strain LW125 membranes. The quenching values shown are the average of the three determinations for each type of experiment with antisubunit c serum and the average of two determinations for the experiments with preimmune serum and antisubunit a serum.

the quenching response (Figure 1). Interference in F_1 binding by the antisubunit c serum was tested at a slightly subsaturating concentration of F_1, i.e., the concentration of F_1 restoring approximately 80% of the maximal quenching response. The results of such an experiment are shown in Figure 2. Preincubation of stripped membranes with antisubunit c serum significantly reduced the restoration of quenching, i.e., from 60% to 24%, whereas preincubation with the preimmune serum had no effect. We next asked whether binding of antibody to an epitope in the $Lys_{34} \rightarrow Ile_{46}$ sequence was responsible for the inhibition of F_1 binding. When the $Lys_{34} \rightarrow Ile_{46}$ peptide was included in the preincubation with the stripped membrane and antisubunit c serum, more F_1 was subsequently bound to the membrane as indicated by the restoration of a nearly complete quenching (Figure 2). That is, the peptide competed with F_0 for binding of the antibody, which when bound to F_0 prevented binding of F_1. In a total of four similar experiments, the antisubunit c serum reduced the reconstitution of quenching response to 52% of the control, and the peptide restored the response to 85% of control. The specificity of this effect is indicated by the lack of effect of peptide on F_1 binding in the control and preimmune control experiments (Figure 2). Finally, an antiserum to subunit a of F_0 also effectively blocked F_1 binding (Figure 2). The $Lys_{34} \rightarrow Ile_{46}$ peptide had no effect in reversing the block in F_1 binding by antisubunit a serum, which again indicates the specificity of the competition.

Discussion

Previous work by Loo and Bragg (1982) and Deckers-Hebestreit and Altendorf (1986) with antisubunit c sera established that an epitope of subunit c was exposed on the F_1-binding side of the inner membrane. The antiserum of Loo and Bragg (1982) inhibited F_1 binding to the F_0 exposed in F_1-stripped inside-out membrane vesicles and partially blocked H^+ translocation by these vesicles. In Deckers-Hebestreit and Altendorf (1986), the binding of antibodies to subunit c was reduced by incubation of antiserum with F_1-stripped, inside-out membrane vesicles, and to a significantly lesser degree by unstripped vesicles. Right-side-out membrane vesicles, and mutant membranes vesicles lacking F_0 proteins, competed much more poorly. In the work reported here, we have confirmed that antisubunit c serum prevents binding of F_1 to F_0 and have established that an epitope in the $Lys_{34} \rightarrow Ile_{46}$ segment of the polar loop composes at least a part of the antibody binding site. The $Lys_{34} \rightarrow Ile_{46}$ cognate peptide competed with F_0 for the binding of antisubunit c antibody and therefore reversed the inhibitory effect of the antibody on binding of F_1 to F_0. We should note that we have also tested the antisubunit c serum generated by Loo and Bragg (1982) in our assay. At equivalent dilution, the Loo and Bragg antiserum gave a somewhat greater inhibitory effect, and the inhibitory effect was also reversed by the peptide.

We would not have been able to localize the epitope in the $Lys_{34} \rightarrow Ile_{46}$ peptide by use of the antipeptide serum alone. The peptide is apparently a weak antigen. Antibodies in the cognate peptide antiserum were bound by F_0 or by subunit c, but the question of sidedness was not convincingly resolved by competition ELISA assays with stripped and unstripped inside-out membrane vesicles etc. Finally, the antipeptide serum did not significantly disrupt the rebinding of F_1 to F_0, as judged by reconstitution of ATP-driven quinacrine quenching, probably because the affinity of the antibody was too low relative to the affinity of F_1 for F_0.

The placement of the polar loop region of subunit c at the inside surface of the inner membrane is supported by several other chemical and genetic observations. Deckers-Hebestreit et al. (1987) have shown that both Tyr_{10} and Tyr_{73} are labeled by tetranitromethane, presumably from the hydrophobic interior of the membrane. Only Tyr_{73} was reducible by treatment of right-side-out membrane vesicles with sodium dithionite, and this observation places the C-terminus of the protein at the outside surface of the membrane. Hoppe et al. (1984) have shown that segments of both the N-terminal and C-terminal ends of the protein are labeled by 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine on photoactivation, with the labeling presumably occurring from the hydrophobic interior of the membrane. These results suggest a hairpin-like folding of the protein in the membrane. Finally, mutations in the conserved Gln_{42} and Pro_{43} residues of the protein have

been shown to disrupt the binding and functional coupling of F_1 to F_0 (Mosher et al., 1985; Miller et al., 1989), and the simplest explanation is to place these residues at the F_0–F_1 interface. This interpretation is supported by the experiments presented here, where an epitope in the $Lys_{34} \rightarrow Ile_{46}$ segment of the protein was placed at the F_1-binding surface of the membrane.

ACKNOWLEDGMENTS

We are indebted to Dr. Larry Kahan (University of Wisconsin, Madison) for helpful advice on immunological procedures and the generous use of his equipment and to Dr. D. H. Rich (University of Wisconsin, Madison) in whose laboratories the peptide synthesis was performed. We thank Dr. Philip Bragg (University of British Columbia) and Drs. Karlheinz Altendorf and Gabriel Deckers-Hebestreit (Universität Osnabrück) for samples of their antisubunit c and antisubunit a antisera.

SUPPLEMENTARY MATERIAL AVAILABLE

Details of the $Lys_{34} \rightarrow Ile_{46}$ peptide synthesis and purification (4 pages). Ordering information is given on any current masthead page.

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Folding of a Peptide Corresponding to the α-Helix in Bovine Pancreatic Trypsin Inhibitor†

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Received November 10, 1988; Revised Manuscript Received February 1, 1989

ABSTRACT: A short peptide corresponding to the α-helical region of BPTI shows partial folding in aqueous solution (pH 7) as judged by circular dichroism (CD). Folding is temperature and denaturant sensitive, and the peptide is monomeric. The difference CD spectrum, obtained from spectra at two temperatures, indicates that the peptide folds as an α-helix. Difference CD spectroscopy provides a sensitive assay for helix formation in peptides exhibiting small amounts of structure. Helix stability in this peptide shows a marked pH dependence which is consistent with stabilizing charged side-chain interactions with the helix dipole and/or salt bridge formation.

Since proteins in general contain sufficient information in their amino acid sequence to enable them to fold spontaneously, it is interesting to ask how much of a protein is required for structure to occur. In other words, can part of a protein (a peptide) exhibit autonomous folding into native-like structure? There are now a few examples of aqueous, monomeric "autonomous folding units" (AFUs). C-Peptide, corresponding to an α-helical region in ribonuclease A, shows ~30% α-helical structure at 0 °C in aqueous solution (Brown & Klee, 1971; Bierzynski et al., 1982; Shoemaker et al., 1987). A nine-residue peptide with a native sequence adopts a reverse turn structure in aqueous solution (Dyson et al., 1985), and a peptide corresponding to another native helical sequence exists as a collection of transient α-helical turns in aqueous solution (Dyson et al., 1988). Short peptides designed de novo with several $i, i + 4$ type salt bridges have as much as ~80% helix content at 0 °C (Marqusee & Baldwin, 1987).

† This research was supported in part by a grant from the National Institutes of Health (GM37241).

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0006-2960/89/0428-4343$01.50/0