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Assembly of the F_o Proton Channel of the *Escherichia coli* F_1F_o ATPase: Low Proton Conductance of Reconstituted F_o Sectors Synthesized and Assembled in the Absence of F_1†

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ABSTRACT: We have previously proposed that during assembly of the *Escherichia coli* F_1F_o ATPase, the proton permeability of the F_o sector of the *E. coli* F_1F_o ATPase is increased significantly by interactions with F_1 subunits [Pati, S., & Brusilow, W. S. A. (1989) *J. Biol. Chem 264*, 2640-2644]. To test this model for F_o assembly, we purified F_o sectors synthesized in the presence and absence of F_1 subunits and measured the abilities of these different preparations to bind purified F_1 ATPase and to conduct protons when reconstituted into liposomes. The results of these studies demonstrated significant differences in proton-conducting abilities of the different F_o preparations. F_o sectors synthesized in the presence of F_1 subunits were more permeable to protons than those synthesized in the absence of F_1 subunits.

The proton-translocating F_1F_o ATPase of *Escherichia coli* consists of an intrinsic membrane-bound sector, F_o, which forms a proton channel across the cytoplasmic membrane and an extrinsic sector, F_1, which catalyzes ATP synthesis or hydrolysis [for reviews, see Schneider and Altendorf (1987) and Futai et al. (1989)]. The F_o consists of three different subunits (a, b, and c), and the F_1 consists of five different subunits (α, β, γ, δ, and ϵ). The subunits are present in different numbers in the complex, and the stoichiometry has been shown to be $a_1b_2c_{10}\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (Foster & Fillingame, 1982). The synthesis, membrane insertion, and assembly of these different subunits into functional F_1, F_o, and F_1F_o complexes must be accomplished without the formation of harmful intermediates which might disrupt the transmembrane proton gradient, deplete cellular ATP levels, or both. A model proposed by Cox et al. (1981; Cox & Gibson, 1987) described an assembly pathway in which certain F_o subunits would not be assembled into the membrane in the absence of certain F_1 subunits, thereby eliminating the possibility of either the F_o or the F_1 assembling in the absence of the other. Subsequent studies from other laboratories, however, demonstrated that reconstitutable F_o could be synthesized and assembled in the absence of F_1 (Aris et al., 1985; Fillingame et al., 1986). Genetic studies by Brusilow (1987) concluded that the presence of F_1 subunits could significantly affect the proton permeability of F_o sectors synthesized from genes on multicopy plasmids, and Pati and

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Brusilow (1989) proposed a model in which the F_o was synthesized and assembled in a closed form which was opened by interaction with F_1 subunits. Humbert and Altendorf (1989), studying aminoglycoside resistance caused by ATPase–dependent proton permeability, also concluded that the proton-conducting ability of the F_o might depend on exposure to F_1 subunits.

To determine whether "closed" F_o sectors are synthesized and assembled in a form which can be purified and reconstituted, and to determine whether the proton conductance of the F_o is affected by exposure to F_1 subunits during assembly, we purified F_o sectors which had been synthesized and assembled in the presence or absence of F_1 subunits and compared those sectors with respect to their proton permeabilities and F_1 binding abilities.

Materials and Methods

E. coli Strains, Plasmids, Growth Media. The strains used in these studies have been described previously, including LE392 (Silhavy et al., 1984), LE392Δ(*uncI-uncC*) (Angov & Brusilow, 1988), and the ATPase-overproducing strain KY7485 (Kanazawa et al., 1979). The plasmids which carry F_o genes, pWSB35 (Brusilow, 1987), and pEA4 (Angov et al., 1991) have been described previously. Plasmid pEA4 is identical with pWSB35 except that it contains none of *uncH*, the gene for the δ subunit. Cells were grown in LB medium (Miller, 1972) containing 0.4% glucose, except for KY7485, which was grown on a minimal medium (Tanaka et al., 1967) containing 1% glucose and supplemented with thiamin (0.5 μg/mL), arginine (84 μg/mL), and guanine (45 μg/mL).

Purification of F_o. F_o was isolated directly from F_1-stripped everted membrane vesicles of different *E. coli* strains as described by Schneider and Altendorf (1984). For removal of F_1, the membranes, which were prepared according to Futai et al. (1974), were washed in 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 10% (v/v) glycerol and incubated overnight at 4 °C in 1 Tris-HCl, pH 8.0, 0.5 mM EDTA, and 10% (v/v) glycerol. After centrifugation (90 min at 220000*g*), the incubation was repeated twice for 1 h at room temperature. After this procedure, the membranes of the ATP synthase overproducing strain KY7485 exhibited less than 1% of the starting ATPase activity. For *E. coli* strain LE392Δ(*uncB-uncD*) containing plasmid pWSB35 (F_o + δ) or pEA4 (F_o only), the F_o preparation was carried out in the same way, although no F_1 complex was present. Protein concentrations were determined by the method of Dulley and Grieve (1975).

Immunoblot Analysis of F_o Preparations. F_o preparations were separated by SDS–PAGE on a 12.5% gel. The proteins were electrophoretically transferred onto nitrocellulose paper (Sartorius, Hayward, CA) and probed with polyclonal antibodies raised against each of the F_o subunits. Immunoblot development was carried out as described previously (Brusilow, 1987). Alternatively, F_o preparations were analyzed by immuno-dot blots. The F_o preparations were serially diluted in cholate-containing buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.2 mM phenylmethanesulfonyl fluoride, and 1% (w/v) sodium cholate] and spotted directly onto dry nitrocellulose paper. These blots were probed with anti-b antibody and developed as described above.

Reconstitution of F_o into Liposomes. Asolectin (Sigma type II-s) was purified by the procedure of Sone et al. (1977). Liposomes were prepared by reconstitution by the method of Okamoto et al. (1977) except that a phospholipid:protein ratio of either 80:1 or 40:1 was used. Purified asolectin (80 mg) was mixed into a solution containing 15 mM Tricine–NaOH, pH 8.0, 7.5 mM DTT (dithiothreitol), 0.2 mM EDTA, 1.6% (w/v) sodium cholate, and 0.8% (w/v) deoxycholate. The suspension was sonicated for 10 min on ice under N_2 gas. F_o samples were diluted to 2 or 4 mg/mL in the cholate-containing buffer. Each reconstitution sample contained 50 μL of F_o or cholate-containing buffer alone and 200 μL of the sonicated asolectin suspension. The samples were then dialyzed overnight at 4 °C in 250 mL of dialysis buffer (10 mM Tricine–NaOH, pH 8.0, 0.2 mM DTT, 0.2 mM EDTA, and 2.5 mM $MgSO_4$).

Proton Translocation Assays. The reconstituted proteoliposomes were loaded with 0.2 M K^+ by the freeze–thaw method of Sone et al. (1981). Reconstituted proteoliposomes were added to an equal volume of 20 mM sodium phosphate, pH 7.0, and 0.4 M KCl in a total volume of 300 μL. This suspension was mixed and sonicated on ice for 20 pulses with a microtip on a Branson sonifier/cell disrupter B15 set on stage 2 and 50% pulsed dry cycle. The sonicated suspension was then frozen in liquid N_2 for 20 min and thawed at room temperature for 20 min. The sonication, freeze, and thaw steps were repeated once. Each sample was sonicated as described above for a third time immediately preceding the proton translocation assay.

Proton flux across the liposome was measured as described by Schneider and Altendorf (1985). Changes in pH were detected on a Radiometer Copenhagen PHM64 pH meter and monitored on a Versagraph Linear 800 series chart recorder. The assay was performed by adding 50 μL of K^+-loaded proteoliposomes to 2 mL of 0.2 mM Tricine–NaOH, pH 8.0, 0.2 M Na_2SO_4, and 5 mM $MgSO_4$ with constant stirring in a 3-mL cuvette. Once a stable base line was achieved, 1 μL of 100 μM valinomycin was added to cause efflux of K^+ ions. Next, 2 μL of 200 μM TTFB [4,5,6,7-tetrachloro-2-(trifluoromethyl)benzimidazole], an uncoupler, was added to ensure that the liposomal vesicles were loaded. Each assay was calibrated by the addition of 5 μL of 1 mM HCl. DCCD (*N,N'*-dicyclohexylcarbodiimide) was added when needed to a final concentration of 40 μM 20 min before the addition of valinomycin.

F_1 Binding Assay, DCCD Treatment. The extent of F_1 binding to reconstituted proteoliposomes was determined as described by Steffens et al. (1984). Reconstituted proteoliposomes (50 μL) were sonicated in a bath-type sonicator (Branson 220) for 30 s. This preparation was incubated with 90 μg of purified F_1 (specific activity = 50–70 units/mg) and 2 mL of binding buffer (50 mM Tricine–NaOH, pH 8.0, 2 mM $MgSO_4$, and 2 mM DTT) for 20 min at 37 °C. The proteoliposomes were pelleted by centrifugation at 240000*g* for 15 min at 4 °C, washed once in binding buffer, and then resuspended in 100 μL of binding buffer. The ATPase activity of these F_1-bound proteoliposomes was measured as described by Arnold et al. (1976). The DCCD sensitivity was determined by incubating the liposomes in 80 μM DCCD for 20 min at 37 °C before the ATPase assay.

Results

We isolated F_o sectors from the inducible λ_{unc} lysogen KY7485, LE392Δ(*uncI-uncC*) (pEA4), LE392Δ(*uncI-uncC*) (pWSB35), and LE392Δ(*uncI-uncC*) (no plasmid). Plasmids pEA4 and pWSB35 carry the genes for the F_o subunits. We used these preparations to compare the proton-translocating abilities of F_o which had interacted with F_1 and F_o which had not. Figure 1 is an immunoblot of these various F_o preparations run on SDS-PAGE. Equal amounts of total protein were loaded in each lane and then probed with anti-a, anti-b, or anti-c antibodies. It appeared that the F_o preparation isolated from KY7485 was more concentrated than the preparations

Table I: Quantitation of F_1 Binding Activity and Proton-Translocating Activity of Reconstituted F_o Preparations[a]

F_o	protein (μg) used in recons	protein (μg) used in ATPase assay	protein (μg) used in H^+-trans assay	ATPase act. of F_1-bound liposomes (nmol of P_i hydrolyzed/min)	H^+-trans rate (nmol of H^+/min)	H^+-trans rate (% of KY7485)
KY7485	100	4	10	7.2	65	100
LE392Δ(*uncI-uncC*) + pEA4 (F_o only)	100	4	10	3.2	4.9	8
	200	8	20	6.0	13	20
LE392Δ(*uncI-uncC*) + pWSB35 (F_o + δ)	100	4	10	2.6	9.7	15
	200	8	20	5.2	24	37
LE392Δ(*uncI-uncC*) + no plasmid	100	4	10			
	200	8	20	0	0	0
no F_o				0	0	0

[a] Either 100 or 200 μg of each F_o preparation (first column) was reconstituted (recons) into liposomes and tested for F_1 binding activity and proton-translocating (trans) ability as described as described under Materials and Methods. The second, third, and fourth columns indicate the amount of protein from each F_o preparation reconstituted into liposomes, how much of the reconstituted liposome preparation was treated with F_1 and assayed for ATPase activity, and how much of each reconstituted preparation was assayed for H^+–translocation activity, respectively. Bound ATPase activity, H^+–translocation rate, and H^+–translocation rate as a percent of KY7485 are indicated.

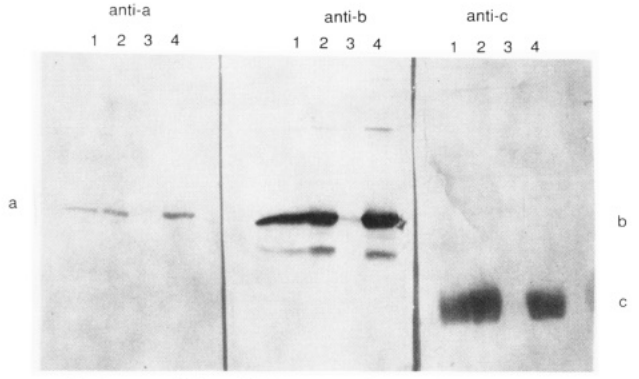

FIGURE 1: Immunoblots of F_o preparations. Twenty micrograms of F_o prepared from (1) an *unc* deletion, LE392Δ(*uncI-uncC*), carrying pEA4 (F_o only), (2) the *unc* deletion carrying pWSB35 (F_o+δ), (3) the *unc* deletion carrying no plasmid, or (4) induced KY7485 (*unc*$^+$) was separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-a subunit (left), anti-b subunit (center), or anti-c subunit (right) antibodies, as described under Materials and Methods. The locations of each subunit are indicated. Due to the alignment of the blots for this photograph, the a subunit appears to migrate closer to the b subunit than it actually does.

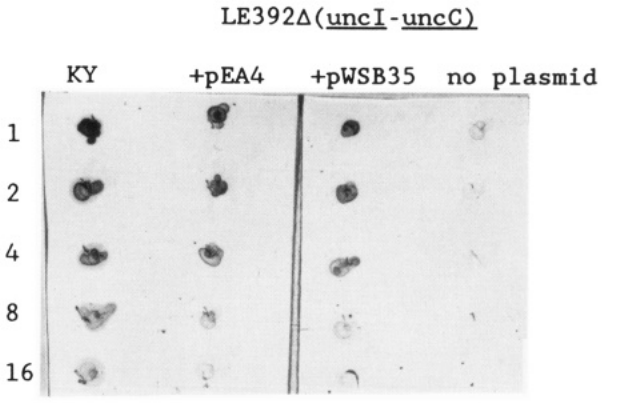

FIGURE 2: Immuno-dot blots of F_o preparations. The F_o preparations described for Figure 1 were diluted to a concentration of 1 mg/mL (top row of each lane) and then serially diluted as indicated on the left. Five microliters of each dilution was spotted on nitrocellulose and probed with anti-b antibody as described under Materials and Methods.

from either of the plasmid-containing strains. It was therefore necessary to quantitate the relative amounts of F_o in each sample. Figure 2 is an immuno-dot blot of various dilutions of the four F_o samples probed with anti-b antibody. From this immunoblot and the F_1 binding studies described below, we estimated that F_o preparations from the KY7485 strain contained approximately twice the amount of F_o per milligram of total protein as did the F_o preparation isolated from plasmid-bearing strains.

F_o was reconstituted into sonicated, asolectin liposomes by the dialysis method of Okamoto et al. (1977). Since the plasmid-derived F_o preparations contained less F_o, reconstitutions with 100 and 200 μg of F_o were performed, corresponding to protein:lipid ratios of 1:80 and 1:40, respectively. We treated these reconstituted preparations with purified F_1 and assayed 4 μg (of the 1:80) or 8 μg (of the 1:40) for bound ATPase activity. Table I summarizes the ATPase activity of the proteoliposomes which had been reconstituted with F_o and then treated with purified F_1. The highest ATPase activity was observed with liposomes containing F_o isolated from KY7485. These liposomes yielded an activity of 7.2 nmol of P_i hydrolyzed/min when the reconstituted F_o (protein:lipid ratio = 1:80) was treated with F_1 (4 μg assayed). We found that the F_1 binding capability of either of the plasmid-derived F_o liposomes was comparable to that of KY7485 when the reconstituted F_o from either pEA4 or pWSB35 (protein:lipid ratio = 1:40) was treated with F_1 (8 μg assayed). This result agreed with the conclusions from our immuno-dot blot about the relative amount of F_o in our various preparations. It appeared as if the KY7485 F_o preparation contained approximately twice the amount of F_o as the F_o from pEA4 or pWSB35. As importantly, the ATPase activity bound to liposomes containing F_o from pEA4 or pWSB35 was significantly greater than the background activity observed with reconstitutions done with no F_o or with a mock F_o preparation from the *unc* deletion strain. It was therefore apparent that the plasmids pEA4 and pWSB35 could produce an F_o which was assembled and could be purified and reconstituted in vitro. Any affects the F_1 subunits might have on F_o function did not involve any gross assembly of the F_o complex. Furthermore, the reconstituted ATPase activities could be inhibited by DCCD by 80–85%, indicating that the ATPase activity was coupled to proton translocation across the liposome (data not shown).

Proton flow was measured after imposing an artificial membrane potential by the addition of valinomycin to K^+-loaded vesicles which causes an efflux of K^+ ions. Protons can move into liposomal vesicles in response to this potential only if an open F_o channel is present. This proton flow results in alkalinization of the medium which can be monitored by a pH meter.

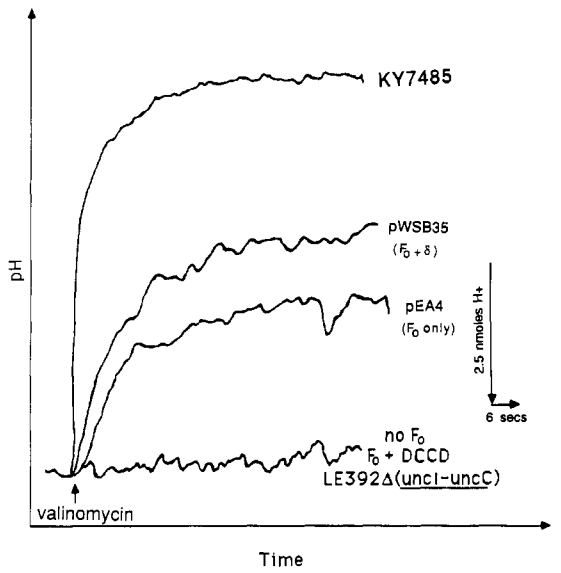

FIGURE 3: Proton translocation by F_0 preparations reconstituted into liposomes. Liposomes were reconstituted with the four F_0 preparations described for Figure 1. We reconstituted either 100 μg of the F_0 preparation from KY7485 or the *unc*-deleted cells, or 200 μg of the F_0 preparations from cells carrying either pWSB35 ($F_0+\delta$) or pEA4 (F_0 only) into liposomes. The amount of protein present in each proton translocation assay was 10 μg of the F_0 preparation from KY7485 or from the *unc*-deleted cells, and 20 μg of the F_0 preparations from cells carrying either pWSB35 or pEA4. An artificial electrochemical gradient was imposed across the proteoliposomal membranes by treatment with K^+ and valinomycin, and alkalinization of the medium, caused by movement of protons into the vesicles, was monitored with a pH meter. pH is indicated on the ordinate, and time (in seconds) is indicated on the abscissa. The addition of DCCD to any of the reconstituted F_0 preparations resulted in a curve indistinguishable from that obtained from the control "F_0 preparation" from an *unc* deletion strain (bottom curve).

The results of these assays are shown in Figure 3, and the initial rates of H^+ translocation are presented in Table I. Because of the differences in the amounts of F_0 in the various preparations described previously, we assayed 10 or 20 μg of reconstituted F_0 protein prepared from plasmid-containing cells and 10 μg of F_0 prepared from induced KY7485. KY7485 is an ATPase-overproducing strain which synthesizes all of the ATPase subunits. Therefore, the F_0 isolated from this strain had been bound to F_1 before being isolated, as compared to the F_0 made from pEA4 or pWSB35, which had never been exposed to F_1 subunits (except for the δ subunit coded for by pWSB35). The F_0 purified from KY7485 displayed the highest rate of H^+ flow with a rate of 65 nmol of H^+ translocated/min. F_0 isolated from the deletion strain carrying either pEA4 or pWSB35 conducted protons at much lower rates of 13 and 24 nmol of H^+ translocated/min, respectively. When DCCD was added to the assay mixture, no proton movement was detected with any of the reconstituted F_0 preparations. Control experiments on liposomes containing no F_0 or liposomes reconstituted with a mock F_0 preparation from the deletion strain carrying no plasmid also displayed no proton conduction.

Since the proton flow rate from pEA4 F_0 and pWSB35 F_0 was DCCD-dependent and was significantly higher than the background H^+ leak rate, the F_0 isolated from these strains was functional. However, neither conducted protons as well as the F_0 purified from KY7485. Furthermore, the rate of proton translocation through the F_0 purified from cells carrying pEA4 was reproducibly lower than the rate of proton translocation through F_0 purified from cells carrying pWSB35. The presence of the δ subunit might be responsible for the small but reproducible differences in conductance of the F_0 sectors isolated from cells carrying pWSB35 ($+\delta$) and pEA4 ($-\delta$). It has been demonstrated that the δ subunit plays a role in opening the newly synthesized but "closed" F_0 (Angov et al., 1991).

Discussion

Past studies have suggested that during the synthesis and assembly of the F_1F_0 ATPase, the proton permeability of the F_0 sector is affected by interactions with F_1 subunits. The genes for the F_0 subunits cloned onto multicopy plasmids have been shown to have no significant effects on the growth of cells deleted for chromosomal *unc* genes (Aris et al., 1985; Brusilow, 1987). Certain F_0-carrying plasmids have been shown to be harmful to *unc*$^+$ *E. coli* but not to *unc*-deleted *E. coli*, and the deleterious effect has been shown to require the presence of both the α and δ genes (Brusilow, 1987; Angov et al., 1991). Lethal proton permeability can be induced in cells carrying F_0-containing plasmids by inducing the expression of the gene for the α subunit, and this lethality is counteracted by also inducing the expression of the gene for the γ subunit (Pati & Brusilow, 1989). Aminoglycoside resistance, which has been shown to be caused by F_0-dependent proton permeability, is diminished significantly by the deletion of certain F_1 genes from F_0-containing plasmids (Humbert & Altendorf, 1989). The only case in which cloned F_0 genes have been shown to affect the growth of *E. coli* involved a high copy number plasmid in which the F_0 genes plus the gene for the δ subunit were cloned behind the inducible *lac* promoter (Fillingame et al., 1986). Therefore, from a variety of observations, a model was proposed in which the F_0 sector is synthesized and assembled in a form which is relatively impermeable to protons (Pati & Brusilow, 1989). This "closed" F_0 is opened by interactions with certain F_1 subunits, most importantly α and δ, but perhaps involving all the F_1 subunits. The studies presented here demonstrate that when the F_0 is purified from cells carrying F_1 genes and reconstituted into liposomes, it is much more permeable to protons than F_0 sectors purified from cells which carry no F_1 genes. These results agree with the proposed model, which accounts for how an F_0 sector can be synthesized and assembled without the generation of harmful intermediates. For protons to flow, a newly synthesized F_0 must interact with F_1 subunits, but under normal in vivo conditions, such interactions probably also result in the blocking of that opened channel. Only by manipulating the composition and expression of ATPase subunits in cells can the process be separated into individual steps.

It has been demonstrated that the presence of F_1 subunits is not necessary for reconstitution from purified subunits of an F_0 complex which is active in proton translocation and F_1 binding (Schneider & Altendorf, 1985). Therefore, the presence of F_1 subunits during biosynthesis and assembly brings about the "education" of the F_0 complex to translocate protons, in the form of a structural change which is maintained even after solubilization and dissociation of the F_0 complex into the individual subunits.

It is interesting that the closed F_0 sectors do bind F_1 and do conduct protons, but at a rate much lower than that for the F_0 isolated from the *unc*$^+$ strain. These results indicate that these F_0 sectors are assembled in the absence of F_1 and that they are not truly impermeable, just not permeable enough to prevent any significant depletion of the proton gradient which might harm the cells. Also, the presence of the δ subunit appears to make the F_0 sectors synthesized from pWSB35 more permeable than the F_0 sectors synthesized and assembled from pEA4, which does not code for the δ subunit. Fluores-

cence quenching experiments comparing the proton permeabilities of cell membranes isolated from *unc*-deleted cells carrying either pWSB35 or pEA4 compared to control membranes from *unc*-deleted cells have supported this conclusion (Brusilow, unpublished results). The studies presented here agree with other studies (Angov et al., 1991) that δ plays a role in increasing the proton permeability of the F_o during synthesis and assembly.

There is uncertainty concerning how well F_o sectors conduct protons. The translocation rate for protons of the reconstituted F_o complex isolated from *unc*$^+$ cells was measured to be 6.5–20 H^+/s per F_o (Schneider & Altendorf, 1982, 1985), which is in good agreement with the values obtained by using everted membrane vesicles from *E. coli* (Fillingame, 1981). Recently, Lill et al. (1986) have calculated the rate of proton translocation through CF_o from EDTA-treated vesicles of thylakoid membranes for spinach chloroplasts. Surprisingly, they arrived at a value of 10^5 H^+/s per CF_o, which is orders of magnitude higher than that observed for the F_o from *E. coli*. However, one has to keep in mind that both rates have been determined by using totally different methods. Furthermore, those high rates could be observed only in a small fraction (3%) of the CF_o complexes tested and only for a limited time after removal of CF_1. The rates of ATP synthesis in everted membrane vesicles of *E. coli* (Scarpetta and Brusilow, unpublished results) indicate that the measured proton translocation rate of purified F_o is too low to meet the necessary requirements for ATP synthesis, assuming an H^+:ATP ratio of 3. Therefore, the question remains whether the removal of EF_1 leads to a so far unrecognized structural change within F_o giving rise to a much lower proton translocation rate. In that case, the rate of proton translocation of the everted vesicles and of purified F_o reconstituted into liposomes might represent only residual activity and not the true in vivo proton-conducting ability of the F_1F_o complex.

It should be noted that these studies investigated the assembly of F_o sectors synthesized from genes on multicopy plasmids or from an induced λ lysogen. The original model of Cox et al. (1981), which proposed that certain F_o subunits were not properly inserted into the membrane in the absence of F_1 subunits, was derived from studies which investigated chromosomally encoded F_o. It is possible that the assembly pathway for the F_o differs depending on whether the genes are present in single copy or multiple copies, but the mechanism for such differences is unclear. In this context, it is worthwhile mentioning that for the assembly of the mitochondrial ATP synthase, different chaperonins have been found to be necessary (Prasad et al., 1990; Ackerman & Tzagoloff, 1990). However, for the prokaryotic ATP synthases, no evidence exists that chaperonins are involved in the assembly process. Mutant analyses have revealed that even the *uncI* gene product does not play such a role (von Meyenburg et al., 1982; Gay, 1984).

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