JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1383–1389
0021-9193/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 176, No. 5

Role of the δ Subunit in Enhancing Proton Conduction through the F_0 of the *Escherichia coli* F_1F_0 ATPase

ROBERT A. MONTICELLO† AND WILLIAM S. A. BRUSILOW*

Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201

Received 7 September 1993/Accepted 13 December 1993

We studied the effect of the δ subunit of the *Escherichia coli* F_1 ATPase on the proton permeability of the F_0 proton channel synthesized and assembled in vivo. Membranes isolated from an *unc* deletion strain carrying a plasmid containing the genes for the F_0 subunits and the δ subunit were significantly more permeable to protons than membranes isolated from the same strain carrying a plasmid containing the genes for the F_0 subunits alone. This increased proton permeability could be blocked by treatment with either dicyclohexylcarbodiimide or purified F_1, both of which block proton conduction through the F_0. After reconstitution with purified F_1 in vitro, both membrane preparations could couple proton pumping to ATP hydrolysis. These results demonstrate that an interaction between the δ subunit and the F_0 during synthesis and assembly produces a significant change in the proton permeability of the F_0 proton channel.

The proton-translocating F_1F_0 ATPase of *Escherichia coli* consists of two sectors, F_1 and F_0. The membrane-integral F_0 sector forms a proton channel across the cytoplasmic membrane, and the membrane-peripheral F_1 sector is an ATPase or ATP synthase capable of interconverting the energy of the transmembrane proton gradient with the synthesis or hydrolysis of ATP. The F_0 consists of three subunits, a, b, and c, and the F_1 consists of five subunits, α, β, γ, δ, and ε. The subunits are encoded by the genes of the *unc* (or *atp*) operon, located at 84 min on the *E. coli* map (20, 25).

Because the F_0 sector is a transmembrane proton channel and because the F_1 sector is an ATPase, the synthesis and assembly of the F_1F_0 ATPase must be accomplished without the production of harmful intermediates which might increase membrane proton permeability or decrease cellular ATP levels. Cox et al. (4, 5) proposed a mechanism of assembly in which the membrane insertion of the b subunit of the F_0 is catalyzed by certain F_1 subunits. The assembly of the F_0 channel would therefore be in concert with F_1 subunits, eliminating the possibility of either sector assembling in the absence of the other.

We have proposed a model for F_0 assembly in which the F_0 is synthesized and assembled in a relatively proton-impermeable form (3, 15). Instead of the F_1 subunits catalyzing membrane insertion of certain F_0 subunits, the F_0 subunits are inserted spontaneously and assemble in an immature form. It is through interactions with F_1 subunits that this immature "closed" channel is opened, and the flow of protons through the complex is then regulated by the activity of the F_1 sector. In this study, we examined the role of the δ subunit of the F_1 sector during assembly of the F_0 sector. The δ subunit is coded for by the *uncH* gene, which is the first F_1 gene in the operon, located immediately following the F_0 genes. Previous genetic studies implicated the δ subunit as causing the F_1-dependent proton permeability of the F_0 (1). Studies of the chloroplast F_1F_0 ATPase have placed the δ subunit at the F_0-F_1 interface (6). In these experiments, we characterized the biochemical differences between membranes of cells carrying plasmids encoding F_0 subunits and membranes of cells carrying plasmids encoding F_0 subunits and the δ subunit.

* Corresponding author. Mailing address: Department of Biochemistry, Wayne State University School of Medicine, Scott Hall, 540 E. Canfield Ave., Detroit, MI 48201. Phone: (313) 577-6659. Fax: (313) 577-2765.

† Present address: Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI 48202.

MATERIALS AND METHODS

Strains and plasmids. These studies used *E. coli* JM103 Δ(*uncB-uncD*), which is strain JM103 (11) with a deletion of seven of the nine *unc* genes, including all the F_0 genes (10). Plasmids pEA5, pWSB30.0, and pWSB33 were described previously (13, 22). pRM1, which carries the F_0 genes plus *uncH* (δ subunit) cloned behind the *lac* promoter, was constructed by digesting pWSB30.0 with *Hin*dIII and *Eco*RI and ligating the resulting fragments with the pUC9 (23) vector, which had been digested with *Hin*dIII and *Eco*RI. pRM1 was digested with *Afl*II, which cuts at the stop codon for *uncH*, treated with mung bean nuclease to form blunt ends, and then ligated with a *Sal*I adapter (5′-TGGTGTCGACACCA-3′) to produce plasmid pRM6. pRM7, which carries the F_0 genes and all of *uncH* fused in frame to a biotinylation sequence, was constructed by ligating the *Sal*I-*Eco*RI fragment from the biotinylation vector YEp352-Bio7 into pRM6, which had been digested with the same enzymes. YEp352-Bio7 was a generous gift from A. Tzagoloff. Treatment with mung bean nuclease and insertion of the *Sal*I adapter removed the stop codon for *uncH* and allowed the biotin attachment sequence to be cloned in frame. This region of the resultant plasmid was sequenced to verify the construction. pRM8, which encodes the *uncH*-biotin attachment fusion gene alone, was constructed by ligating the *Nru*I-*Eco*RI fragment from pRM7 into pWSB33, which had been digested with *Nru*I and *Eco*RI. pRM8 is identical to pWSB33 but codes for the synthesis of the biotinylated δ fusion protein.

Growth and induction of cloned genes. Cells were grown in LB medium (12) containing 100 mg of ampicillin per liter, and growth was measured by monitoring cell turbidity (optical density at 550 nm [OD_{550}]). At an OD_{550} of 0.4, transcription from the *lac* promoter was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. To maintain plasmids, the ampicillin concentration was increased to 400 mg/liter. Cells were grown at 37°C with vigorous shaking.

Membrane preparation. Cells were harvested as they entered stationary phase ($OD_{550} \approx 1.0$), chilled, pelleted by centrifugation at 10,000 × *g* for 10 min, and suspended in 50 mM morpholinepropanesulfonic acid (MOPS)–10 mM $MgCl_2$ (pH 7.0) (MOPS-Mg buffer) to a final concentration of 0.25 g of wet cells per ml. Cells were lysed at 16,000 lb/in^2 in a French pressure cell. Unlysed cells were removed by centrifugation at 5,000 × *g* for 10 min. The membrane-containing supernatant fractions were centrifuged at 100,000 × *g* for 1 h, washed once with MOPS-Mg buffer, and then resuspended in the same buffer to a final concentration of 20 to 40 mg of membrane protein per ml.

Fluorescence quenching. The use of 9-amino 6-chloro 2-methoxyacridine (ACMA) fluorescence quenching as an assay of membrane proton permeability was previously described (14). ACMA was obtained from Molecular Probes Inc. (Eugene, Oreg.). For NADH-driven quenching experiments, 0.5 mg of membrane protein was assayed with 2 ml of 20 mM Tris-HCl (pH 7.8)–200 mM KCl–5 mM $MgSO_4$ (fluorescence quenching buffer). ACMA was added to a final concentration of 5 μM, and fluorescence was monitored until a stable baseline was maintained. NADH was then added to a final concentration of 500 μM to induce respiration. Respiration-driven quenching was halted by the addition of 1 mM KCN. For ATP-driven quenching experiments, the final concentrations of ACMA, ATP, and NH_4Cl were 2.5 μM, 250 μM, and 20 mM, respectively. Membrane suspensions were excited at 410 nm, and emission was measured at 490 nm with an SLM model 8000 fluorimeter.

Assays of F_1 and DCCD binding, ATPase, and ATP synthase. One milligram of membrane protein was incubated with 1, 2, or 4 U of purified F_1 ATPase (specific activity, 30 U/mg) and an equal volume of 2× fluorescence quenching buffer for 15 min at 30°C. The membranes were then pelleted by centrifugation at 100,000 × *g* for 1 h, washed once with 5 ml of MOPS-Mg buffer, suspended in 200 μl of the same buffer, and assayed. To test the sensitivity of the membranes or reconstituted membranes to dicyclohexylcarbodiimide (DCCD), 40 μM DCCD in 100% ethanol was added directly to the membranes, incubated for 15 min at 30°C, and then assayed. The same volume of ethanol alone had no effect on any of the assayed activities. For ATP-dependent fluorescence quenching, 0.25 mg of reconstituted membranes was assayed directly without washing. In vitro ATPase and ATP synthase assays were conducted as described previously (17).

SDS gels and immunoblots. Sodium dodecyl sulfate (SDS) gel analysis of whole cells and crude membranes, electrophoretic transfer to nitrocellulose, and immunoblot analysis were carried out as described previously (2) with the following modifications: 2 μg of membrane protein per lane was loaded onto a Bio-Rad minigel system and, after electrophoresis, transferred to nitrocellulose paper for 15 min at 100 V and 4°C. The nitrocellulose paper was blocked with 1% bovine serum albumin (BSA) for 15 min and then treated with primary anti-F_1F_0 antibody for 1 h. The blot was then washed three times with 0.2% Tween 20–0.9% NaCl–10 mM Tris (pH 7.4), incubated with secondary biotinylated goat anti-rabbit antibodies (GIBCO BRL) for 30 min, and incubated with streptavidin-alkaline phosphatase (GIBCO BRL) for 30 min. The blot was then probed for alkaline phosphatase activity following GIBCO BRL instructions. For detection of biotinylated fusion proteins, after the nitrocellulose paper was blocked with BSA, the blot was treated with streptavidin-alkaline phosphatase and developed as described above.

Polyclonal antibodies to F_1F_0 ATPase. The F_0 ATPase was purified as described by Schneider and Altendorf (18). This preparation, which contained primarily F_0 subunits but was contaminated with F_1 subunits, was injected into rabbits for the purpose of raising polyclonal antibodies against F_0 subunits. Initial antibody preparations were found to be primarily against the α, β, γ, and b subunits. We then boosted the rabbits with a preparation which was more specific for the other subunits. The F_1F_0 ATPase was purified as described by Foster and Fillingame (9). The complex was subjected to the dissociation conditions described by Schneider and Altendorf for the dissociation of purified F_0 (19), and the products were separated by exclusion chromatography. One of the peaks from the column contained primarily the a, c, and δ subunits, with a small amount of b subunit and no other detectable ATPase subunits. This fraction was used to boost the antibody response in a rabbit which was already producing antibodies against the α, β, γ, and b subunits. The resulting antibody preparation reacted with all the ATPase subunits in immunoblots of purified F_1 and purified F_1F_0. In immunoblots of wild-type membranes compared with membranes isolated from an *unc* deletion strain, we could identify all the ATPase subunits except for ε in the wild-type membranes. Reactivity to the a subunit was relatively weak compared with reactivity to the other subunits. Also, despite preabsorption of antiserum with membranes isolated from the *unc* deletion strain, many cross-reacting proteins were detected in membranes isolated from the *unc* deletion strain.

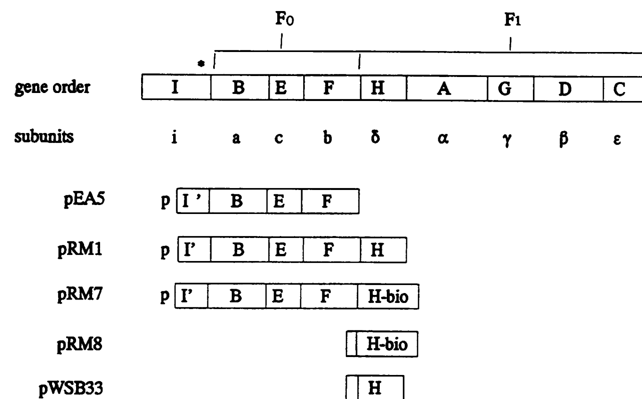

FIG. 1. Plasmids constructed for this study. The boxes at the top indicate the genes in the *unc* operon; the genes for the F_0 and F_1 sectors are indicated above the boxes, and the subunits coded for by the genes are shown below the boxes. The plasmids are listed on the left, and the F_1 or F_0 genes cloned in each plasmid are indicated by the boxed gene designations. The location of the *lac* promoter in these plasmids, determined by use of pUC vectors, is indicated by the letter "p". The asterisk indicates the location of a putative secondary *unc* promoter. H-bio is the designation for the gene containing *uncH* fused in frame to a biotinylation sequence. Gene I, the partial gene I (I′), and the product of gene I (the i polypeptide) have no known function.

RESULTS

Synthesis of F_0 subunits in an *unc* deletion strain. The plasmids used in this study are shown in Fig. 1. With the exception of the F_0+δ plasmids, these plasmids were described previously (13). pRM1 contains all the genes (*uncBEF*) for the F_0 sector in addition to the gene for the δ subunit of F_1 (*uncH*). It is identical to plasmid pBP101 studied by Fillingame et al. (8). We previously described plasmid pEA5, which is the equivalent of pRM1 lacking *uncH* (13). By comparing the effects of pEA5 and pRM1 on cell growth, membrane proton

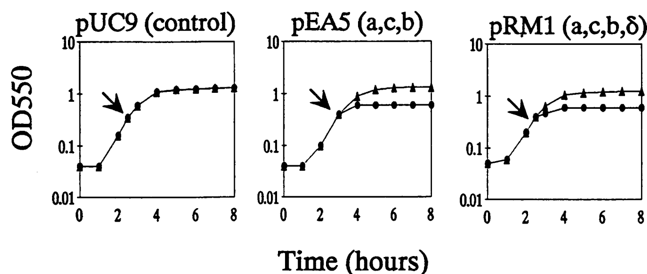

FIG. 2. Growth curves for induced and uninduced cultures. *E. coli* JM103 Δ(*uncB-uncD*) carrying each of the indicated plasmids was grown with shaking at 37°C in 250-ml cultures of LB medium containing 100 μg of ampicillin per ml to an OD_{550} of approximately 0.4. At the arrow, each culture was treated with 1 mM IPTG to induce transcription from the *lac* promoter and 300 μg of ampicillin per ml was added to prevent plasmid loss. The OD_{550} was monitored for an additional 6 h. Symbols: ●, induced; ▲, uninduced.

permeability, and F_0 function, we attempted to assess the role of the δ subunit in F_0 function.

As was the case for cells carrying pEA5 (13), IPTG-induced synthesis of the F_0 and δ subunits from plasmid pRM1 resulted in significant growth inhibition after approximately 30 min (Fig. 2). However, as described previously for the induced synthesis of F_0 subunits from plasmid pEA5 (13), this growth inhibition was probably not the result of unblocked F_0 channels but rather was nonspecific growth inhibition caused by overexpression of a gene(s) for membrane-bound proteins. Membranes isolated from such induced cultures contain abundant F_0 subunits, but when treated with purified F_1, they exhibit very poor energy-coupling activity. The F_0 subunits synthesized from the inducible *lac* promoter are therefore not properly assembled. Uninduced cultures of the same cells produce F_0 subunits, presumably from a secondary *unc* promoter (13), in amounts comparable to those found in membranes isolated from wild-type cells. These F_0 subunits can be reconstituted with purified F_1 to produce functional F_1F_0 complexes. Therefore, our analyses of membrane proton permeability and F_0 function were conducted on membranes isolated from uninduced cultures of the *unc* deletion strain carrying pRM1 (F_0+δ); we compared these membranes with membranes isolated from uninduced cultures of the *unc* deletion strain carrying pEA5 (F_0). We found that the uninduced cultures of the deletion strain carrying pRM1 grew more poorly than those of the same strain carrying pEA5, but we were able to grow both cultures to an OD_{550} of 1 for these analyses.

Immunoblot analysis of F_1F_0 subunit synthesis. To demonstrate that F_0 subunits coded for by these various plasmids were being synthesized and inserted into membranes, we analyzed whole cells and purified membranes for the presence of F_1F_0 subunits by immunoblotting. Figure 3 shows an immunoblot of whole cells and membranes isolated from cultures of the *unc* deletion strain carrying pUC9 (control), pEA5 (F_0), and pRM1 (F_0+δ). Significant amounts of subunits b and c were present in cells containing pEA5, and subunits b, c, and δ were present in cells carrying pRM1. Densitometric analysis of these lanes showed that the plasmids produced 70 to 80% of the wild-type levels of the F_0 and δ subunits. This immunoblot does not show subunit a because of the presence of a cross-reacting band in the deletion strain. However, other blots including purified anti-subunit a antibody (a gift from Karlheinz Altendorf) showed that these membranes contained nearly wild-type levels of subunit a (data not shown).

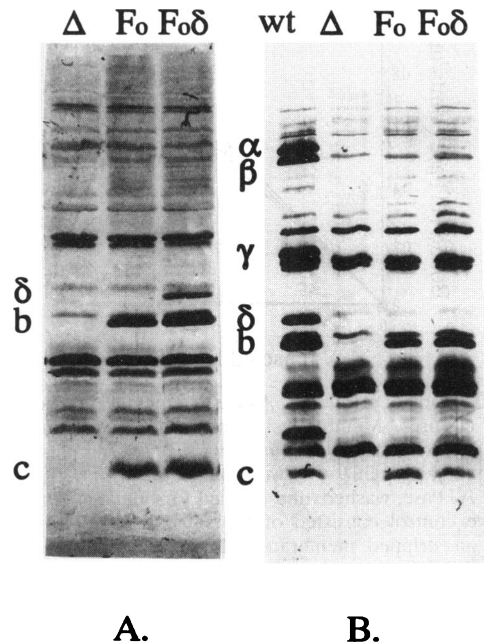

FIG. 3. Immunoblots of SDS gels of whole cells and membranes isolated from plasmid-containing cultures. (A) Whole-cell lysates from the *unc* deletion strain (Δ) or the deletion strain carrying pEA5 (F_0) or pRM1 (F_0δ) harvested at an OD_{550} of ≈1. (B) Membranes isolated from the wild-type strain (wt), the deletion strain (Δ), or the deletion strain carrying pEA5 (F_0) or pRM1 (F_0δ). Two micrograms of membrane protein was loaded into each lane. The immunoblots were visualized with antibodies to the F_1 and F_0 subunits as described in Materials and Methods. The locations of the subunits are indicated.

F_1 binding assays. Membranes isolated from cells carrying pUC9, pEA5, or pRM1 were incubated with purified F_1, and membrane-bound ATPase activity was measured after the membranes were washed to remove unbound F_1. As a control, membranes isolated from wild-type JM103 were stripped to remove F_1 and then reconstituted with different amounts of purified F_1. Figure 4 shows that membranes isolated from cells carrying either pEA5 or pRM1 bound F_1 equally well and at the same levels as stripped wild-type membranes. Membranes from the plasmid-bearing cells, therefore, contain F_0 subunits and are capable of binding F_1 at levels comparable to those found in wild-type cells.

Measurement of reconstituted membrane-bound ATPase activity. To assess the functionality of the F_0 sectors synthesized from these plasmids, the membranes described above were reconstituted with purified F_1 and assayed for F_1F_0-dependent energy-coupling abilities. ATP-driven fluorescence quenching measures the extent to which the membranes can hydrolyze ATP and couple this energy to the movement of protons. The resulting proton gradient is reflected in a decrease in the relative fluorescence of the dye ACMA after the addition of ATP. Figure 5 shows that membranes isolated from *unc* deletion cells carrying pRM1 (F_0+δ) and reconstituted with F_1 generated almost 70% of the wild-type levels of ATP-driven fluorescence quenching. Membranes isolated from the same cells carrying pEA5 were capable of similar levels of reconstituted ATP-dependent proton pumping (13). The deletion strain which contained no F_1F_0 acquired no proton-pumping activity when treated with purified F_1.

We also assayed the ability of the reconstituted membranes to catalyze respiration-dependent ATP synthesis. Table 1

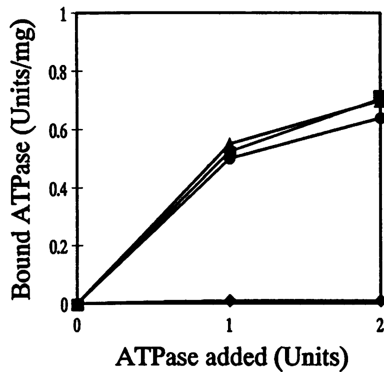

FIG. 4. Binding of purified F_1 ATPase to membranes isolated from uninduced cultures. Membranes (1 mg of protein) isolated from uninduced cultures of JM103 Δ(*uncB-uncD*) carrying plasmid pRM1 (▲), pEA5 (●), or pUC9 (◆) were incubated with 0, 1, or 2 U of purified F_1 ATPase, washed, and assayed for bound ATPase activity. The positive control consisted of the same F_1 binding experiment conducted on stripped membranes (■): membranes were isolated from unc^+ JM103, stripped of F_1 by three incubations in stripping buffer (1 mM Tris [pH 8], 0.5 mM EDTA, 10% glycerol), and centrifuged at 100,000 × g for 1 h. The final specific activities of the F_1-reconstituted membranes (units per milligram of membrane protein) are plotted on the ordinate as a function of added F_1 (abscissa). Each point represents the average for duplicate samples. The error was typically less than 5%. In this assay, untreated and unstripped wild-type (unc^+) membranes typically contain 0.8 to 1.0 U/mg.

presents the in vitro ATP synthase activities of the same reconstituted membranes as those shown in Fig. 4. When reconstituted with purified F_1, membranes isolated from cells carrying either pEA5 or pRM1 were capable of 15 to 20% of wild-type ATP synthase activity. As described previously (13), in our assays, reconstituted membranes carry out energy coupling in the direction of ATP synthesis less effectively than energy coupling in the direction of ATP-driven proton pumping. Table 1 shows that even membranes isolated from wild-type cells and stripped of F_1 have only about 15% of wild-type ATP synthase activity when reconstituted with purified F_1.

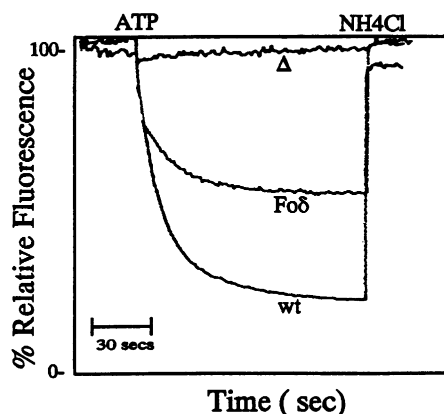

FIG. 5. ATP-dependent fluorescence quenching of F_1-reconstituted membranes. Membranes isolated from JM103 unc^+ (wt; positive control), JM103 Δ(*uncB-uncD*) (Δ; negative control), and JM103 Δ(*uncB-uncD*) carrying pRM1 ($F_0\delta$) were reconstituted with 2 U of purified F_1 and assayed for ATP-dependent fluorescence quenching as described in Materials and Methods. $F_0\delta$ membranes were isolated from uninduced cultures at an OD_{550} of approximately 1. Relative fluorescence is plotted versus time.

TABLE 1. ATP synthase activities[a]

F_1-reconstituted membranes	ATP synthase activity (nmol/min/mg)
JM103 Δ(*uncB-uncD*) plus:	
pUC9	0
pRM1	20 ± 2
pEA5	15 ± 1
pRM1I	5 ± 1
JM103 unc^+	90 ± 10
Stripped (without F_1)	0
Stripped (with F_1)	13 ± 1

[a] Membranes isolated from the *unc* deletion strain carrying the indicated plasmids were reconstituted with purified F_1 and assayed for in vitro ATP synthase activity. Membranes were isolated from uninduced cultures of the deletion strain carrying either pRM1 or pEA5 at an OD_{550} of approximately 1. pRM1I membranes were isolated from the deletion strain carrying pRM1 60 min after induction (at an OD_{550} of 0.4) with IPTG. Controls consisted of membranes isolated from *unc* deletion strain JM103 Δ(*uncB-uncD*), JM103 unc^+, and JM103 unc^+ stripped of membranes and then reconstituted with 0 or 4 U of purified F_1 as described in the legend to Fig. 4.

The immunoblots demonstrated that membranes from plasmid-bearing cells contained approximately 80% of wild-type levels of F_0 subunits. The activity assays demonstrated that when these membranes were reconstituted with purified F_1, they had substantial coupled ATPase activities and their ATP synthase activities were indistinguishable from that of wild-type membranes stripped of F_1 and then reconstituted. We therefore conclude that structurally intact, functional, reconstitutable F_0 can be synthesized and assembled in *unc* deletion cells carrying either pEA5 (F_0) or pRM1 (F_0+δ).

Proton permeability of F_0 made in the presence or the absence of the δ subunit. In past genetic studies, we had observed that the presence of the *uncH* gene in combination with uninduced F_0 genes and other F_1 genes resulted in significant growth inhibition which could be overcome by the addition of the F_0 proton channel blocker DCCD (1). We measured respiration-dependent fluorescence quenching to determine the relative proton permeabilities of F_0 sectors made in vivo in the presence or the absence of the δ subunit (Fig. 6). Membranes isolated from the *unc* deletion strain carrying pUC9, pEA5 (F_0), or pWSB33 (δ) were all capable of generating essentially the same large proton motive force, as measured by a substantial decrease in ACMA fluorescence when the membranes were incubated with NADH. The same assay of membranes isolated from the deletion strain carrying pRM1 (F_0+δ), however, revealed a much lower response to NADH, indicating that the F_0 sectors synthesized and assembled in the presence of the δ subunit were significantly more proton permeable than F_0 sectors assembled alone.

As a further demonstration that this increased proton permeability was caused by an assembled and leaky proton channel, we incubated F_0+δ membranes with either DCCD or purified F_1, both of which block the F_0 proton channel. Figure 7 shows that both of these treatments significantly increased respiration-driven fluorescence quenching, indicating that they both blocked proton conduction. Also, when DCCD was added to F_0+δ membranes reconstituted with purified F_1, the bound F_1 ATPase activity was inhibited to an extent comparable to that seen in wild-type cells (results not shown).

Is the δ subunit bound to the F_0? These results clearly demonstrate a δ-dependent effect upon the proton permeability of the F_0. We therefore attempted to demonstrate that the

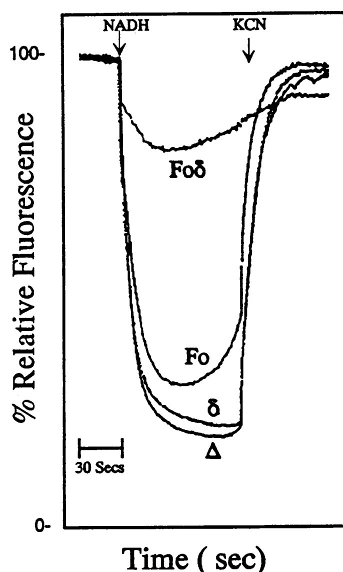

FIG. 6. NADH-dependent ACMA fluorescence quenching of membranes containing the indicated F_1F_0 subunits. Membranes isolated from the *unc* deletion strain carrying the control vector pUC9 (Δ), pWSB33 (δ), pEA5 (F_0), or pRM1 (F_0δ) were prepared from uninduced cultures grown to an OD_{550} of ≈1. Membranes were incubated with NADH in the presence of ACMA, and the resulting fluorescence quenching was measured. After 1 min or after stable fluorescence was measured, KCN was added to inhibit further respiration. Relative fluorescence is plotted versus time.

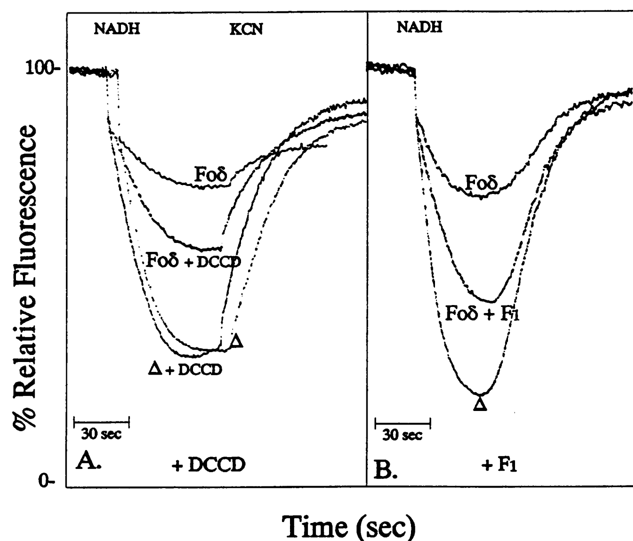

FIG. 7. NADH-dependent ACMA fluorescence quenching of membranes treated with DCCD or with purified F_1. Membranes isolated from uninduced cultures of the deletion strain carrying pRM1 (F_0δ) or the vector pUC9 (Δ) were treated with 40 μM DCCD (A) or 2 U of purified F_1 (B) and assayed for NADH-dependent fluorescence quenching as indicated in Materials and Methods. NADH and KCN were added at the indicated times. Relative fluorescence is plotted versus time.

δ subunit was actually bound to membranes in an F_0-dependent fashion. An immunoblot of whole-cell lysates clearly showed that the δ subunit was synthesized in cells carrying pRM1 (Fig. 3A), but the membrane preparation from cells containing pRM1 did not contain the δ subunit (Fig. 3B). We also prepared membranes in buffers of higher ionic strength—MOPS-Mg buffer and 100 mM MOPS–20 mM $MgCl_2$—and these conditions did produce marginally better F_0-dependent membrane association of the δ subunit, but the results were not striking, and increasing the ionic strength of the preparation buffers did not result in amounts of bound δ comparable to those seen in wild-type membranes.

To further test whether in cells containing the genes for F_0 and δ the δ subunit associates with the F_0, we constructed a biotinylated δ fusion protein. As described in Materials and Methods, we fused the DNA sequence encoding the biotin attachment site to the 3′ end of *uncH* to create plasmid pRM7. This plasmid is identical to pRM1, except that *uncH* has been modified to produce a δ subunit with an additional 75 amino acids attached at the carboxyl terminus. Membranes isolated from cells carrying this plasmid contain the biotinylated δ fusion protein, even in the lower-ionic-strength buffer (Fig. 8B, lane 3). However, membranes isolated from cells carrying pRM8, which makes the biotinylated δ protein alone, also contain the biotinylated δ fusion protein, even in the absence of the F_0 (Fig. 8B, lane 4). In both cases, the amount of biotinylated δ fusion protein on the membranes represents a small fraction of the amount seen in immunoblots of whole cells, since at least two of the background proteins are enriched in the membranes, while the amount of the fusion protein is decreased.

DISCUSSION

Our results indicate that F_0 made in the presence of the δ subunit is functional and is significantly more permeable to protons than F_0 made in the absence of F_1 subunits. Previous studies had indicated that the presence of the δ subunit in combination with F_0 and other F_1 subunits produced growth inhibition (1). The present studies demonstrate a direct effect of the δ subunit on the F_0 which produces increased proton permeability. The δ subunit has not such effect on membranes in the absence of the F_0.

We used a combination of anti-δ subunit antibodies and a biotinylated δ derivative to attempt to localize the δ subunit to membranes, but we found that although the δ subunit was bound to membranes which contained the F_0, the δ subunit alone or its biotinylated derivative also bound equally well, albeit at low levels, to membranes in the absence of the F_0. Therefore, despite the obvious effects of δ on the biochemistry of the F_0, it appears as though either the association between F_0 and δ alone is relatively weak and cannot withstand the membrane preparation treatment in the absence of other F_1 subunits or, in the absence of other F_1 subunits, δ is very susceptible to proteolysis and is therefore degraded shortly after binding to and opening of the F_0 proton channel. We were unable to identify the δ subunit in the supernatant fraction after centrifugation of membranes isolated from cells carrying pRM1, suggesting that it may have been proteolyzed.

Studies on the chloroplast δ subunit have concluded that the δ subunit functions at the interface between ATP synthesis and proton conduction (6). Our studies demonstrate that the δ subunit has a direct effect on the F_0. Exactly which F_0 subunit or subunits δ binds to is not known. It has been speculated that δ binds to the elongated b subunit, on the basis of computer modeling studies (24), because δ is required to bind F_1 to F_0 in vitro (21), and because the removal of the extended hydrophilic region of the b subunit eliminates F_1 binding to stripped

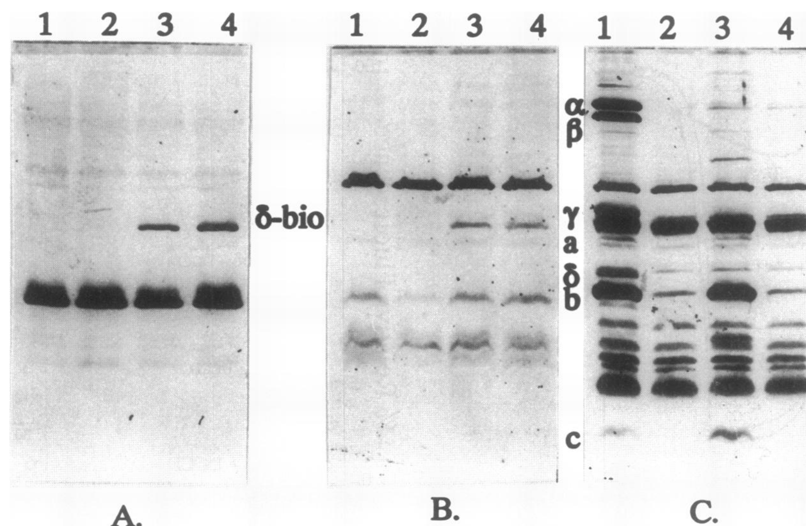

FIG. 8. Analysis of association of δ subunit and F_0. Whole-cell lysates and membranes were prepared from wild-type cells (lanes 1), *unc* deletion cells (lanes 2), *unc* deletion cells carrying pRM7 (F_0-biotinylated δ) (lanes 3), and *unc* deletion cells carrying pRM8 (biotinylated δ alone) (lanes 4). (A) Immunoblot of whole-cell lysates developed with streptavidin-alkaline phosphatase. (B) Immunoblot of purified membranes (2 μg per lane) developed with streptavidin-alkaline phosphatase. (C) Immunoblot of purified membranes (2 μg per lane) probed with primary anti-F_1F_0 antibody and secondary biotinylated antibodies and then developed with streptavidin-alkaline phosphatase. The locations of the biotinylated δ fusion protein (δ-bio) and the F_1F_0 subunits are indicated.

membranes (16). However, a direct interaction between δ and any of the F_0 subunits has not been demonstrated. These results, which show an influence of δ on proton conductance, do not rule out a δ-b interaction, since b is necessary for F_0 assembly (18), but also raise the possibility that δ interacts with either the a or the c subunit or both, since both are believed to participate in transmembrane proton conductance (7).

As was the case with a plasmid carrying only the F_0 genes cloned behind the *lac* promoter, inducing the transcription of F_0 and δ subunit genes inhibited cell growth. However, we demonstrated previously that this growth inhibtion was not related to increased proton permeability and was probably caused by overexpression of the membrane-bound a subunit (13). Uninduced cultures of pRM1 (F_0+δ) grew more slowly than uninduced cultures of pEA5, even though the amounts of the F_0 subunits in the membranes were comparable. This effect on growth probably was related to the increased membrane proton permeability seen in fluorescence quenching experiments and, as our experiments demonstrate, can be attributed to the effects of the δ subunit on proton conductance by the F_0.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant DMB-9096159 and by American Heart Association grant-in-aid 93007630. W.S.A.B. is the recipient of U.S. Public Health Service Research Career Development Award NIH KO4-AI00882.

REFERENCES

1. **Angov, E., T. C. N. Ng, and W. S. A. Brusilow.** 1991. Effect of the δ subunit on assembly and proton permeability of the F_0 proton channel of *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. **173:**407–411.
2. **Brusilow, W. S. A.** 1987. Proton leakiness caused by cloned genes for the F_0 sector of the proton-translocating ATPase of *Escherichia coli*: requirement for F_1 genes. J. Bacteriol. **169:**4984–4990.
3. **Brusilow, W. S. A.** 1993. Assembly of the *Escherichia coli* F_1F_0-ATPase, a large multimeric membrane-bound enzyme. Mol. Microbiol. **9:**419–424.
4. **Cox, G. B., J. A. Downie, L. Langman, A. E. Senior, G. Ash, D. R. H. Fayle, and F. Gibson.** 1981. Assembly of the adenosine triphosphate complex in *Escherichia coli*: assembly of F_0 is dependent on the formation of specific F_1 subunits. J. Bacteriol. **148:**30–42.
5. **Cox, G. B., and F. Gibson.** 1987. The assembly of the F_1F_0-ATPase in *Escherichia coli*. Curr. Top. Bioenerg. **15:**163–175.
6. **Engelbrecht, S., and W. Junge.** 1990. Subunit δ of H^+-ATPases: at the interface between proton flow and ATP synthesis. Biochim. Biophys. Acta **1015:**379–390.
7. **Fillingame, R. H., M. E. Girvin, D. Fraga, and Y. Zhang.** 1993. Correlations of structure and function in H^+ translocating subunit c of F_1F_0 ATP synthase. Ann. N.Y. Acad. Sci. **671:**323–334.
8. **Fillingame, R. H., B. Porter, J. Hermolin, and L. K. White.** 1986. Synthesis of a functional F_0 sector of the *Escherichia coli* H^+-ATPase does not require synthesis of the alpha or beta subunits of F_1. J. Bacteriol. **165:**244–251.
9. **Foster, D. L., and R. H. Fillingame.** 1979. Energy-transducing H^+-ATPase of *Escherichia coli*. J. Biol. Chem. **254:**8230–8236.
10. **Klionsky, D. J., W. S. A. Brusilow, and R. D. Simoni.** 1983. Assembly of a functional F_0 of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **258:**10136–10143.
11. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9:**309–321.
12. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Monticello, R. A., E. Angov, and W. S. A. Brusilow.** 1992. Effects of inducing expression of cloned genes for the F_0 proton channel of the *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. **174:**3370–3376.
14. **Nieuwenhuis, F. J. R. M., B. I. Kanner, D. L. Gutnick, P. W. Postma, and K. Van Dam.** 1973. Energy conservation in membranes of mutants of *Escherichia coli* defective in oxidative phosphorylation. Biochim. Biophys. Acta **325:**62–71.
15. **Pati, S., and W. S. A. Brusilow.** 1989. The roles of the α and γ subunits in proton conduction through the F_0 sector of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **264:**2640–2644.
16. **Perlin, D. S., D. N. Cox, and A. E. Senior.** 1983. Integration of F_1 and the membrane sector of the proton-ATPase of *Escherichia coli*. J. Biol. Chem. **258:**9793–9800.
17. **Scarpetta, M. A., C. A. Hawthorne, and W. S. A. Brusilow.** 1991. Characterization of semi-uncoupled hybrid *Escherichia coli-Bacil-*

lus megaterium F_1F_0 proton translocating ATPases. J. Biol. Chem. **266**:18567–18572.

18. **Schneider, E., and K. Altendorf.** 1984. Subunit b of the membrane moiety (F_0) of ATP synthase (F_1F_0) from *Escherichia coli* is indispensable for H^+ translocation and binding of the water-soluble F_1 moiety. Proc. Natl. Acad. Sci. USA **81**:7279–7283.
19. **Schneider, E., and K. Altendorf.** 1985. All three subunits are required for the reconstitution of an active proton channel (F_0) of *Escherichia coli* ATP synthase (F_1F_0). EMBO J. **4**:515–518.
20. **Senior, A. E.** 1990. The proton-translocating ATPase of *Escherichia coli*. Annu. Rev. Biochem. **19**:7–41.
21. **Smith, J. B., and P. C. Sternweiss.** 1975. Restoration of coupling factor activity to *Escherichia coli* ATPase missing the δ subunit. Biochem. Biophys. Res. Commun. **62**:764–771.
22. **Solomon, K. S., and W. S. A. Brusilow.** 1988. Effect of an *uncE* regulatory mutation on the synthesis and assembly of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **263**:5401–5407.
23. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
24. **Walker, J. E., M. Saraste, and N. J. Gay.** 1982. *E. coli* F_1-ATPase interacts with a membrane protein component of a proton channel. Nature (London) **298**:867–869.
25. **Walker, J. E., M. Saraste, and N. J. Gay.** 1984. The *unc* operon. Nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta **768**:164–200.