JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 815–821
0021-9193/82/110815-07$02.00/0

Vol. 152, No. 2

In Vitro Membrane Association of the F_0 Polypeptides of the *Escherichia coli* Proton Translocating ATPase

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Received 14 May 1982/Accepted 3 August 1982

The F_0 polypeptides *a*, *b*, and *c* of the H^+-translocating ATPase associated with membranes when synthesized in vitro. This association occurred when the membranes were present either cotranslationally or post-translationally. In addition, the F_0 polypeptides associated with liposomes. The membrane association seemed to be an insertion process since there was protection of polypeptides *a* and *c* from proteolysis. The in vitro insertion of the F_0 polypeptides *a*, *b*, and *c* was independent of the synthesis of each polypeptide and of the F_1 polypeptides.

The proton-translocating ATPase of *Escherichia coli* is a complex of eight polypeptides (6, 7, 9, 10). Five of the polypeptides (α, β, γ, δ, and ε), in the F_1 portion of the complex, are loosely bound to the cytoplasmic membrane and thought to be peripheral to it. The remaining three polypeptides (*a*, *b*, and *c*) comprise the F_0 portion and are assumed to be integral membrane proteins (6–8). Physiologically, the F_1F_0 complex catalyzes the transfer of P_i to ADP to synthesize ATP at the expense of energy derived from the proton gradient generated by the respiratory chain. Under anaerobic conditions, the complex hydrolyzes ATP to form a proton gradient (26), which is essential for other energy-dependent cellular functions, such as solute transport (24, 25).

Extensive studies have established that, in *E. coli*, the genes which specify the polypeptides of the ATPase are organized in an operon called *unc*. The gene-polypeptide relationships have been established within this operon (5, 11, 15, 17, 23). Our studies have used in vitro protein synthesis directed by cloned *unc* genes. This in vitro approach will eventually allow us to describe the steps that occur between gene expression and the assembled functional complex. Toward this end, we have attempted to first demonstrate which, if any, of the ATPase subunits become associated with membranes during in vitro synthesis. It has previously been reported (1, 4, 5) that some of the ATPase polypeptides synthesized in vitro sediment with membranes. In this study, we examined the membrane associations of the F_0 polypeptides in more detail. We demonstrate that these polypeptides associate with membranes or liposomes present either cotranslationally or post-translationally. These experiments suggest that the F_0 polypeptides are capable of associating with membranes independently of the synthesis of each polypeptide and of the F_1 polypeptides. Proteolysis experiments indicated that this association probably represents insertion of the proteins into the membrane.

MATERIALS AND METHODS

Chemicals. L-[^{35}S]methionine (1,100 Ci/mmol) was obtained from New England Nuclear Corp. Biochemicals, bovine serum albumin, phenylmethylsulfonyl fluoride, *N-p*-tosyl-L-lysine chloromethyl ketone, and crude soybean phospholipids (type II-S) were purchased from Sigma Chemical Co. All other chemicals were obtained from commercial sources.

Bacterial strains. *E. coli* A19 (RNase I_{19}, Hfr, *met* λ^+ Δ(*ton-trp*)) (12) was used to prepare the cell-free protein synthesis extract (S-30) for initial experiments as previously described (16). An S-30 extract from a mutant strain defective in ATPase was also used to support protein synthesis to compare the membrane association of polypeptides in the absence of preformed ATPase. The mutant strain was then used in the preparation of S-30 extract, membrane-free extract (S-100), ribosomes, and membranes for all subsequent experiments. This strain (designated RH93), which contains a Mu phage insertion in the *uncE* gene of the *unc* operon, is described elsewhere (R. Humbert and R. Simoni, submitted for publication). A membrane-free bacterial cell extract (S-100) was prepared as described by Daniels et al. (3). Ribosomes were prepared as described by Godson and Sinsheimer (14). Protein concentration was determined by the method of Lowry et al. (22).

In vitro protein synthesis. In vitro protein synthesis reaction mixtures were prepared by previously described techniques (16). Synthesis extracts (S-30 or S-100) were added to template DNA, buffer, and cofactor mixes to provide a cell-free protein synthesis system. Synthesis was initiated by addition of 10 μCi

of L-[^{35}S]methionine (specific activity, 1,100 Ci/mmol). Reaction volumes were 50 μl (unless otherwise stated), and incubations were carried out at 37°C for 30 min. Reactions were terminated by the addition to each sample of 5 μl of a solution containing 1 mg of RNase and 1 mg of DNase per ml, followed by incubation on ice for 5 min. To minimize sample proteolysis during the subsequent fractionation step, we added 50 μg of bovine serum albumin with the nuclease solution to increase the protein concentration. In addition, the protease inhibitors phenylmethylsulfonyl fluoride and *N-p*-tosyl-L-lysine chloromethyl ketone were added immediately after the nuclease solution to achieve a final concentration of 0.0002 M to inhibit proteolysis.

Plasmid DNA was prepared as previously described (1). Plasmid pRPG54 contains the genes coding for all eight known polypeptides of the ATPase complex, and the genes are in the correct sequence. Plasmid pRPG45 codes for F_0 polypeptides *a*, *b*, and *c*, whereas plasmid pRPG51 codes for polypeptides δ and *b*, and plasmid pRPG58 codes for polypeptide *c*.

Membrane and liposome preparation. The membrane pellet derived from the centrifugation of the S-30 cell extract prepared from strain RH93 was washed and suspended in buffer (0.01 M Tris-acetate [pH 8.2], 0.014 M magnesium acetate, 0.06 M potassium acetate, 0.001 M DL-dithiothreitol). Liposomes were prepared from soybean phosphatidylcholine. The crude phospholipids were dissolved in chloroform at a concentration of 40 mg/ml, dried under nitrogen, extracted with ether, and suspended in an equal volume of 0.01 M Tris-acetate buffer (pH 8.2). The solution was blended with a Vortex mixer and then sonicated under argon with a microtip probe Sonifier (Branson Instruments Co.), at an output of 20 A for 6 min.

Fractionation of in vitro products. After protein synthesis reactions were terminated, half of each incubation mixture was precipitated by the addition of acetone (10 volumes) and the resulting pellet, obtained by centrifugation, was suspended in sample buffer for gel electrophoresis. The remaining half of each incubation mixture was fractionated by isopycnic sucrose density sedimentation by layering it over a step gradient composed of 50 and 100 μl of sucrose in 0.01 M Tris-acetate buffer (pH 8.2) at concentrations of 30 and 80% (wt/vol), respectively (ρ = 1.1263 and 1.4085, respectively). The tubes were centrifuged in an Airfuge (Beckman Instruments, Inc.) at 160,000 × *g* for 30 min, and fractions were collected as follows. A 35-μl fraction, which contained soluble proteins, was taken from the top of the gradient. The 15 μl below that fraction was discarded. The third fraction (65 μl) contained the interface region between the 30 and 80% sucrose and also contained membranes. The remaining liquid in the tube was discarded, and the final fraction contained material which pelleted. The first supernatant fraction was diluted twofold with 0.01 M Tris-acetate buffer (pH 8.2) and centrifuged at 160,000 × *g* for 10 min. The supernatant fraction of this second centrifugation was precipitated with acetone and suspended in sample buffer for gel electrophoresis. The interface fraction was diluted threefold with the same buffer and centrifuged at 160,000 × *g* for 10 min. The resulting pellet was suspended in gel electrophoresis sample buffer for subsequent gel analysis. The fraction containing insoluble material that had pelleted through 80% sucrose was resuspended in 80% sucrose and centrifuged. The pellet from this centrifugation was suspended in sample buffer. The sucrose gradient fractionation procedure was also used to separate proteins produced in the S-100 extract in the absence or presence of membranes. The F_0 subunits were shown by the sucrose gradient technique to be membrane associated when synthesis was conducted in the presence of membranes. Therefore, for most experiments, synthesis samples were fractionated by a simple sedimentation procedure rather than with the sucrose gradient. In vitro reaction mixtures were diluted twofold with 0.01 M Tris-acetate buffer (pH 8.2) and centrifuged at 160,000 × *g* for 10 min in the Airfuge. The supernatant solution was removed, and the pellet was resuspended in the same buffer. Both supernatant and pellet fractions were centrifuged at 160,000 × *g* for 10 min to minimize cross contamination and prepared for gel electrophoresis.

Gel electrophoresis. Samples were suspended in sample buffer (10% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 6% [wt/vol] sodium dodecyl sulfate, 0.125 M Tris-hydrochloride [pH 6.8]) and sonicated in a bath Sonifier (Branson) for 20 s. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the samples was carried out by the method of Laemmli (21) with 12.5% polyacrylamide gels. The proteins labeled with L-[^{35}S]methionine from the in vitro synthesis system were detected by autoradiography of dried gels with Kodak XAR5 film. Typical exposure times were 1 to 3 days. The amount of each polypeptide was determined by scanning the autoradiogram with a Joyce-Lobel densitometer. The relative amount of each polypeptide determined in this way was independent of exposure time.

RESULTS

Incorporation of F_0 polypeptides *a*, *b*, and *c* into *E. coli* membranes. Several of the ATPase subunits are produced in sedimentable form during in vitro protein synthesis (1, 4, 5). To determine whether these proteins were membrane associated or simply insoluble as might be expected for hydrophobic membrane proteins, the S-30 reaction mixture was fractionated on a sucrose gradient. To determine where in the gradient membrane-associated proteins would migrate, a preparation of *E. coli* membranes was fractionated, and the fractions were assayed for the proton-translocating ATPase. The results, shown in Table 1, indicate that the membranes were located at the interface between the 30 and 80% sucrose layers. ATPase activity was negligible in the uppermost layer (soluble proteins) and in the lowest layer (insoluble material). Approximately 87% of the protein and 95% of the ATPase activity was recovered from the interface fraction. The specific activity of the interface fraction was typical for the membrane-bound activity. Separate experiments with heat-denatured insoluble albumin showed that insoluble proteins were completely pelleted under the conditions employed in fractionation.

TABLE 1. ATPase assay of membranes fractionated by sucrose gradient density centrifugation[a]

Fraction	Protein (mg)	ATPase activity	
		% of total	Specific (U/mg)
Total	0.85	100	0.78
Supernatant	0.11		ND[b]
Interface	0.68	95	0.93
Pellet	0.01		ND

[a] Fractionation of *E. coli* membranes by sucrose gradient density centrifugation was conducted as described in the text. ATPase assays were conducted as previously described (25). One unit of ATPase activity is defined as micromoles of P_i produced per minute per milligram of ATP.

[b] ND, Not detectable.

This fractionation was first performed on the proteins synthesized by the S-30 extract which contained membranes. Plasmid pRPG54, which codes for all eight of the ATPase polypeptides, was used as template. The amount of each ATPase polypeptide was measured by densitometry, and recovery was determined. The average recovery of each polypeptide after fractionation was ≥70%. The results of the fractionation (Fig. 1a) demonstrate that the F_1 polypeptides were predominantly in soluble form under these synthesis conditions, whereas the F_0 polypeptides were almost exclusively membrane associated. Small, variable amounts of some of the F_1 polypeptides were membrane bound. This observation confirms earlier observations (1, 4, 5) that the F_0 polypeptides synthesized in vitro are sedimentable but, in addition, demonstrates that the sedimentability is due to membrane association and not to simple insolubility.

It is possible that the membrane association of the F_0 polypeptides described above could be due to the interaction of newly synthesized polypeptides with endogenous ATPase polypeptides present in the synthesis extract. To test this possibility, we prepared an S-30 extract from strain RH93, which has a Mu phage insertion in the gene (*uncE*) coding for polypeptide *c*. This gene is the second in the *unc* operon, and the effect of the Mu phage insertion is to stop expression of all of the genes distal to the insertion, owing to transcriptional polarity (13). Thus, this strain was expected to produce only polypeptide *a*. Crude cell extracts prepared from RH93 did not contain ATPase activity, nor did isolated membranes from this strain exhibit quenching of acridine dye fluorescence upon addition of ATP, although they exhibited the normal fluorescence quenching in the presence of NADH. This behavior was consistent with the interpretation that RH93 lacks a functional ATPase. A comparison of the association of the F_0 polypeptides with membranes of the normal strain (A19) and RH93 is shown in Fig. 1. The subunits of the ATPase exhibited the same pattern of membrane association regardless of the strain used to prepare the S-30 extract. The F_0 polypeptides associated with the membrane fraction, and the F_1 polypeptides were soluble. A reproducible difference in the behavior of the δ subunit was noted, however. If the normal (A19) S-30 extract was used to support protein synthesis, roughly 50% of the δ subunit associated with membranes, but when the RH93 extract was used, no δ subunit was detected in the membrane fraction. This suggests that the δ subunit associates with preexisting ATPase polypeptides when synthesis is conducted with the normal S-30 extract.

Synthesis of ATPase polypeptides in a membrane-free system. It has been shown that some proteins are capable of association with mem-

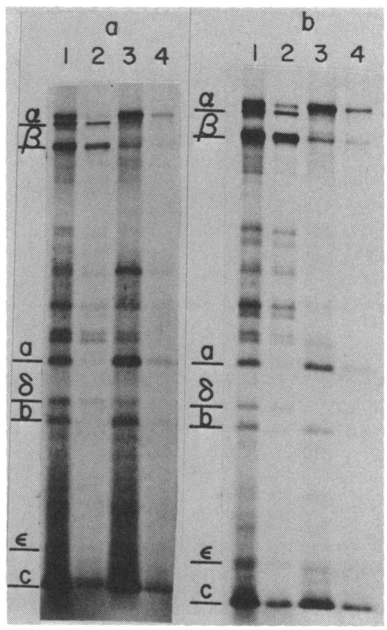

FIG. 1. Association of F_0 polypeptides produced in vitro with membranes. The products from the S-30 reactions were fractionated by isopycnic sucrose gradient centrifugation as described in the text. Each fraction was then analyzed by gel electrophoresis and autoradiography. (a) Synthesis and fractionation of *unc* gene products produced by an S-30 extract prepared from strain A19. Lane 1, Unfractionated sample; lane 2, supernatant fraction containing soluble proteins; lane 3, interface fraction containing membrane proteins; and lane 4, pellet fraction containing insoluble proteins. (b) Synthesis of *unc* gene products with an S-30 extract prepared from strain RH93. The lanes are the same as in (a). In both (a) and (b), the volume of the unfractionated sample (lane 1) was equivalent to the volume of the samples loaded in the other lanes.

branes only during translation (20), whereas other proteins may associate with membranes either during or after protein synthesis (27). To establish when membrane association of the F_0 polypeptides occurs, we used a protein synthesis system (S-100) which lacked membranes. Membrane preparations were then added to this synthesis system either during protein synthesis or after synthesis was terminated by nuclease addition. The reaction mixture was then fractionated to determine which subunits were membrane bound. The S-100 extract, membranes, and ribosomes were prepared from RH93. The data presented in Fig. 2 indicate that all of the ATPase polypeptides were produced in soluble form when no membranes were present during synthesis. When membranes were present co-

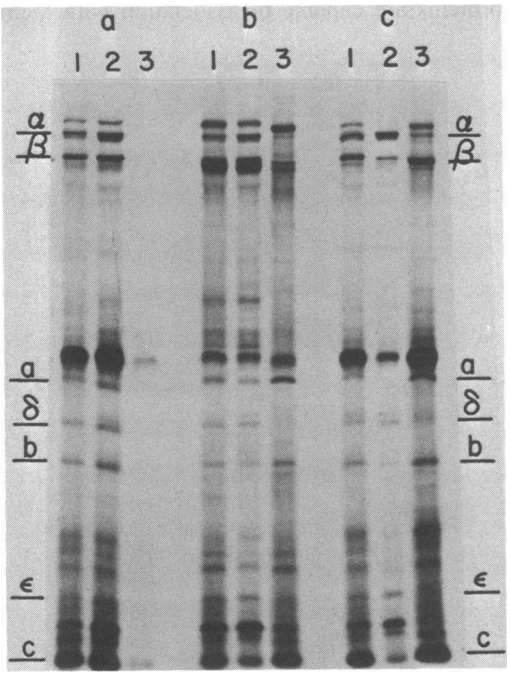

FIG. 2. Association of F_0 polypeptides with membranes either cotranslationally or post-translationally. Membrane-free S-100 extract was used to direct synthesis (30 min) of the *unc* gene products. Proteins were then fractionated by simple sedimentation to separate membrane-bound proteins from soluble proteins as described in the text. (a) Synthesis in the absence of membranes. (b) Synthesis in the presence of membranes. (c) Synthesis as described in (a), except that after synthesis was terminated purified membranes were added, and the mixture was incubated for an additional 30 min. In each panel, lane 1 represents the total synthesis mixture, lane 2 represents the soluble proteins, and lane 3 represents the sedimentable membrane-containing fraction. In each panel, the sample volume of unfractionated material (lane 1) was one-half that of the fractionated material (lanes 2 and 3).

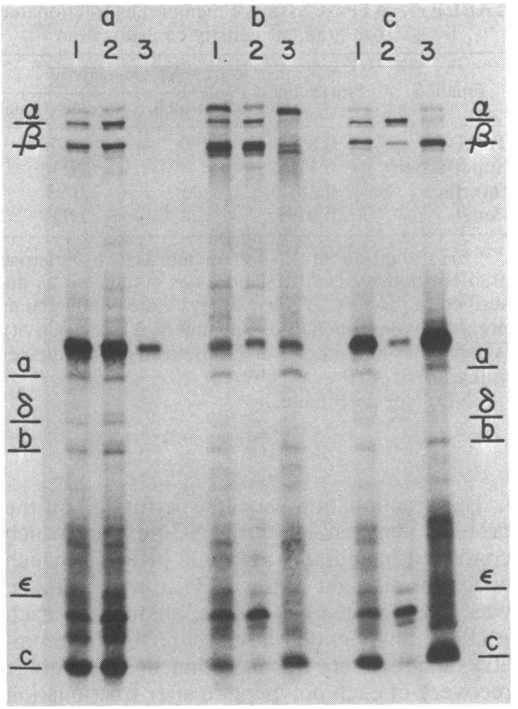

FIG. 3. Association of F_0 polypeptides with liposomes either cotranslationally or post-translationally. An S-100 extract was used to direct protein synthesis (30 min) in the absence or presence of liposomes, and the products were separated by sedimentation as described in the text. (a) Results of synthesis conducted in the S-100 system. (b) Synthesis was conducted in the presence of liposomes (80 μg of lipid), and the mixture was then fractionated. (c) Synthesis was conducted in the absence of liposomes as described in (a), and, after the reaction was terminated, liposomes were added (80 μg of lipid), and the mixture was incubated for an additional 30 min. In each panel, lane 1 represents the unsedimented material and contains one-half the volume present in the other two lanes. Lanes 2 and 3 represent the supernatant and pellet fractions, respectively.

translationally, F_0 polypeptides *a*, *b*, and *c* became membrane associated. The F_0 polypeptides also became membrane bound when membranes were added after protein synthesis was terminated by the addition of nucleases. Thus, the F_0 polypeptides can associate with membranes either cotranslationally or post-translationally.

A similar experiment in which liposomes were substituted for *E. coli* membranes was performed. The purpose of this experiment was to determine whether the F_0 proteins could associate with these model membranes, which do not contain the proteins present in *E. coli* membranes. Protein synthesis was conducted in the presence or absence of liposomes, and the prod-

ucts were fractionated as described above. The F_0 polypeptides associated with liposomes when liposomes were added either during translation or after protein synthesis was terminated (Fig. 3).

Nature of the membrane association. The ATPase polypeptides which were synthesized by the S-100 extract or by an S-100 extract to which membranes had been added cotranslationally were subjected to protease digestion to determine if the polypeptides became integrated into the membrane or attached to it. The results (Fig. 4) indicate that F_0 components produced in soluble form were susceptible to chymotryptic digestion to various degrees. When the polypeptides were membrane associated, polypeptide *a* and polypeptide *c* became more resistant to proteolysis. Polypeptide *b* was not protected to any significant extent (see below).

The possible effect of F_1 polypeptides on the partitioning of F_0 polypeptides into membranes was investigated. This was done by examining the membrane association of the F_0 polypeptides when these proteins were synthesized from plasmids coding for selected polypeptides. When plasmid pRPG45, which codes for F_0 polypeptides *a*, *b*, and *c* and F_1 polypeptide δ was used to direct in vitro synthesis, F_0 polypeptides *a*, *b*, and *c* were again recovered predominantly in the membrane fraction (Fig. 5). To ascertain if individual F_0 polypeptides required the presence of the other two F_0 polypeptides, plasmid pRPG51, which codes for polypeptides *b* and δ, and plasmid pRPG58, which codes only for polypeptide *c*, were used to direct protein synthesis. Polypeptide *b* became membrane bound when synthesized in the absence of other F_0 polypeptides, as did polypeptide *c*. These experiments were also done by the sucrose gradient sedimentation procedure to separate the reaction products, and the F_0 proteins were shown to be associated with the membrane fraction (data not shown). In each case, the same percentage of each of the F_0 polypeptides was membrane associated as when all of the ATPase polypeptides were produced. With the possible exception of polypeptide *a*, which is presumably present in membranes of RH93, which was used to prepare the synthesis extracts, it appeared that the F_0 polypeptides associated with membranes independent of the synthesis of the F_1 polypeptides.

DISCUSSION

When the polypeptides of the H^+-ATPase complex were synthesized in vitro and separated into soluble and membrane fractions, the F_0 polypeptides *a*, *b*, and *c* were found to be associated almost exclusively with the membrane fraction. This association with *E. coli* membranes occurred either during translation or after translation was complete. The F_0 subunits were also capable of associating with liposomes when the liposomes were added either cotranslationally or post-translationally. This association did not depend on preexisting ATPase polypeptides in the extract, as indicated by results obtained with extracts from RH93, which should not contain any ATPase polypeptide except polypeptide *a*. However, there remains the possibility that polypeptide *a*, presumed present in the membranes of RH93 (by genetic evidence),

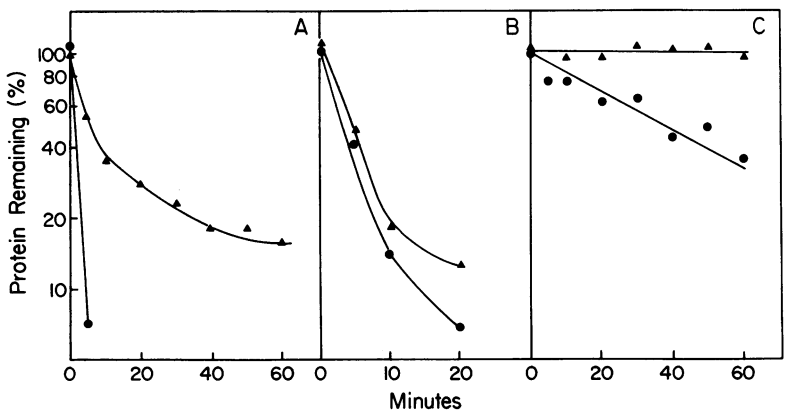

FIG. 4. Resistance of F_0 polypeptides *a* and *c* to proteolysis when membrane associated. Protein synthesis was carried out with 306 μl of S-100 reaction mixture, and, in parallel, with 306 μl of S-100 reaction mixture to which 0.42 mg of *E. coli* membranes was added. After 30 min, the reactions were stopped by the addition of nucleases, and chymotrypsin was added to a final concentration of 7.9 μg/ml. Samples were then incubated, and portions were removed at the indicated times and prepared for gel electrophoresis. The data show the rate of proteolysis of the polypeptides when synthesized in the absence (●) or presence (▲) of membranes. A, B, and C, Rates of proteolysis of polypeptides *a*, *b* and *c*, respectively.

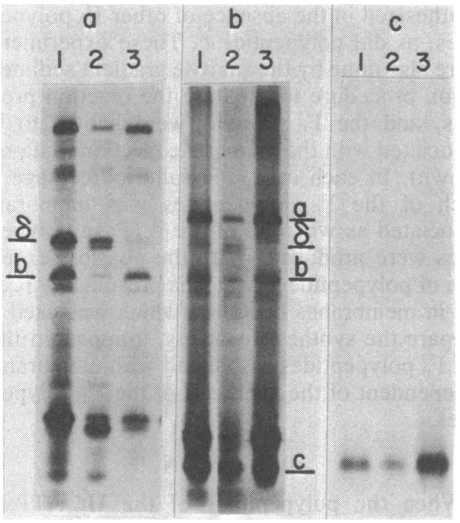

FIG. 5. Effect of other ATPase polypeptides on the membrane association of the F_0 polypeptides. Plasmids pRPG45 (coding for polypeptides *a*, δ, *b*, and *c*), pRPG51 (coding for polypeptides δ and *b*), and pRPG58 (coding for polypeptide *c*) were used to direct protein synthesis with the S-30 extract prepared from RH93. The polypeptides produced were subjected to sedimentation and separated by gel electrophoresis. (a) Synthesis and analysis of polypeptides from pRPG51. (b) Results of synthesis of polypeptides from pRPG45. (c) Synthesis and analysis of polypeptides produced from pRPG58. In each panel, lane 1 represents the unfractionated sample, lane 2 represents the soluble portion, and lane 3 represents the pelleted membrane material. In (a), lane 1, corresponding to the total mixture, contains twice as much material as lanes 2 and 3, whereas in (b) and (c), lane 1 contains one-half as much material as the lanes corresponding to the fractionated material.

or the gene product of the putative *unc* gene 1 proposed by Gay and Walker (11) may be regulating the membrane association of the remaining F_0 polypeptides. The plasmids used in this study did not contain the *unc* promoter or the putative *unc* gene 1. Therefore, it may be that the absence of the *unc* promoter or of *unc* gene 1 affected the association of the F_0 subunits with membranes.

When plasmids coding for different subsets of ATPase polypeptides were used to direct protein synthesis, the F_0 polypeptides were shown to be membrane associated. This finding suggests that synthesis of other ATPase polypeptides is not required for the association to occur. In addition, the ability of the F_0 polypeptides to integrate into liposomes indicates that non-ATPase membrane proteins are not required for insertion. These results appear to differ from the recent evidence presented by Cox et al. (2), that F_1 polypeptides α and β are required for the insertion of polypeptide *b* into the membrane in vivo. A comparison of the results of the two studies must be undertaken cautiously, since we analyzed the sequence of assembly of the ATPase complex in vitro, whereas Cox et al. (2) examined the ATPase complex as produced by various *unc*-containing mutants in vivo.

The nature of the attachment of the F_0 polypeptides to the bacterial membranes was examined. This association resulted in protection of polypeptides *a* and *c* from proteolysis, suggesting some insertion of these polypeptides into the membrane. F_0 polypeptide *a* was more resistant to chymotrypsin digestion when it was allowed to associate with membranes but was digested rapidly by the same concentration of protease when in soluble form. Polypeptide *b* was not protected from digestion by chymotrypsin when it became membrane associated. Polypeptide *c* appeared to be more resistant to this protease than either *a* or *b*, but it was clearly protected upon insertion into the membrane. This may be due in part to aggregation into its multimeric state in vitro, resulting in inaccessability to protease. These patterns of protease sensitivity are qualitatively consistent with estimates of how each protein may be associated with the membrane. Inspection of the primary structure of each polypeptide as deduced from the nucleotide sequence (11, 18, 19, 23) permits a logical prediction of the membrane configuration of each polypeptide. Polypeptide *b* is very hydrophilic, with only a small N-terminal hydrophobic region capable of spanning the bilayer. Thus, extensive proteolysis of *b* would be expected. Polypeptide *c* is extremely hydrophobic and is probably completely buried within the bilayer. The hydrophobicity of polypeptide *a* seems to be between these two extremes, and polypeptide *a* may have as much as 50% of its structure exposed.

The F_0 proteins can associate with membranes both during or after translation and in the absence of the other ATPase polypeptides. Additional experiments are necessary to determine if the polypeptides inserted into the membrane in vitro assume the correct orientation with respect to the membrane and interact with each other to form a functional F_0 complex. It also remains to be demonstrated whether our in vitro results represent the in vivo assembly process.

ACKNOWLEDGMENTS

We thank Richard Humbert for providing strain RH93. We also thank Elizabeth Haynes for preparation of this manuscript.

This work was supported by Public Health Service grant GM 18539 and Research Career Development Award GM 00225 to R.D.S. from the National Institutes of Health. K.P.D. was supported by the Medical Scientist Training Program. W.S.A.B. was supported by postdoctoral fellowship

GM 07598, and R.P.G. was supported by doctoral training grant GM 07781, both from the National Institutes of Health.

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