The assembly of the $F_{0}$ sector of the *Escherichia coli* ATP synthase has been studied using both structural and functional criteria for assembly. Cross-linking $E. coli$ minicell membranes containing only the $F_{0}$ subunits $a$, $b$, and $c$ with dithiobis(succinimidy1 propionate) (DSP) produces $b_{2}$ and $c_{3}$ dimers that are generated by cross-linking membranes containing the assembled holoenzyme. Five plasmids carrying the genes specifying the $F_{0}$ polypeptides in a bacterial strain resembling holoenzyme. Five plasmids carrying the genes show a good correlation between $F_{0}$ function and the amount of the membrane-bound $F_{0}$ polypeptides. In this report we revise a conclusion reached previously (Klionsky, D. J., Brusilow, W. S. A., and Simon, R. D. (1983) *J. Biol. Chem.* 258, 10136–10143) and present evidence that the $F_{0}$ subunits alone are sufficient to assemble a functional proton pore.

Proton-translocating adenosinetriphosphatases (EC 3.6.1.3) are present in the energy-transducing membranes found in bacteria, plant, and animal cells. The H+-ATPase, or ATP synthase, catalyzes the synthesis of ATP from ADP and P$_{i}$ at the expense of a hydrogen ion potential. The *Escherichia coli* ATP synthase is composed of two domains termed the $F_{0}$ and the $F_{1}$. The $F_{0}$ is the membrane extrinsic catalytic domain. The $F_{1}$ sector is located in, and conducts protons across, the cytoplasmic membrane. The $F_{1}$ and $F_{0}$ polypeptides are coded for by the operon uncBEFHAGDC ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and $\zeta$ subunit(s)) and extensive study has revealed much about its structure and function (for reviews, see Refs. 3 and 4). The $F_{0}$ consists of three subunits, $a$ ($M_{r} = 30,300$), $b$ ($M_{r} = 17,200$), and $c$ ($M_{r} = 8,300$), which probably occur in a ratio of 1:2:10, or 1:2:12:5, respectively (5, 6). The structure and function of the $E. coli$ $F_{0}$ sector are areas of investigation that have experienced significant progress recently (for review, see Ref. 7). The very hydrophobic $c$ polypeptide, or DCCD$^+$-binding protein, probably folds into a helical hairpin which traverses the membrane bilayer. The $b$ polypeptide consists of a short hydrophobic amino terminus, which probably inserts into the bilayer, connected to a membrane extrinsic hydrophilic portion (9, 8). Relatively little is known about the $\alpha$ subunit, which probably folds six or seven $\alpha$ helical segments across the bilayer leaving hydrophilic portions of its length to either side of the membrane bilayer (9–12). Proton translocation requires all three subunits in vivo (13) and in vitro (14).

The assembly of the *E. coli* ATP synthase has been analyzed using mutations in the unc operon. Cox et al. (15) have observed that a mutation in uncD (coding for $\beta$) resulted in the absence of the $b$ polypeptide from the membrane. An assembly sequence was proposed on the basis of the analysis of this and a number of other unc mutations (15). One feature of the proposed assembly sequence is coordinated assembly of the $F_{0}$ and $F_{1}$ sectors: completion of $F_{0}$ assembly (membrane insertion of $b$) requires $F_{1}$ subunit synthesis. We and others find that the $b$ subunit inserts into the cytoplasmic membrane in the absence of the $\beta$ or other $F_{1}$ subunits (13, 16, 17). In addition, it is not clear that $b$ exists or accumulates in the cytoplasm in the uncD mutant (15). It has been suggested that the membrane insertion of $b$ in the absence of $\beta$ may be an artifact of the overproduction of the $b$ polypeptide from multicopy plasmids containing the uncF gene (18). In the present paper, we address some of these uncertainties in $F_{0}$ assembly using both structural and functional criteria for $F_{0}$ assembly.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Assembly of the $F_{0}$, Sector in Minicells Detected by DSP Cross-linking—In our initial experiments, the combination DK3/pRPG45 (uncB-D/abc$\beta$) was unable to promote the formation of a functional $F_{0}$ in the absence of the $F_{1}$ subunits $\alpha$ and $\beta$ (16). The cross-linking experiment described below was undertaken to establish whether or not the inability to measure $F_{0}$ function was due to lack of assembly of the $F_{0}$.
subunits. Do the F, polypeptides form interactions in the absence of the F, subunits?

We have previously used the cleavable cross-linking reagent DSP on the purified F,F, complex and on a minicell lysate to detect interactions between F, polypeptides (10). The cross-linking procedure in this report has been modified in two ways compared to our previous report. An unlabeled methionine chase (1 mM) is conducted at the end of the [35S]methionine-labeling period to permit synthesis of increased amounts of the F, polypeptides. Cross-linking is performed on membrane suspensions instead of minicell lysates.

DSP cross-linking of the DK3/pRPG45 (ΔuncB-D/acbd) minicell membranes results in the detection of the b,dimer and the c,dimer at a DSP concentration of 0.5 mM (Fig. 2A, spots 1 and 2). The apparent molecular weights of the b, and c, dimers are 35,000 and 13,000, corresponding to the predicted values of 34,400 and 13,800, respectively (the apparent molecular weight for c in the gel system is 6,800). A DSP concentration of 0.05 mM detects only the b,dimer (Fig. 2B, spot 1). DSP cross-linking of the F,F, complex in minicell membranes from DK3/pRPG45 (ΔuncB-D/acbd) shows the b, and c, dimers (Fig. 2C, spots 1 and 2) at a concentration of 0.5 mM DSP. Without DSP, no cross-links are generated (Fig. 2D). The amount of the cross-linked F, polypeptides in Fig. 2, A and B, is low due to the nature of the cross-linking methodology but compares favorably with the amount of the cross-linked products in Fig. 2C, as well as with the amount of the cross-linked products obtained with purified F,F, holoenzyme (10). The formation of DSP cross-links between F, subunits has not been found to be quantitative in experiments performed by us as well as others (10, 11). Treatment of the cross-linked membranes with 20 mM iodoacetate and 20 mM N-ethylmaleimide in sample buffer (containing no reducing agent) for 20 min at 50 °C has no effect on the presence of the b, dimer spot (data not shown). This indicates that the b,dimer detected with DSP cross-linking does not result from thiol group exchange or the formation of a cystine bridge between b molecules during electrophoresis.

An interaction between the b and c subunits has been detected using an immunoprecipitation technique. Antiserum specific to the b polypeptide (10) co-immunoprecipitates the c polypeptide from an Aminoxid WS 35-solubilized membrane preparation from DK3/pRPG45 (ΔuncB-D/acbd) minicells (data not shown).

Although interactions between a and b have been detected in vivo (11) and with the purified F,F, (10), it has been difficult to detect this interaction in the minicell system (10). The stoichiometry of a,b,c in membranes from DK3/pRPG45 (ΔuncB-D/acbd) minicells labeled with [35S]methionine is 1:0.1:9:12:6, respectively. This stoichiometry was determined by electrophoresing a sample of the DK3/pRPG45 minicell membranes, performing autoradiography, and quantitating the radioactivity present in each band densitometrically. The close correspondence of this ratio with the probable in vivo ratio of a:2:b:6,c:15 (5, 6) suggests that the a subunit has properly integrated into the F, sector.

Dimerization of b Polypeptides in the Absence of Other F, Subunits—The interactions between the F, subunits detected above suggest that the information necessary for the assembly of the F, resides in the individual subunits and that the individual subunits may exhibit assembly interactions when isolated from one another. We have detected such an interaction in the analysis of the cross-linking properties of the b polypeptide in a membrane containing no other F, or F, polypeptides.

Minicell membranes from DK3/pRPG51 (ΔuncB-D/bb) containing 35S-labeled b protein produce a prominent b,dimer when treated with DSP (Fig. 3A, spot 1). Cross-linking also occurs in membranes from the strain DK6/pJPA17 (ΔuncB-C/b') between nonfunctional b' polypeptides carrying the missense mutation which substitutes Asp-131 for Gly-131 (Fig. 3B, spot 2).

We have attempted to identify the residue(s) in the b polypeptide chain that are responsible for the b-b interaction by cross-linking truncated b polypeptides with DSP. For this purpose we have used three b polypeptides that have missing carboxyl-terminal portions due to the introduction of nonsense mutations into the uncF gene (19). The three truncated b polypeptides studied have sizes of 13,700, 11,600, and 10,400 daltons corresponding to 122, 105, and 95 amino acids, respectively, compared to 156 amino acids residues in the full-length b. These truncated b polypeptides are designated b(14), b(12), and b(10), respectively. The b(14), b(12), and b(10) polypeptides in minicell membranes from DK3/pJPA14, DK3/pJPA12, and DK3/pJPA10, respectively, are cross-linked with DSP to form dimers (Fig. 4, spots 1-3). The efficiency of cross-linking appears to diminish somewhat with the absence of the carboxyl-terminal residues. Two shorter truncated b polypeptides of sizes 7,900 and 9,300 daltons have been subjected to similar DSP cross-linking analysis and appear to form dimers as well (data not shown).

The a, b, and c Subunits Are Sufficient to Form a Functional F,—On the basis of the results from the structural analysis of F, assembly above, we decided to re-examine the function of the F, sector in membrane suspensions using a fluorescence quenching assay. Membrane suspensions were carefully prepared and assayed as quickly as possible from fresh bacterial cultures without freezing of cell or membrane samples (see "Experimental Procedures"). The fluorescence quenching activity of a membrane sample from a given strain/plasmid combination varied only 5 to 10% between different membrane preparations.

Membranes from DK3/pRPG54 (ΔuncB-D/acbd) contain a complete ATP synthase complex and show high NADH-driven and ATP-driven fluorescence quenching activity (Fig. 5C). The amount of fluorescence quenching observed corresponds to the membrane potential generated by the electron transport chain, and the ATP synthase at the expense of the substrates NADH and ATP, respectively. The electron transport poison KCN and the protonophore CCCP abolish proton-dependent fluorescence quenching. The plasmid pDKJ19 codes for the F, subunits a, b, and c and a partial α polypeptide (α'). The function of the F, from DK3/pDKJ19 (ΔuncB-D/acbd') is measured in two ways in the fluorescence assay. Membranes show a low level of NADH-driven fluorescence quenching (Fig. 5A). This reduced level of quenching is due to the dissipation of the NADH-dependent proton gradient by the functional proton-translocating F, sector. Membranes from DK3/pDKJ19 (ΔuncB-D/acbd') do not contain a functional F, and produce no ATP-driven fluorescence quenching (Fig. 5A). Purified F, may be added back to the DK3/pDKJ19 membrane preparation to reconstitute ATP-driven fluorescence quenching (Fig. 5B). F,-reconstituted membranes also show restored NADH-driven quenching because proton translocation through the F, is blocked by the attached F,.

Five Plasmids with Different Levels of F, Activity—In order to investigate the basis for differences of F, function, we have examined five plasmids which produce a range of F, activity. The plasmids we have used are pDKJ19 (acb'), pDKJ20 (acb), pJPA1 (acb), pRPG23 (acb'), and pRPG45 (acb'). Plasmids pDKJ19 and pDKJ20 are derivatives of pRPG45.
Assembly of the E. coli F\textsubscript{o} Subunits

**Fig. 2. Cross-linking F\textsubscript{o} proteins in minicell membranes.** DK3 (\(\Delta\text{uncB-D}\)) minicells containing pRPG45 (\(\text{acbd}\gamma\delta\alpha\)) or pRPG54 (\(\text{acbd}\delta\gamma\beta\alpha\)) were radiolabeled and a membrane suspension was prepared. The membrane sample was reacted with DSP at the final concentrations below. First dimension from left to right, 8–16% gradient SDS-PAGE without 2-mercaptoethanol treatment. Second dimension, 14% SDS-PAGE under reducing conditions. Uncross-linked subunits form a curve in the two-dimensional SDS-PAGE system and are indicated by letters. Spots to the left of the curve represent 2-mercaptoethanol resolved cross-linked polypeptides. A, DK3/pRPG45 (\(\Delta\text{uncB-D/}\text{acbd}\)) treated with 0.5 mM DSP shows the \(b_2\) dimer (spot 1) and the \(c_2\) dimer (spot 2). B, DK3/pRPG45 treated with 0.05 mM DSP shows the \(b_2\) dimer. C, DK3/pRPG54 (\(\text{acbd}\delta\gamma\beta\alpha\)) treated with 0.5 mM DSP shows the \(b_2\) and \(c_2\) dimers. (Autoradiography time for C was half of that for A and B.) D, DK3/pRPG45, no DSP. CAT, chloramphenicol acetyltransferase.

They were constructed in order to take advantage of the intermediate synthesis efficiency characteristic of the plasmid pRPG23 and to examine the effects of the absence of the \(\delta\) and \(\alpha\) subunits on F\textsubscript{o} function.

The range of F\textsubscript{o} activity is demonstrated by both the ATP-driven fluorescence quenching of the F\textsubscript{1}-reconstituted mem-
branes, and the NADH-driven fluorescence quenching of un
reconstituted membranes (Fig. 6). Fluorescence quenching traces F and G are control samples. The corresponding membrane samples were isolated from strain DK3 (uncB-D) carrying plasmids encoding only one or two of the F, genes: plasmid pDJK4 promotes synthesis of a and c only; pRPG51 codes for b and d. Without all three F, subunits present in one membrane bilayer there is no measurable F, activity (Fig. 6, F and G). Although plasmid pDJK4 (ac) codes for the uncF gene (16), the b polypeptide is not detected in minicell labeling experiments unless a plasmid such as pDJK35 (bacyt) is present in the minicell. The reason for this is unclear.

The plasmids responsible for the range of F, activity may be ordered from least to most active: pRPG45 (acb), pJPAl (acb), pRPG23 (acbdcac), pDJK19 (acbcos), and pDJK20 (acbc). As expected, we find in general, the higher the reconstituted ATP-driven fluorescence quenching, the lower the NADH-driven fluorescence quenching. The one exception occurs when comparing the pDJK19 and pDJK20 samples (Fig. 6, A and B). Neither the d subunit, nor the combination of the δ and α subunits, appear to have an in vivo effect on F, function.

The ATP-driven fluorescence quenching obtained with F, reconstituted membranes from DK6/pJPAl (uncB-C/abc) was identical to that obtained with F, reconstituted membranes from DK3/pJPAl (uncB-D/abc) (data not shown). This indicates that the c subunit, coded for by uncC, also does not play an obligate role in F, assembly.

Significantly, DK3/pDJK19 (uncB-D/abcos) grew with a doubling time of 52 min, which compares favorably with the 49-min doubling time observed for the control combination DK3/pDJK4 (uncB-D/abc). Likewise, DK3/pRPG23 (uncB-D/abcac) doubled in 50 min. Thus, the levels of F, function we have measured do not appear to be deleterious to the growth rate of the bacteria in rich medium.

The Amount of Membrane-bound F, Subunits Correlate with F, Activity—In order to characterize this F, activity and demonstrate its authenticity, the relationship between the amount of the membrane-bound F, subunits and the activity measured in the fluorescence assay was investigated. It was important to show, for example, that the F, activity was not due to an excess of one or more of the F, subunits.

Membrane samples assayed by fluorescence were examined using a technique that allows the F, proteins to be identified among the large number of other proteins in a membrane sample. Membrane suspensions (of equal protein content) were diluted with a low ionic strength buffer (ST1) and sedimented. The resulting membrane pellets were resuspended in ST1 buffer, stirred in the cold, and sedimented as before. This procedure removes extrinsic proteins from the membrane. Electrophoresis was carried out on an aliquot of the resultant "stripped" membrane samples (Fig. 7). Subunits a, b, and c are identified by co-migration with the F, polypeptides present in a sample of purified ATP synthase and by comparison of membrane samples with and without the F,.
Assembly of the E. coli F, Subunits

Fig. 5. Measurement of F, function in the fluorescence quenching assay. Membrane samples were prepared, assayed, and reconstituted with purified F, as detailed under "Experimental Procedures." Arrows indicate the addition of NADH, KCN, ATP, and CCCP. ACMA was added before NADH and the initial fluorescence was adjusted to 100%. Quenching of fluorescence (15%) and elapsed time scales are indicated. A, DK3/pDJK19 (ΔuncB-D/acba') membranes prepared with high ionic strength (HM) buffer. B, DK3/pDJK19 membranes prepared with HM buffer and reconstituted with 40 µg of F,. C, DK3/pRPG54 (ΔuncB-D/acba'γβ) membranes prepared with HM buffer.

Fig. 6. Comparison of the F, activity from five strain/plasmid combinations. Measurement of fluorescence quenching was performed as in Fig. 5. All membrane samples were prepared with HM buffer. For the ATP-driven fluorescence quenching portion of the assay, membranes prepared with HM buffer were reconstituted with 40 µg of F,. A, DK3/pDJK19 (ΔuncB-D/acba'). B, DK3/pDJK20 (acba'). C, DK3/pRPG22 (acbb'). D, DK3/pPA1 (acbb). E, DK3/pRPG45 (acbab). F, DK3/pDJK4 (acbb). G, DK3/pRPG51 (bb).

subunits. The low ionic strength treatment of membranes does not completely resolve the b subunit from a protein in the DK3 strain which is not part of the ATP synthase complex (Fig. 7).

In order to more accurately quantify the amount of the b subunit present in the membrane, a procedure was developed for the extraction of b from low ionic strength washed membranes with the detergent Aminoxid WS 35. As described above, the membrane samples are diluted with ST1 buffer and sedimented. Extraction of b is achieved by resuspending the membrane pellet in ST1 buffer containing 10 mM Aminoxid. After sedimentation, the supernatant contains solubilized b subunit. We have characterized this procedure by comparing the polypeptides solubilized from DK3/pDJK4 (ΔuncB-D/ac) and DK3/pDJK19 (ΔuncB-D/acba') membranes. The solubilized b polypeptide can be seen in the Aminoxid supernatant from DK3/pDJK19, but not from DK3/pDJK4 (Fig. 8, lanes 4 and 5). The b polypeptide was identified by co-migration with the b subunit in a sample of purified ATP synthase (data not shown). Using the Aminoxid procedure, electrophoretic quantification of the b subunit present in the membrane samples was performed (Fig. 9).

The results from the electrophoretic quantification of all three F, subunits using the two procedures described above are summarized in Table I. The masses of the F, subunits in each of the membrane samples are expressed as a per cent of the amount present in the membrane of the wild-type strain DS410. These data are derived from densitometric analysis of the gels presented in Figs. 7 and 9. The amounts of the a and b subunits correlate, in general, with the level of F, activity observed in the fluorescence assay. A comparison of the amount of the a and b subunits with the corresponding F, activity is facilitated by the quotient: F, activity divided by amount of b polypeptide (Table I, values for Q). This quotient, Q, is a rough measure of F, "assembly efficiency". Although the correspondence between activity and mass of F, polypeptides is not exactly quantitative, there is only a small amount of variation in the quotient Q (Table I). Furthermore, the Q value for the wild type strain DS410 compares favorably with the values for strain DK3 with the plasmids. The generally good correlation between amount of polypeptide and F, function indicates that the different levels of F, function are due
to different amounts of $F_o$ sectors in the membranes and not to different efficiencies of $F_o$ assembly. The $Q$ value for DS410 strain because the ATP-driven fluorescence quenching activity was increased relative to the values for the plasmid-bearing polypeptides synthesized from these plasmids to differences in the promoters in the plasmid vector sequences (see "Experimental Procedures").

The stoichiometry of the $a$ and $b$ subunits may be calculated from the absolute staining intensity data obtained from the densitometer analysis (data not shown). The $a:b$ stoichiometries based on the densitometer scan data are: 1:1.6 (pRPG45), 1:2.1 (pPA1), 1:2.5 (pRPG23), 1:1.4 (pDK19), 1:1.9 (pDK20), and 1:1.6 (DS410). Although the relative staining efficiency for the $F_o$ subunits is unknown, we believe it is significant that these ratios are in agreement with the probable in vivo ratio of $a:b_o$ ($5, 6$).

The amount of membrane-bound c subunit shows no consistent relationship to $F_o$ function and even appears to be inversely related to activity in samples with intermediate levels of activity (Table I). Considering the extreme hydrophobicity of the c subunit, it is unlikely that c is lost from the membrane during manipulations. The discrepancy may be due to inaccuracies in the electrophoresis and staining of c related to the proximity of the c band to the electrophoretic front.

**Figure 7. Determination of the relative amounts of the membrane-bound $F_o$ subunits.** Membranes (0.5 mg of protein) from the strain and plasmid indicated were stripped, electrophoresed, and silver-stained as described under "Experimental Procedures." The lower half of the gel is shown. Lane 1, 10 $\mu$g of pure $F_o$. Lane 2, DK3 ($\Delta uncB-D$). Lane 3, DS410 (unc$^+$). Lane 4, DK3/pDK4 (ac). Lane 5, DK3/pRPG45 (ac). Lane 6, DK3/pPA1 (ac). Lane 7, DK3/pRPG23 (ac). Lane 8, DK3/pDK19 (ac). Lane 9, DK3/pDK20 (ac).

**Figure 8. Characterization of the $b$ polypeptide solubilization procedure.** Membrane samples from the strain and plasmid indicated were washed with the low ionic strength buffer ST1, solubilized with Aminoxid WS 35, and centrifuged as described under "Experimental Procedures." The total amount of protein solubilized by Aminoxid (present in the supernatant fraction) was electrophoresed in lanes 3-5. Only 2.5% of the amount of protein remaining in the pellet fraction was electrophoresed in lanes 1 and 2. Lane 1, DK3/pDK4 ($\Delta uncB-D/ac$) membranes after extraction. Lane 2, DK3/pDK19 (unc$^+$) membranes after extraction. Lane 3, supernatant from DK3/pDK19 membranes treated with ST1 buffer without Aminoxid. Lane 4, Aminoxid-solubilized proteins from DK3/pDK4 membranes. Lane 5, Aminoxid-solubilized proteins from DK3/pDK19 membranes.

*The $F_o$ Subunits Synthesized from Plasmids Do Not Over-accumulate*—One possible explanation for the observation of a functional $F_o$ in membranes from bacteria containing multicopy plasmids is that the large number of copies of the genes coding for the $F_o$ polypeptides results in an excess of membrane-bound $F_o$ subunits. An excess of $F_o$ subunits in the membrane might allow a low percentage of the $F_o$ subunits to assemble by an inefficient, nonphysiological pathway. We have addressed this possibility in the experiments above. Electrophoretic quantification of the $F_o$ subunits from a wild-type bacterium without a plasmid, strain DS410 (unc$^+$), was performed alongside the experiments with the plasmids (Figs. 7 and 9). None of the plasmids we have studied results in an excess of membrane-bound $F_o$ subunits, with the exception of the amount of c present in DK3/pDK20. DS410 membranes contain more of the $a$ and $b$ subunits than the membranes from strain DK3 ($\Delta uncB-D$) containing the plasmids pDK20 (ac) or pDK19 (ac$^+$) (Table I). Hence, overaccumulation of the $F_o$ subunits in the membrane is not the reason we observe a functional $F_o$.

We have attempted to delete the genes uncH, -A, -G, -D, and -C, coding for the $F_i$ polypeptides, from the chromosomal operon in strain DS410, leaving only the unc promoter, uncI, and the genes coding for the $F_o$ polypeptides in the chromo-
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FIG. 9. Determination of the relative amounts of membrane bound b using the Aminoxid procedure. Lanes 2-9 show the proteins solubilized with Aminoxid as described in Fig. 8. Lanes 1-9 are exactly as in Fig. 7. The b subunit sometimes migrates as a pair of bands. In lanes 1 and 9 the upper band of the doublet has become flared at the edge during electrophoresis. The lower half of the gel is shown.

TABLE I

| Strain| Plasmid| Relative amount of membrane-bound subunit | \( Q' \) | Lane |
|-------|--------|------------------------------------------|--------|------|
| DK3   | pDKJ4  | g g g 94 4                                 | 3      | 3    |
| DK3   | pRPG45 | 19 20 11 0 0.34 5                         | 5      | 5    |
| DK3   | pJPAI  | 31 41 18 88 0.29 6                        | 6      | 6    |
| DK3   | pRPG23 | 46 73 45 75 0.23 7                        | 7      | 7    |
| DK3   | pDKJ19 | 85 76 71 63 0.32 8                        | 8      | 8    |
| DK3   | pDKJ20 | 83 98 82 144 0.27 9                       | 9      | 9    |
| DS410 | 100 100 100 100 0.45 2                    | 2      | 2    |

\( ^a \)The data are expressed as a per cent of the value obtained for the wild-type strain DS410.

\( ^b \)Strains and plasmids listed in order of observed F\textsubscript{6} activity.

\( ^c \)Data obtained by densitometric analysis of Fig. 7.

\( ^d \)Data obtained by densitometric analysis of Fig. 9.

\( ^e \)\( Q = \text{ATP-driven fluorescence quenching units (\%)} \) (from Fig. 6) divided by values for b (this table; from Fig. 7).

\( ^f \)Corresponding lane numbers in Figs. 7 and 9 from which the densitometric measurements were made.

\( ^g \)This value (>0) is too close to the DK3 control value to accurately determine.

some. Southern analysis of chromosomal DNA from the strain lacking the uncH-C genes indicates that the uncB, -E, and -F genes in the chromosome are intact, but fluorescence assays and electrophoretic analysis of membrane preparations show that the F\textsubscript{6} polypeptides are not present in the membrane (data not shown). Perhaps, efficient synthesis of the F\textsubscript{6} polypeptides from the unc promoter in a strain deleted for uncH-C impairs cell growth and selects for a mutation in the unc promoter region.

The F\textsubscript{6} Subunits Have a Small Effect on F\textsubscript{6} Activity—We have further addressed the possibility that plasmid-directed formation of a functional F\textsubscript{6} represents an inefficient, non-physiological assembly process by (i) testing the sensitivity of the F\textsubscript{6} from DK3/pRPG45 (\( \Delta \text{uncB-D/abcB} \)) to DCCD and (ii) examining the role of the F\textsubscript{6} subunits in F\textsubscript{6} assembly.

One easily assayed characteristic of an authentic F\textsubscript{6} is the inhibition of proton translocation by DCCD. The F\textsubscript{6}, synthesized in DK3/pRPG45, which demonstrates a low level of activity, is sensitive to DCCD (Fig. 10, A and C). Reaction of the F\textsubscript{6} from other plasmids with DCCD gives the same fluorescence quenching trace as for DK3/pRPG45 (data not shown).

The role of the F\textsubscript{6} in F\textsubscript{6} assembly was examined by comparing the function of the F\textsubscript{6} from cells synthesizing only the F\textsubscript{6} polypeptides to the F\textsubscript{6} function from cells synthesizing both the F\textsubscript{6} and F\textsubscript{6} polypeptides. If the F\textsubscript{6} resulting from DK3/pRPG45 is assembling inefficiently due to the absence of the F\textsubscript{6}, then the addition of F\textsubscript{6} during synthesis of the F\textsubscript{6} should increase F\textsubscript{6} function substantially. We compared membranes from DK3/pRPG45 (\( \Delta \text{uncB-D/abcB} \)) to membranes from DK3/pRPG45/pDKJ35 (\( \Delta \text{uncB-D/abcB/hr} \)). The membranes were treated with low ionic strength (ST2) buffer to strip F\textsubscript{6} subunits away from the F\textsubscript{6} sector. Purified F\textsubscript{6} was added back to membrane samples using the reconstitution procedure in order to measure ATP-driven fluorescence quenching. The F\textsubscript{6} activity in the absence of the F\textsubscript{6} was 65% of the activity in the presence of the F\textsubscript{6} (Fig. 10, D and E). Similarly, the F\textsubscript{6} activity from DK3/pDKJ19 (abcB') is about 65% of that from DK3/pRPG45 (abcB/abcB/hr) (Fig. 5). The 35% increase in F\textsubscript{6} activity due to simultaneous synthesis of F\textsubscript{6} and F\textsubscript{6} subunits is a relatively small increase which may result from the protection of the F\textsubscript{6} from degradation in vivo. If the F\textsubscript{6} protects the F\textsubscript{6}, then the labile F\textsubscript{6} in vivo should be detectable in the absence of the F\textsubscript{6}. Consistent with this, we find that strain DK3/pRPG45 incubated for 12 h in stationary phase loses F\textsubscript{6} activity (Fig. 10, B and C). This result may explain our previous findings with the plasmid pRPG45: we were unable to detect formation of a functional F\textsubscript{6} unless the F\textsubscript{6} subunits were also synthesized (16). In our previous experiments, bacterial cultures were incubated at 37 °C for about 2 h in stationary phase before harvesting (16). In the absence of the F\textsubscript{6} subunits, the labile F\textsubscript{6} may have been degraded during the stationary phase, preventing the detection of F\textsubscript{6} activity.

DISCUSSION

Cross-linking experiments demonstrate b-b and c-c interactions characteristic of the assembled F\textsubscript{6} sector. The c cross-linking pattern observed in membranes from cells bearing the

FIG. 10. The F\textsubscript{6} subunits have a small effect on F\textsubscript{6} activity. Fluorescence quenching was measured as in Fig. 5. Membranes (0.5 mg of protein) prepared with HM buffer (A-C) or with ST2 buffer (D and E) are reconstituted with F\textsubscript{6} A, DK3/pRPG45 (\( \Delta \text{uncB-D/abcB} \)) treated with DCCD. B, DK3/pRPG45 incubated 12 h in stationary phase. C, DK3/pRPG45. D, DK3/pRPG45. E, DK3/pRPG45/pDKJ35 (\( \Delta \text{uncB-D/abcB/hr} \)).
plasmid-borne uncB, -E, and -F genes from a very efficient effect in studies of the expression of the F, genes from a plasmid-borne polypeptide in a strain carrying the polar uncD436 allele may account for the inability to detect the decrease in the amount of the F, subunits in the membrane. Comparing the wild-type bacterium with the plasmid-bearing strains indicates that plasmid-directed synthesis of the a, b, and c polypeptides does not result in an excess of membrane-bound polypeptides. This is probably because the promoter in the vector sequences of the five plasmids studied are not as strong as the unc operon promoter, which appears to be similar to the lac promoter in strength (20). Expression of the plasmid-borne uncB, -E, and -F genes from a very efficient promoter may have an inhibitory effect on growth rate that we did not observe. Fillingame (21) has observed such an effect in studies of the expression of the F, genes from a plasmid-borne lac promoter.

The F, subunits do not appear to contribute significantly to F, assembly, although the F, may protect the F, subunits from degradation in vivo. In our previous examination of F, assembly, we concluded that the a and b subunits were essential for F, assembly (16). Currently, we interpret these results to mean that the a and b subunits are capable of protecting the F, sector, perhaps the b polypeptide specifically, from degradation. Similarly, the lack of protection of the b polypeptide in a strain carrying the polar uncD436 allele may account for the inability to detect the b polypeptide in the membranes from this strain (15). Hoppe et al. (22) have shown that in strains producing reduced amounts of the F, polypeptides, or none at all, the amount of membrane-bound b subunit decreases with decreasing synthesis of the a and b subunits. Furthermore, the mass of subunit b was reduced in strains harvested in stationary phase compared to those harvested in log phase growth (22). Similarly, the presence of b in logarithmic growth phase and its disappearance in stationary phase in an unc mutant with an F, binding affinity of only 30% that of the wild type shows the sensitivity of b towards endogenous proteolysis (13). The susceptibility of b to degradation by exogenous protease has been well characterized and has revealed much about the structure and function of b (13, 17, 22, 23).

In addition to the precautions taken to prevent or reduce degradation during membrane preparation, the bacterial strain used may have contributed to the measurement of proton translocation activity in this report. The minicell strain DK3 (ΔuncB-D) transformed with the F, plasmids produced the highest F, activity values of the strains studied. The strains 1100 and MC4100, deleted for the uncB-D genes and transformed with the F, plasmids, showed 25% or less of the F, activity measured in DK3 with identical plasmids. Recently, Schneider and Altendorf (14) have reported the reconstitution of the F, in vitro from fractionated F, subunits. Similarly, Perlin et al. (23) have been able to reconstitute the F, in vivo by mixing two membrane samples in the presence of asolectin. The ability to reconstitute a functional F, in the absence of the F, subunits supports our findings that the F, subunits are sufficient to assemble a functional F,.

Klionsky and Simon (24), in the accompanying report, show that the F, subunits are capable of assembling in the cytoplasm independent of the F, polypeptides. This suggests an assembly pathway in which the F, and F, sectors assemble separately and bind to one another only after each portion is complete. However, the demonstration that the F, and F, subunits are sufficient to assemble functional F, and F, domains does not preclude the interaction of subunits from each sector during assembly in vivo. An F, subunit, or the intact F, may bind to a membrane integral F, subunit, perhaps b, before the attached F, subunit becomes integrated into the assembled F,. Although the assembly of the ATP synthase may involve interactions between the F, and F, sectors, it seems clear that the F, and F, subunits possess the information necessary to specify the proper assembly of each sector, respectively, in the cytoplasmic and membrane compartments in vivo.

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REFERENCES
1. Gunsalus, R. P., Braslow, W. S. A., and Simon, R. D. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 829-834.
2. Gys, N. J., and Walker, J. E. (1981) Nucleic Acids Res. 9, 3919-3926.
3. Dunn, S. D., and Heppel, L. A. (1981) Arch. Biochem. Biophys. 210, 421-436.
4. Futai, M., and Kanazawa, H. (1986) Curr. Top. Bioenerg. 10, 181-215.
5. Foster, D. L., and Fillingame, R. R. (1982) J. Biol. Chem. 257, 2609-2615.
6. von Meyenburg, K., Jorgensen, B. B., Nielsen, J., Hansen, F., and Michel-ensen, O. (1982) Tokai J. Exp. Clin. Med. (Special Symposium Issue) 7, 23-31.
7. Hoppe, J., and Sebald, W. (1984) Biochim. Biophys. Acta 765, 1-27.
8. Hoppe, J., Montecucco, C., and Friedl, P. (1980) J. Biol. Chem. 255, 2882-2885.
9. Senior, A. E. (1983) Biochim. Biophys. Acta 726, 81-95.
10. Aris, P. J., and Simon, R. D. (1980) J. Biol. Chem. 255, 14590-14609.
11. Hermolin, J., Gallant, J., and Fillingame, R. H. (1983) J. Biol. Chem. 258, 14650-14655.
12. Foster, D. L., Boulik, M., and Kack, H. R. (1983) J. Biol. Chem. 258, 31-34.
13. Friedl, P., Hoppe, J., Gunsalus, R. P., Michelsen, O., von Meyenburg, K., and Schairer, H. U. (1983) EMBO J. 2, 19-25.
14. Schneider, E., and Altenendorf, K. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7279-7283.
15. Cox, G. B., Dowre, J. A., Langman, L., Senior, A. E., Ash, G., Faye, D. R. H., and Gibson, F. (1981) J. Bacteriol. 148, 39-42.
16. Klionsky, D. J., Braslow, W. S. A., and Simon, R. D. (1983) J. Biol. Chem. 258, 19136-19143.
17. Decker, R. P., Braslow, W. S. A., Gunsalus, R. P., and Simon, R. D. (1982) J. Bacteriol. 152, 815-821.
18. Jans, D. A., Finn, A. L., Hatch, L., Gibson, F., and Cox, G. B. (1984) J. Biol. Chem. 259, 14590-14609.
19. Porter, A. C. G., Kusneto, C., Aldape, K., and Simon, R. D. (1985) J. Biol. Chem. 260, 8182-8187.
20. Porter, A. C. G., Braslow, W. S. A., and Simon, R. D. (1983) J. Bacteriol. 155, 1271-1278.
21. Fillingame, R. H. (1984) in H."-ATPase (ATP Synthase) Structure, Function, Bioenergetics. The F, complex of coupling membranes. (Papa, S., Altenendorf, K., Ernster, L., Packer, L., eds.) pp. 109-118, for ICSQ Press by Adriatica Editrice, Bari, Italy.
22. Hoppe, J., Friedl, P., Schairer, H. U., Sebald, W., von Meyenburg, K., and Jorgensen, B. B. (1983) EMBO J. 2, 115-120.
23. Perlin, D. S., Cox, D. N., and Senet, A. E. (1983) J. Biol. Chem. 258, 9799-9800.
24. Klionsky, D. J., and Simon, R. D. (1985) J. Biol. Chem. 260, 11200-11206.

Additional references are found on p. 11215.
Supplementary Material to

**EXPERIMENTAL PROCEDURES**

**Materials**

Dithiothreitol (500 mg/ml) was obtained from Pierce Chemical Co. (Rockford, Ill.). NADH (500 mg/ml) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction endonucleases were from New England Biolabs (Beverly, Mass.). E. coli RNA polymerase was a gift of Dr. H. U. Schaller. All other chemicals and media were of a commercially available high purity.

**Bacterial strains and plasmids**

**pBR322** is the E. coli minimal producing strain with a wild-type unc operon sequence (1). pOS1 is a derivative of pBR322 which is deleted for the umuC genes (2). pDK12 is a derivative lacking the umuC genes (3).

**Preparation of culture**

**pDK12** was constructed as follows (Fig. 1). The plasmid pDK12 was reduced by 2.4 kilobases by digestion with NalI, which cuts in the gene coding for delta and epsilon, and religated to give the plasmid pDK12. An identical approach was used to produce pDK14 reductions in plasmids pRK101.4, pRK101.7, and pRK101.43 to give pRK101.4, pRK101.7, and pRK101.43, respectively. The restriction enzyme recognition sites are indicated. The regions of the plasmids containing the resulting minicells were constructed using the vector pBR322 and confer resistance to ampicillin. Plasmid pOS145 is a derivative of pOS145 which cuts twice in the alpha polypeptide (Klopp et al. 1983) and confers resistance on E. coli to 2.0 ml of methionine for 45 min. The minicell producing strain was chosen for the purpose of preparing the strain so that minicells could be used to verify the synthesis of polypeptides encoded by the plasmid under study. When minicells and a sample prepared from harvested cells and minicells were resuspended in 50 mM HEPES (pH 7.5), 2 M guanidine derivatized with 0.2% glucose.

**Characterization of the alpha polypeptide (alpha)**

The alpha polypeptide (alpha) was eliminated in order to detect the off-minus sense transcript (as described by Klopp et al. 1983) by hybridization of the 2.5 kilobase plasmid, pRK101.4, which contains the alpha polypeptide (y) gene.

**Preparation of minicell lysates**

**A** approach was used to produce RNA at 37°C by swirling on ice. We found that minicell could be used to verify the synthesis of polypeptides encoded by the plasmid under study. When minicells and a sample prepared from harvested cells and minicells were resuspended in 50 mM HEPES (pH 7.5), 2 M guanidine derivatized with 0.2% glucose. The resulting supernatant fraction was centrifuged at 100,000 g for one hour. The membrane protein was then isolated with cold NM buffer, sediected at 0°C, and centrifuged in 0.5 ml of NM buffer and resuspended in 0.5 ml of NM buffer. To produce minicell lysates, the lysates were centrifuged at 20,000 g for one hour. The membrane pellet was then isolated and used in 100 ml of NM buffer.

**Quantification of the alpha polypeptide (alpha)**

Quantification of the amount of the alpha polypeptide present in each sample was accomplished using a Quickanhar 8 and 9 densitometer and a Haemocyt stock liquid scintillation counter.

**Minicell labeling and membrane preparation**

**pJK19** and **pJK20** are plasmids which do not contain the plasmid pOS145 which contains the alpha polypeptide (alpha) which is the component of the alpha polypeptide (alpha) which is the component of the alpha polypeptide (alpha). To construct the plasmid pJK19, plasmid pOS145 was digested using EcoRI, which cuts twice in the alpha polypeptide (alpha) and confined to the alpha cistron. The regions of the plasmids were constructed using the vector pBR322 and confer resistance to ampicillin. Plasmid pOS145 was constructed using the vector pBR322 and used to convert the plasmids pOS145. The minicells were labeled at 37°C with 5 μc of [35S]methionine for 45 min. Labeling minicells was performed as described (2).

**Preparation of minicell lysates**

**pJK19** and **pJK20** are plasmids which do not contain the plasmid pOS145 which contains the alpha polypeptide (alpha). To construct the plasmid pJK19, plasmid pOS145 was digested using EcoRI, which cuts twice in the alpha polypeptide (alpha) and confined to the alpha cistron. The regions of the plasmids were constructed using the vector pBR322 and confer resistance to ampicillin. Plasmid pOS145 was constructed using the vector pBR322 and used to convert the plasmids pOS145. The minicells were labeled at 37°C with 5 μc of [35S]methionine for 45 min. Labeling minicells was performed as described (2).

**Preparation of minicell lysates**

**pJK19** and **pJK20** are plasmids which do not contain the plasmid pOS145 which contains the alpha polypeptide (alpha) which is the component of the alpha polypeptide (alpha). To construct the plasmid pJK19, plasmid pOS145 was digested using EcoRI, which cuts twice in the alpha polypeptide (alpha) and confined to the alpha cistron. The regions of the plasmids were constructed using the vector pBR322 and confer resistance to ampicillin. Plasmid pOS145 was constructed using the vector pBR322 and used to convert the plasmids pOS145. The minicells were labeled at 37°C with 5 μc of [35S]methionine for 45 min. Labeling minicells was performed as described (2).

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