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Characterization of the Purified Membrane Attachment (δ) Subunit of the Proton Translocating Adenosine Triphosphatase from *Escherichia coli*†

Paul C. Sternweis* and Jeffrey B. Smith

ABSTRACT: Some of the physical and functional characteristics of the purified δ subunit obtained from the proton-translocating ATPase of *Escherichia coli* (ECF_1) have been examined. The subunit has a molecular weight of about 18 500 as measured by sodium dodecyl sulfate electrophoresis and by sedimentation equilibrium either with or without 6 M guanidine hydrochloride. δ therefore exists as a monomer and the apparent high molecular weight of about 33 000 obtained from molecular sieve chromatography suggests that the protein is a rather elongated molecule with a calculated f/f_0 of 1.4. Circular dichroism spectra indicate that δ has a high degree of secondary structure with an α-helix content of about 55-70%. The amino acid composition was determined. δ attaches δ-deficient ECF_1 to inverted membrane vesicles depleted of ECF_1 and restores oxidative phosphorylation. About 1 part δ by weight in the presence of excess ECF_1 lacking δ reconstituted depleted membranes to the same extent as 20 parts of completely reconstitutive ECF_1. This indicates that only one δ of mol wt 18 500 is needed per functional ECF_1 molecule of mol wt 370 000. δ associates rapidly with δ-deficient enzyme to yield a stable five-subunit complex which was separated from excess δ by molecular sieve chromatography and was fully active in reconstituting depleted membranes. δ has no effect on activities in depleted or partially reconstituted membrane vesicles and binds only poorly, if at all, to these membranes. It appears then that reconstitution with δ is an ordered process in which δ first combines with δ-deficient ECF_1 to yield a complex which then binds to membranes.

The Mg^{2+}-dependent ATPase located in the cytoplasmic membrane of *Escherichia coli* is the catalytic unit which reversibly transduces the energy between an electrochemical gradient and ATP.[1] The complete enzyme is composed of several nonidentical polypeptides which belong to one or the other of two morphologically and biochemically distinct portions. The portion designated F_0, which is intrinsic to the membrane, acts as a proton ionophore and a receptor for the other portion referred to as F_1. The F_1 portion, which is peripheral to the membrane, contains the catalytic site as well as the high affinity binding sites for nucleotides. F_1 dissociates readily from the F_0 in the membrane as a water-soluble protein. The catalytic and structural features of the F_0-F_1 complex from *E. coli* are remarkably similar to those found for the proton-pump ATPases of mitochondria, chloroplasts, and other bacteria (see reviews by Pedersen, 1975; Racker, 1976; Harold, 1977).

The purified F_1 portion of the ATPase contains five different polypeptides (α, β, γ, δ, and ϵ) which, in the case of *E. coli*, range in size from about 60 000 for the α subunit (Bragg and Hou, 1972) to 16 000 for ϵ (Smith and Sternweis, 1977). The authenticity of each of these polypeptides as an F_1 subunit is suggested by their occurrence in all preparations of F_1 capable of reconstituting a variety of energy transducing reactions catalyzed by the complete F_0-F_1 complexes. However, definitive evidence requires one to show that each polypeptide is indeed a functional unit of F_1. This is especially critical when considering the two smaller F_1 subunits (δ and ϵ) since they represent a relatively minor amount of the total F_1 molecule, which is comprised mostly of α and β chains. ϵ is believed to

† From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853. *Received April 4, 1977.* This work was supported by National Science Foundation Grant No. BMS 75-20287, Grant No. AM-11789 from the National Institutes of Health, and a National Research Service Award (IF 32GM02419-01) to J.B.S.

[1] Abbreviations used are: ATP, adenosine triphosphate; F_0, the portion of the proton-translocating ATPase which is an integral component of the membrane; F_1, the portion of the proton-translocating ATPase which is peripheral to the membrane; α, β, γ, δ, ϵ, separate polypeptides of F_1 molecules in order of decreasing size; CF_1, F_1 from chloroplasts; ECF_1, F_1 from *E. coli;* 4-subunit F_1, F_1 containing only the α, β, γ, and ϵ subunits; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NADH, reduced nicotinamide adenine dinucleotide; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

be a regulatory subunit since it was shown to be an inhibitor of ATPase activity in CF_1 (Nelson et al., 1972) and ECF_1 (Smith and Sternweis, 1977).

The first evidence suggesting that δ was a functional component of the ATPase was obtained with a F_1 from *E. coli* which was missing this subunit. Enzyme containing α, β, γ, and ε polypeptides did not reconstitute energy transducing reactions in membranes depleted of F_1 (Bragg et al., 1973). Subsequently, it was shown that the enzyme lacking δ did not even bind to F_1-depleted membranes (Futai et al., 1974), a result which also holds for δ-deficient F_1 from *Streptococcus faecalis* (Abrams et al., 1976a) and chloroplasts (Younis et al., 1977). This result suggested that δ was an attachment factor. The suggestion was confirmed when a preparation of δ free of other ATPase subunits was obtained after a pyridine treatment of ECF_1 and found to have the capability of completely restoring membrane attachment and energy coupling activities to the δ-deficient enzyme (Smith et al., 1975). δ subunits have recently been purified to apparent homogeneity from CF_1 and ECF_1 and shown to restore full coupling activity to δ-deficient enzyme from chloroplasts (Nelson and Karny, 1976) and bacteria (Smith and Sternweis, 1977). The functional necessity of this subunit has also been confirmed by reconstitution work using isolated subunits of the F_1 from a thermophilic bacterium. All five subunits including δ were needed to restore ATP-stimulated proton translocation in liposomes formed with F_0 and lipid (Kagawa, 1976).

Previously, we reported the purification of the δ and ε subunits of *E. coli* and some of the activities associated with these subunits (Smith and Sternweis, 1977). Here we describe the physical characterization of the δ subunit and some evidence for its mechanism and location in the pathway for the assembly of ECF_1 from subunits in vitro. The characterization of the purified ε subunit of ECF_1 will be reported in a subsequent paper.

Experimental Procedure

Preparations. Both the ML308-225 and K12(λ) strains of *E. coli* were grown in minimal media (Tanaka et al., 1967) supplemented with 1 μg/mL thiamin and 1% glycerol with the pH maintained at about 7 by the continuous addition of sodium hydroxide during growth. Cells were harvested in the late log phase of growth and stored at −90 °C.

Five-subunit ECF_1 was prepared from strain ML308-225 as previously described (Smith and Sternweis, 1977) by a modified procedure of Futai et al. (1974). Four-subunit ECF_1 (lacking δ) was obtained from K12(λ) grown on minimal media as described above and the same preparative procedure. Often, total loss of δ occurred during purification from this strain. Any δ retained by the purified enzyme was removed by molecular sieve chromatography at pH 9.4 as previously described for five-subunit preparations of the same strain (Smith et al., 1975). By contrast, the ML strain always produces five-subunit enzyme and shows only partial stripping of δ from the complex when subjected to the alkaline gel chromatography. This apparent difference in the affinities of δ for ECF_1 exhibited from the two strains provides a convenient way of preparing the two different forms of enzyme. However, it also points out the caution with which procedures should be applied to different strains when preparing specific forms of the enzyme and may explain variable or unsuccessful attempts to purify reconstitutive F_1 from certain strains of *E. coli*.

Inverted membrane vesicles were prepared from the ML308-225 strain either as previously described (Futai et al., 1974) or by the method of Hertzberg and Hinkle (1974). Vesicles were depleted of ECF_1 as described (Futai et al., 1974) with the exception that the 1-h incubation in the releasing medium was at 2 °C rather than room temperature.

Analytical Methods. The assay of ATPase (Futai et al., 1974), oxidative phosphorylation (Hertzberg and Hinkle, 1974), ATP-coupled transhydrogenase (Smith and Sternweis, 1977), protein (Lowry et al., 1951), and Ouchterlony immunodiffusion (Ouchterlony, 1968) were performed by published procedures. In the case of the δ subunit, protein determined by Lowry was corrected by a factor determined from amino acid analysis.

Amino acid analysis was performed with a Beckman Model 120C analyzer at high sensitivity. About 100-μg samples were hydrolyzed with 3 N mercaptoethanesulfonic acid (Pierce) at 110 °C for varying times (Penke et al., 1974). Cysteine content was determined as cysteic acid following the performic acid oxidation procedure described by Moore (1963).

To prepare antibodies to the purified δ subunit, about 40–60 μg of the protein was sonicated with 0.5–0.7 mL of Freund's Bacto-complete adjuvent (Difco Laboratories) and injected into the leg muscles of a rabbit. Five injections, spaced 4–8 days apart, were made. Serum drawn before the last two injections was tested for antibody levels and a final bleeding for antibody serum was performed 8 days after the last injection.

Sedimentation equilibrium of δ was carried out with a Beckman Model E ultracentrifuge equipped with absorption optics and a computer linked scanning system for data collection and analysis (Crepeau et al., 1974). Purified δ was run in either 100 mM potassium phosphate buffer (pH 7.0) and 0.1 mM EDTA or the same buffer but with 6 M guanidine hydrochloride added. Protein distribution in Yphantis cells was measured using absorption at 280 nm after overnight equilibration at 40 000 rpm and 4 °C.

Results

The δ subunit was purified from ML 308 ATPase by the pyridine treatment described previously (Smith and Sternweis, 1977). On a small scale, pure δ could be obtained after a single G-75 Sephadex column. However, quantitative collection of δ peaks from several preparations and larger scale preparations contained some impurities. These could be removed by rerunning the combined peaks through the G-75 column. The combined subunit preparations were judged pure since they gave only a single band on sodium dodecyl sulfate gel electrophoresis and produced only a single precipitin line by Ouchterlony double diffusion with δ antisera.

Molecular Weight. Our purification of the δ subunit employs molecular sieve chromatography. Though δ shows a molecular weight on sodium dodecyl sulfate gels only slightly larger than that of ε, 18 500 vs. 16 000, the subunit elutes from the G-75 column with an apparent molecular weight of about 33 000 well resolved from the ε which elutes with its expected molecular weight of 16 000. In Table I, we show that the true molecular weight of δ is about 18 500. Polyacrylamide gel electrophoresis under reducing and denaturing conditions by the sodium dodecyl sulfate method of Weber and Osborn (1969) and the sodium dodecyl sulfate-urea method of Swank and Munkres (1971) yielded molecular weights of 18 300 and 18 800, respectively.

To find out if δ was a dimer in its native state, its molecular weight was determined by sedimentation equilibrium in the analytical ultracentrifuge. Linear plots of log OD (optical density) vs. r^2 indicated a single molecular weight species when δ was examined in either its native, active form or in the presence of 6 M guanidine hydrochloride. Table I shows that both conditions yielded essentially identical molecular weights of about 18 000. This demonstrates that active δ is a monomer

TABLE I: Molecular Weight of the δ Subunit.

Method	Mol wt	Trials	Av error
G-75 Sephadex[a]	33 000	3	400
$NaDodSO_4$ gel electrophoresis[b]			
Weber-Osborn	18 300	3	700
Swank-Munkres ($NaDodSO_4$-urea)	18 800	1	
Sedimentation equilibrium[c]			
In phosphate buffer	18 500	4	1300
In phosphate buffer + 6 M guanidine hydrochloride	17 600	2	2000

[a] Described previously (Smith and Sternweis, 1977). [b] The methods described by Weber and Osborn (1969) and Swank and Munkres (1971) were adapted for use with 1-mm slab gels. Results were obtained with gels formed from 12.5% acrylamide and either 0.4 or 0.8% *N,N'*-methylenebisacrylamide for the straight sodium dodecyl sulfate or sodium dodecyl sulfate-urea systems, respectively. [c] Method described in Experimental Procedure. The partial specific volume was calculated from the amino acid composition (Table II) by the method described by Cohn and Edsall (1943). The calculated value of 0.736 $cm^3\ g^{-1}$ was used for measurements in guanidine hydrochloride as well as for measurements of the native subunit.

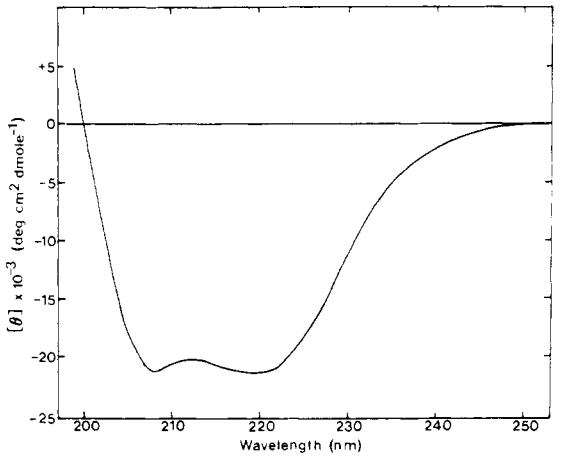

FIGURE 1: Circular dichroism spectrum of δ. This spectrum was measured on a Cary 60 recording spectropolarimeter with circular dichroic (CD) accessory at a protein concentration of 0.77 mg/mL and a cell path length of 1 mm.

and that the higher molecular weight obtained by gel chromatography is probably due to deviation from a standard spherical shape. The Stoke's radius for δ, calculated from a calibrated G-75 column, is about 25 Å as compared to a calculated minimum radius of about 18 Å if δ was assumed to be a perfect sphere.

Optical Properties. The ultraviolet absorption spectrum of δ is typical of proteins containing tryptophan as the dominant chromophore. It exhibited a broad maximum from 275 to 280 nm and a shoulder at about 290 nm. However, its extinction coefficient, $E_{1cm}^{1\%}$, at 280 nm was calculated to be only about 3.4, a rather low value.

Fluorescence spectra of δ confirmed the presence of tryptophan. An emission peak with a maximum at 328 nm was obtained when the purified subunit was excited at either 270 or 285 nm.

Figure 1 shows a circular dichroism spectrum of the subunit. The spectrum is defined by two rotational minima of about equal intensity at 208 and 220 nm, a qualitative property characteristic of the rotation produced by α-helical structure.

TABLE II: Amino Acid Analysis of the δ Subunit of ECF_1.

Amino acid	mol %	Suggested residue composition/18 300 mol wt
Trp[a]	0.67	1
Lys	4.70	8
His	1.18	2
Arg	5.99	10
Asp	8.73	15
Thr	2.42	4
Ser	7.34	12
Glu	14.94	25
Pro	2.44	4
Gly	5.33	9
Ala	14.87	25
Cys[b]	1.26	2
Val	8.18	14
Met	3.36	6
Ile	4.73	8
Leu	9.66	16
Tyr	0.81	1
Phe	3.33	6
		168
% nonpolar	47.6	

[a] The mole percent reported here is that determined directly from the amino acid analysis. Interpretation of alkaline UV spectra by the method of Goodwin and Morton (1946) predicts that tryptophan and tyrosine are present in equimolar amounts of about one each per subunit. [b] Cysteine was determined as cysteic acid in performic acid oxidized samples (see Experimental Procedure).

The amplitude of the minima also suggests that δ contains a large amount of α helix. Analysis of the spectrum by a method described by Chen et al. (1974) predicts a composition of 55–70% α helix, the range depending on whether infinite or short-length α helices are assumed.

Amino Acid Content. The amino acid composition of δ is shown in Table II. The presence of tryptophan, predicted by δ's spectroscopic properties, was confirmed. Both this analysis and interpretation of ultraviolet (UV) spectra by the method of Goodwin and Morton (1946) suggest that there is one tryptophan per δ molecule. This contrasts with the apparent absence of tryptophan found for the whole F_1 molecules of mitochondria (Penefsky and Warner, 1965) and chloroplasts (Farron, 1970). The presence of two cysteine residues in each δ subunit was also somewhat unexpected as no cysteine has been reported for the δ subunits of the mitochondrial or chloroplast F_1.

Reconstitution of Oxidative Phosphorylation. Figure 2 demonstrates that δ restores the ability of δ-deficient ECF_1 to reconstitute oxidative phosphorylation in inverted vesicles depleted of ECF_1. This complements previous results (Smith and Sternweis, 1977) which show restoration of coupling in the opposing direction by measurement of ATP-dependent transhydrogenase. The maximum extent of coupled phosphorylation obtained here was the same when either δ and four-subunit ECF_1 (●) or five-subunit ECF_1 (○) were used for reconstitution. In both cases, the restored phosphorylation was completely sensitive to the uncoupler CCCP. Reconstitution with four-subunit ECF_1 or five-subunit enzyme had a sigmoidal dependence on the amount of δ or ECF_1, respectively, as expected for the known bifunctional role of the F_1 as an inhibitor of proton leakage through F_0 and as the unit catalyzing phosphorylation. Hence, the efficiency of phosphorylation presumably increases as more ECF_1 becomes attached. The sigmoidal response is not due to cooperative binding of F_1 to F_0 since binding showed a linear dependence on δ (Smith and Sternweis, 1977). The reconstitution of

TABLE III: Effect of Purified Subunits on Oxidative Phosphorylation.

Additions[a]	P/O: ECF_1-depleted memb. ves.	P/O: ECF_1-depleted memb. ves. + 10 μg of ECF_1[b]	P/O: Nondepleted memb. ves.
1. None	<0.01	0.07	0.21
2. δ (4.8 μg)		0.08	0.23
3. ε (6.0 μg)		0.07	0.23
4. δ (4.8 μg) + ε (6.0 μg)	<0.01	0.07	0.24
5. δ (2.5 μg) + ε (3.0 μg) + four-subunit ATPase (30 μg)	0.31	0.38	0.41
6. Additions as in 5 + 8.3 μM CCCP		<0.01	

[a] Membrane vesicles (0.92 mg of protein) or depleted membrane vesicles (0.68 mg of protein) were mixed with the indicated amount of purified subunits and $MgCl_2$ to a final concentration of 10 mM and incubated for 10 min at 37 °C and 20 min at room temperature before the assay of oxidative phosphorylation as described in Figure 3. Where indicated, four-subunit enzyme was added to the mixture of membranes and subunits after the first incubation at 37 °C. Respiration rates averaged about 390 nmol of O min^{-1} mg^{-1} and 370 nmol of O min^{-1} mg^{-1} for depleted membrane and whole membrane samples, respectively, with deviations of no greater than 25%; 600–650 nmol of total O was used for each assay causing the formation of up to a maximum of 260 nmol of ATP. [b] Depleted membrane vesicles were partially reconstituted with ECF_1 before treatment with the purified subunits.

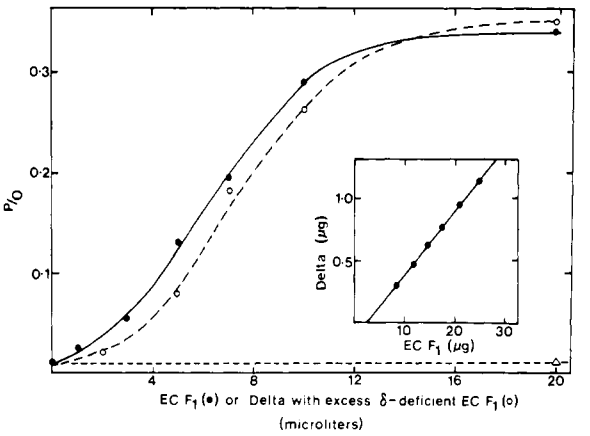

FIGURE 2: Reconstitution of oxidative phosphorylation. Depleted membrane vesicles (0.68 mg of protein) were mixed with either a saturating amount of δ-deficient ECF_1 and increasing amounts of purified δ (●) or increasing amounts of complete ECF_1 (○). The magnesium concentration was adjusted to 10 mM and the samples were incubated on ice for 1–3 h. Oxidative phosphorylation was then assayed as described by Hertzberg and Hinkle (1974) using NADH as the respiratory substrate but in the absence of BSA. Respiration rates of 185–350 nmol of O min^{-1} mg^{-1} were observed. The lower rates appear at longer incubation times in the Tris, EDTA, and dithiothreitol buffers which are added with the subunit and ATPase. Added Mg^{2+} is needed to maintain these rates and the problem can be further alleviated by shortening the preincubation time and using higher temperatures as in Table III. About 600 nmol of O was consumed in each assay. The respiratory rate had no apparent effect on coupled phosphorylation. Complete sensitivity to the uncoupler CCCP at 8.3 μM (△) was still observed in the slowest assays. Insert: At chosen P/O ratios, the amounts of δ needed in the presence of saturating four-subunit ECF_1 are plotted against the amount of five-subunit ECF_1 needed. The plotted points cover a P/O range of 0.05 to 0.30 at 0.05 intervals.

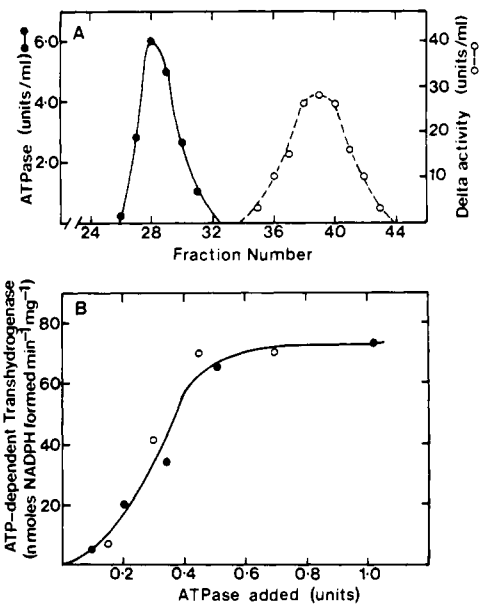

FIGURE 3: Complex formation between δ and four-subunit ECF_1. (A) Elution profile from G-75 Sephadex. About 40 units of ECF_1 devoid of δ and about 70 μg of pure δ were mixed, incubated, and passed through the column as described under Results. (B) Reconstitution of ATP-dependent transhydrogenase in depleted membrane vesicles (0.16 mg of protein) with enzyme from part A (○) or with four-subunit enzyme and an excess of pure δ (●).

ATP-driven transhydrogenase had a similar sigmoidal response curve (Smith and Sternweis, 1977).

The insert to Figure 2 compares the amount of δ in the presence of saturating four-subunit ECF_1 and the amount of five-subunit ECF_1 which result in the same extent of restored coupling as measured by the P/O ratio. The slope of the line is the weight ratio of δ to ECF_1 which gave stoichiometric reconstitution. Thus, 1 part of δ is required per 20 parts of ECF_1. As δ has a molecular weight of 18 500, it follows that 1 δ is sufficient for a completely functional ECF_1 of mol wt 370 000. This molecular weight for the enzyme is comparable to previously determined values (Davies and Bragg, 1972; Hanson and Kennedy, 1973) and indicates that, in *E. coli*, there is only 1 δ subunit per F_1 molecule. To support this result, the five-subunit ECF_1 used for this experiment was shown to be completely reconstitutive by virtue of its complete binding to depleted membranes by itself and its associated insensitivity to stimulation by added δ during reconstitution. That the line in the insert does not go through the origin indicates that the added four-subunit ECF_1 has a slight reconstituting effect. This can be calculated to be due to less than 5% of the enzyme and is probably due to a small amount of five-subunit ECF_1 in the δ-deficient enzyme.

Table III illustrates that δ has no effect on coupling by itself. Previously (Smith and Sternweis, 1975), it was shown that a fraction containing chiefly δ and ε was not capable of restoring aerobic transhydrogenase activity in depleted membrane vesicles or of reconstituting oxidative phosphorylation. Table III (column 1) shows the same result for the purified δ plus ε. Columns 2 and 3 demonstrate that the δ subunit had no effect on coupling in partially reconstituted particles. Though δ acts to attach the ATPase to the membrane, δ by itself neither fulfilled any detectable structural role nor competed effectively for membrane binding sites with bound ATPase or ATPase added during reconstitution (line 5). The inclusion of ε in this table illustrates that it also had no effect on oxidative phosphorylation or its reconstitution by δ plus four-subunit enzyme.

Complex Formation between δ and Four-Subunit ECF_1. Figure 3 shows that δ associates readily and tightly with δ-

deficient enzyme. ECF_1 lacking δ and an excess of the purified δ subunit were mixed in the same buffers in which they were purified. After a 10-min incubation at room temperature to ensure time for binding, this mixture was passed through the same G-75 column as was used to purify the δ subunit (part A). In this case, the column was equilibrated and eluted at 4 °C with buffer containing 40 mM Tris-HCl (pH 7.3), 1 mM EDTA, 0.1 mM ATP, 0.1 mM dithiothreitol, and 10% glycerol. A slightly smaller fraction size accounts for the apparent shift in the position of free δ peak. ATPase elutes with the excluded volume of the column and is clearly separated from an included peak containing free δ subunit. Part B of Figure 3 shows that the enzyme complex prepared in this manner is now capable of restoring coupled functioning to depleted membrane vesicles. Reconstitution of ATP-dependent transhydrogenase activity showed the same dependence on added ATPase using either the isolated complex (open circles) or δ-deficient enzyme with a large excess of δ. The latter should represent the maximum reconstitution possible.

To test that there was a stoichiometric complex formation between four-subunit ECF_1 and δ, 0.24 unit of the isolated complex was used to partially reconstitute depleted membrane vesicles. Either four-subunit ECF_1 or δ was added with the same amount of complex in duplicate reconstitutions. Neither of these extra additions stimulated reconstitution by the complex. This indicates that the isolated complex contained no measurable excess of either free δ or four-subunit enzyme.

Discussion

Results with the purified δ subunits from ECF_1 (Smith and Sternweis, 1977) and CF_1 (Nelson and Karny, 1976) clearly show that δ attaches F_1 to F_0 in the membrane. We refer to δ as the attachment protein because no function besides this structural one has been found for it in the isolated F_1. The absence of δ has no effect on the ATPase activity of the F_1 or its sensitivity to the inhibitory subunit (Smith and Sternweis, 1977). Furthermore, there is no requirement for δ in reconstituting ATPase activity from inactive dissociated subunits (Vogel and Steinhart, 1976; Kagawa, 1976).

Recently, Abrams et al. (1976b) proposed that the α subunits are directly involved in attaching ATPase to the membrane. Treatment of the *S. faecalis* F_1 with chymotrypsin selectively removed a small segment from the α subunit without altering the catalytic activity of the enzyme. This altered enzyme no longer bound to depleted membranes, even in the presence of added δ activity. Although the δ polypeptide was still present after the protease treatment, as seen by sodium dodecyl sulfate gels, it may no longer be bound to the rest of the F_1 molecule. Thus, α may be indirectly involved in membrane attachment by containing a site which binds δ to the rest of F_1. The close proximity of α and δ is indicated by their ability to cross-link to each other in ECF_1 (Bragg and Hou, 1976) and CF_1 (Baird and Hammes, 1976).

As δ is an absolute requirement for F_1-F_0 interaction, it has been proposed to be part of the stalk region seen in electron micrographs of membrane-bound F_1 (Younis et al., 1977; Smith et al., 1976). As the connection between F_1 and F_0, δ would act to transmit the energy of an electrochemical gradient to the catalytic sites either as a conformational transmission or as a channel for protons (Smith and Sternweis, 1977; Nelson and Karny, 1976). In this view, δ might be able to interact with F_0 by itself. We checked for effects of the δ subunit by itself on coupled activities in membrane vesicles. Free δ had no detectable effect on proton permeability through F_0 (no F_1 bound to it) as measured by the efficiency of oxidative phosphorylation in partially reconstituted membrane vesicles (Table III). Further, the subunit did not seem to compete effectively with F_1 for F_0 sites during reconstitution (Table III, Figure 3B). As F_1 would probably be a much better competitor for F_0 than δ, we also looked directly for binding of δ to ECF_1-depleted membranes (data not shown). Under a variety of conditions, including those normally used for reconstitution, some binding was observed but it did not exceed 25% of what would be needed to saturate the F_0 sites on the membrane. We also have no clear indication that this small amount of binding is specific for F_0. Yoshida et al. (1977) have reported that the δ subunit from the thermophile F_1 binds to F_0 liposomes. However, this binding has also not been shown to be specific for F_0. Due to the importance of this question in describing a complete role for δ, we are currently pursuing this binding and its specificity with more sensitive techniques. However, the current results indicate that in our reconstitution systems, δ by itself binds only weakly to the membrane F_0, if at all.

Opposing this lack of response to F_0, δ binds very tightly to δ-deficient ECF_1 (Figure 3). In view of this information, we propose that the δ reconstitution in our system is an ordered two-step process as follows:

$$\delta + ECF_1 \text{ (no } \delta) \underset{\text{pH 9}}{\overset{\text{pH 7}}{\rightleftharpoons}} ECF_1\text{-}\delta \quad (1)$$

$$ECF_1\text{-}\delta + F_0 \text{ (memb)} \underset{\text{EDTA}}{\overset{Mg^{2+}}{\rightleftharpoons}} ECF_1\text{-}\delta\text{-}F_0 \text{ (memb.)} \quad (2)$$

In this scheme the formation of a five-subunit enzyme is an obligatory reaction preceding binding to the membrane and the restoration of energy coupling. Though Mg^{2+} plays a role in attaching ECF_1 to F_0 in the membrane (step 2), no exogenous Mg^{2+} is required to bind δ to the rest of ECF_1 (step 1). It also seems unlikely that endogenous Mg^{2+} is involved in the binding of δ to ECF_1 as it would have had to survive purification of both the δ and δ-deficient enzyme in the presence of EDTA.

The high molecular weight observed for δ by gel chromatography as opposed to sodium dodecyl sulfate electrophoresis has also been observed for δ activity in a preparation called nectin from *S. faecalis* (Abrams et al., 1976b; Baron and Abrams, 1971). Though nectin was proposed to be a dimer (Abrams et al., 1976a), it will be of interest to see whether purified δ from *S. faecalis* proves to be a monomer with an elongated shape like the δ subunit from *E. coli*.

δ contains a rather large proportion of α helix (55–70%), a property also found for the δ subunit of the thermophile F_1 (Y. Kagawa, personal communication). The significance of this unusual property is not clear. Its purpose may be something as simple as providing structural rigidity to the protein to aid in its function as an attachment structure. Alternatively, as δ has been suggested to be the connection between F_0 and F_1, the α-helical structure may fulfill a role in energy transduction by conducting a conformational change or proton movement between F_1 and F_0.

For several years, there has been some controversy over the stoichiometry of the subunits in F_1. Attempts to quantitate the subunits using F_1 purified from bacteria grown with radioactive amino acids have yielded a stoichiometry of only about 0.5 δ per F_1 molecule obtained from either *E. coli* (Bragg and Hou, 1975) or the thermophile, PS3 (Kagawa et al., 1976). However, an F_1-F_0 complex was also isolated from the thermophile and yielded a stoichiometry close to one. These fractional stoichiometries are not surprising in view of the ease with which δ is lost during purification. Results obtained by reconstitution in this paper clearly indicate a stoichiometry of 1 δ per ECF_1 molecule.

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