Purification of Membrane Attachment and Inhibitory Subunits of the Proton Translocating Adenosine Triphosphatase from *Escherichia coli*†

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ABSTRACT: The portion of *Escherichia coli* adenosine triphosphatase (ATPase) which is peripheral to the membrane (ECF_1) is composed of five separate polypeptides referred to as α, β, γ, δ, and ϵ. Treating purified ECF_1 with pyridine precipitated the three larger polypeptides (α, β, and γ), but the two smaller ones (δ and ϵ), which represent only about 10% of ECF_1, remained in solution. After removing the pyridine, both δ and ϵ were active and both were obtained in essentially pure form after chromatography on a single molecular-seive column. ϵ strongly inhibited the ATPase activity of ECF_1, indicating that ϵ has a regulatory role in the enzyme. ϵ inhibited ECF_1 missing δ, indicating that δ is not required for inhibition by ϵ. However, enzyme containing just the α and β subunits, which was prepared by treating ECF_1 with a protease, was fully active hydrolytically but not at all sensitive to inhibition by ϵ. This result suggests that the γ polypeptide is required for the inhibition of the ATPase by ϵ. δ restored the capacity of ECF_1 missing δ to recombine with ECF_1-depleted membrane vesicles. The ECF_1, which became attached to the vesicles by the added δ, was functional in energy transduction, as evidenced by the coupling of ATP hydrolysis to the transhydrogenase reaction in the vesicles. The rebinding of ECF_1 missing δ was directly proportional to the amount of δ added until all the ECF_1 receptors in the membranes were occupied. δ may be a stalk which connects the F_1 headpiece to the membrane, since the attachment of ECF_1 to the membrane exhibited an absolute dependence on δ. Although δ is known to have an apparent molecular weight of about 20 000 by gel electrophoresis in the presence of sodium dodecyl sulfate, the active δ eluted from a molecular-seive column with an apparent molecular weight of about 35 000, suggesting that in the active form δ is a dimer or rather elongated in shape. The active ϵ subunit eluted from the same column with an apparent molecular weight of about 16 000.

The membranes of bacteria, mitochondria, and chloroplasts contain a Mg^{2+}-dependent ATPase[1] that reversibly translocates protons in accordance with the chemiosmotic hypothesis of Mitchell (1961). All of the proton translocating ATPases which have been examined have been found to have remarkably similar catalytic and structural properties and appearance under the electron microscope. A highly distinctive feature of this type of ATPase is that it consists of two kinds of subunits which differ greatly with respect to their level of integration with the phospholipid bilayer of the membrane. One class of subunits compose a lipoprotein complex called F_0, which is an intrinsic part of the membrane. The other class of subunits compose a portion of the enzyme referred to as F_1, which is rather peripheral to the membrane proper and becomes soluble in water once it has been detached from F_0. The F_1 portion of the enzyme catalyzes the practically irreversible hydrolysis of ATP, which is in contrast to the ATPase activity of the complete F_0F_1 complex which is reversible. Negatively stained F_1 has the unusual appearance in electron micrographs of knobs that project from one side of the membrane via a stalk. Besides being a specific receptor for F_1, F_0 contains a polypeptide that reacts covalently with dicyclohexylcarbodiimide, which selectively inhibits only the membrane-bound ATPase and inactivates the proton-ionophore activity of F_0 (see reviews by Mitchell and Moyle, 1974; Racker, 1970; Beechey, 1974; Pedersen, 1975; Abrams and Smith, 1974).

Currently, we are investigating the subunits of the *E. coli* F_1 by separating them from one another in order to study their individual properties and interactions with the rest of the F_1 molecule. Highly purified F_1 from *E. coli* contains five separate polypeptides (α, β, γ, δ, and ϵ) when examined by gel electrophoresis in the presence of sodium dodecyl sulfate (Bragg and Hou, 1972). While the two larger polypeptides (α and β) are apparently sufficient for the expression of the hydrolytic activity of the enzyme (Nelson et al., 1974), only ECF_1 preparations that contain all five of the polypeptides have been found to be capable of carrying out the energy-transducing reactions associated with the membrane vesicles (Bragg and Hou, 1972; Bragg et al., 1973). Preparations of the bacterial F_1 which are deficient in the δ polypeptide have been found to be unable to recombine with F_0, suggesting that δ is required for attaching F_1 to the membrane (Futai et al., 1974; Abrams et al., 1976). Moreover, we recently reported that impure δ isolated from the *E. coli* F_1 restored the capacity of F_1 missing δ to recombine with F_0 and to function in membrane energy transductions (Smith and Sternweis, 1976; Smith et al., 1975).

Now, the δ polypeptide of the *E. coli* ATPase has been purified to homogeneity. Experiments with the homogeneous δ show conclusively for the first time that this subunit has a structural function, since it attaches the ATPase to the membrane. The purification to homogeneity of the smallest or ϵ polypeptide of the *E. coli* F_1 was also achieved. ϵ has a regulatory function, since it is a potent inhibitor of the hydrolytic

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[1] Abbreviations used are: F_1, the portion of the proton-translocating ATPase which is peripheral to the membrane; ECF_1, F_1 from *E. coli*; F_0, the portion of the proton-translocating ATPase that is an integral component of the membrane; α, β, γ, δ, ϵ, separate polypeptides of ECF_1 in order of decreasing size; DTT, dithiothreitol; ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; EDTA, (ethylenedinitrilo)tetraacetic acid.

activity of ECF_1 as suggested by previous results (Smith et al., 1975). While ϵ is homologous to the known inhibitory subunits of the mitochondrial and the chloroplast F_1 (Pullman and Monroy, 1963; Nelson et al., 1972), this is the first time that an inhibitory subunit has been obtained in pure form from a bacterial proton-translocating ATPase.

Materials and Methods

Bacteria and Growth. The ML308-225 strain of *E. coli* was grown in minimal medium (Tanaka et al., 1967) supplemented with 1 μg/ml of thiamine and 1% glycerol with the pH maintained at about 7 by the continuous addition of sodium hydroxide during growth. *E. coli* K12 (λ) was grown in a medium containing 3% casein hydrolysate, 1.5% yeast extract, 1.5% NaCl, 0.3% K_2HPO_4, and 0.07% KH_2PO_4. Cells were harvested during the late-log phase of growth and stored at −90 °C.

Enzyme and Membrane Preparations. Five-subunit ECF_1 from either strain was prepared by a modification of the procedure of Futai et al. (1974). About 200 g (wet weight) of frozen cells was washed once in 5 l. of 10 mM Tris-HCl (pH 8.0) and suspended in 500 ml of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and passed twice through a Gaulin Laboratory Homogenizer at about 8000 lbs of pressure. Cell debris was removed by centrifugation at 8000*g* for 10 min, and the supernatant was centrifuged at 70 000*g* for 2 h to collect the membrane fraction. To wash the membranes, they were suspended in 500 ml of 10 mM Tris-SO_4, pH 7.5, 10 mM EDTA, and 10% glycerol, and recentrifuged under the same conditions. ECF_1 was either immediately released from the washed membranes by extraction with 200 ml of 1 mM Tris-HCl, 0.5 mM EDTA, and 10% glycerol (pH 7.5) at room temperature for 1 h or the washed membranes were stored at −90 °C as a thick suspension in the buffer used for extracting ECF_1 from the membranes. After removing the enzyme-depleted membranes by centrifugation at 70 000*g* for 2.5 h, the solubilized ECF_1 in the supernatant was purified as previously described (Futai et al., 1974).

The δ polypeptide was removed from ECF_1 purified from the K12 (λ) strain by molecular-seive chromatography at pH 9.4 as previously described (Smith et al., 1975). ECF_1 missing δ is sometimes referred to as four-subunit enzyme, since it contains only the α, β, γ, and ϵ peptides. Inverted membrane vesicles were prepared from ML308-225 and depleted of ECF_1 as previously described (Futai et al., 1974).

Assay of ATP-Coupled Transhydrogenase. The energy-dependent reduction of $NADP^+$ by NADH was measured in the presence of cyanide, which blocks respiration. ATPase-depleted membranes (0.16 mg of protein) were incubated with ATPase lacking the δ subunit and δ fraction in a volume of 50 μl containing 50 mM Tris-Cl (pH 8) and 10 mM $MgCl_2$ for 10 min at 37 °C. One milliliter of a buffer containing 50 mM Tris-Cl (pH 8), 10 mM $MgCl_2$, 0.1 mM DDT, and 0.5% ethanol was added followed by 25 μl of 3.0 mM NAD^+, 50 μl of 16.3 mM $NADP^+$, and 10 μl of 1 M NaCN. After incubating for 15 min at room temperature, 50 μl of 4 mg/ml of alcohol dehydrogenase was added to start the reaction. Then the rate of increase in absorbance at 340 nm was recorded with a Gilford 2400 spectrophotometer. ATP-coupled transhydrogenase was measured as the increase in the rate of NADPH production after adding 10 μl of 60 mM NaATP, pH 7. One unit of δ activity is defined as tlhe amount of δ required to give a rate of NADPH reduction, which is half of the maximum rate obtained with an excess of the subunit.

ATPase Inhibitor. A sample of purified ECF_1 (0.01–0.02 unit) was incubated for 10 min at room temperature in 0.1 ml of 50 mM Tris-HCl, pH 8.0, containing 1.0 mM EDTA and 0.1 mM DTT with or without the ϵ subunit fraction as indicated. Then 0.2 ml of 2 mM Tris-HCl, pH 8, with 20 μg/ml of defatted bovine serum albumin was added, and the ATPase activity was assayed by adding 0.3 ml of a solution prewarmed to 37 °C containing 50 mM Tris-HCl, pH 8, 4.0 mM $MgCl_2$, and 8.0 mM NaATP. The bovine serum albumin was added to prevent the loss of ATPase activity which occurs when the enzyme is subjected to extreme dilution. After 10 min at 37 °C, 0.3 ml of 1 M trichloroacetic acid was added and the amount of inorganic phosphate liberated was determined by the method of Tansky and Shorr (1953). A unit of ϵ is the amount which half-maximally inhibits the enzyme under these conditions.

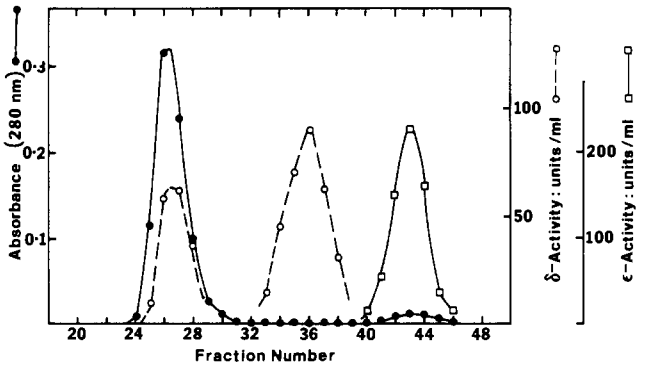

FIGURE 1: Elution of δ and ϵ from a Sephadex G-75 column. The units of δ and ϵ activity are defined under Methods in terms of the transhydrogenase assay for δ and ATPase inhibition for ϵ.

Protein was determined by the method of Lowry et al. (1951).

Results

Purification of the δ and ϵ Subunits. A fraction containing active δ and ϵ subunit of ECF_1 was prepared by treating the enzyme with pyridine as described previously (Smith and Sternweis, 1975). About 20 mg of purified enzyme from ML308-225 was precipitated by adding solid ammonium sulfate to 65% saturation. The precipitate was dissolved in 5 ml of 10 mM glycine–NaOH and 1 mM EDTA at pH 9.0 and 5 ml of pyridine was added with stirring at room temperature. The mixture was allowed to stir for 10 min at room temperature before being diluted with 15 ml of water. Then 26 μl of saturated ammonium sulfate was added and the suspension was incubated at 4 °C for 4–5 h. All of the following steps were performed at 4 °C. The protein, which was precipitated by the pyridine treatment, was removed by centrifugation at 20 000*g* for 15 min. The pyridine supernatant was dialyzed against 5 l. of 50 mM Tris-HCl, 1 mM EDTA, and 0.1 mM DTT at pH 8.0 overnight to remove the pyridine and then concentrated by ultrafiltration using a UM-10 filter (Amicon Corp.) to about 2 ml. Active δ and ϵ subunits were purified from this fraction by molecular-seive chromatography. The δ–ϵ fraction was applied to a Sephadex G-75 (Pharmacia) column (85 × 1.5 cm) which was equilibrated with 50 mM Tris-HCl, 1 mM EDTA, and 0.1 mM DTT at pH 8.0. The column was eluted with the same buffer at a flow rate of 8 ml/h. A representative elution profile is shown in Figure 1.

Most of the protein eluted in a single peak at or very near the void volume of the column. This peak contained nearly all of the contaminants of the δ–ϵ fraction, which included other ECF_1 subunits and some of the minor impurities in the purified

TABLE I: Purification of δ and ϵ Subunits of the Proton-Pump ATPase from *E. coli*.

Subunit	Total Protein (mg)	Total Act. (unit)[a,b]	Sp Act. (units/mg)	Purification (-fold)	Yield (%)
δ					
Pyridine supernatant	5.3	2210	420		
Sephadex G-75 column,					
void	2.9	340	120	0.3	15
included	0.1	660	7500	18	30
ϵ					
Pyridine supernatant	5.3	1700	320		
Sephadex G-75 column	0.2	1250	6250	20	75

[a] δ activity is defined in terms of its ability to restore coupling activity to enzyme lacking the δ subunit (see Methods). [b] One unit of ϵ is the amount required to half-maximally inhibit the hydrolytic activity of purified ECF_1 under these conditions specified under Methods.

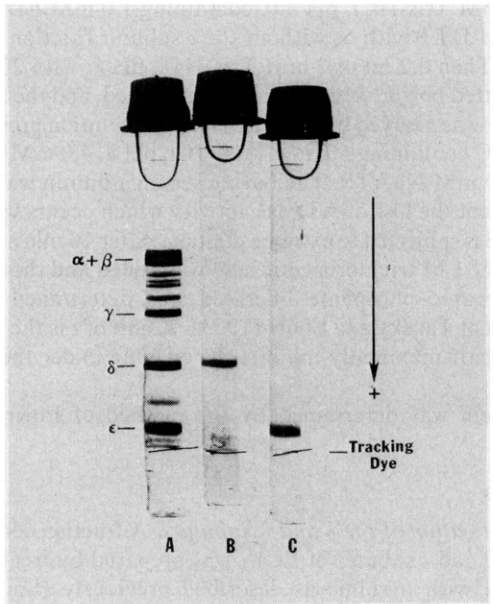

FIGURE 2: Discontinuous gel electrophoresis of the pyridine supernatant (A) and the purified δ (B) and ϵ (C) subunits. The samples were incubated in 1% sodium dodecyl sulfate and 2% β-mercaptoethanol for 3 min in a boiling water bath and electrophoresis was carried out for about 500-V h in 11% polyacrylamide gels according to the procedure of Laemmli (1970). Gels were stained with Coomassie blue. About 30, 3, and 4 μg of protein were applied to gels A, B, and C, respectively. The pin near the bottom of each gel indicates the position of the bromphenol blue tracking dye.

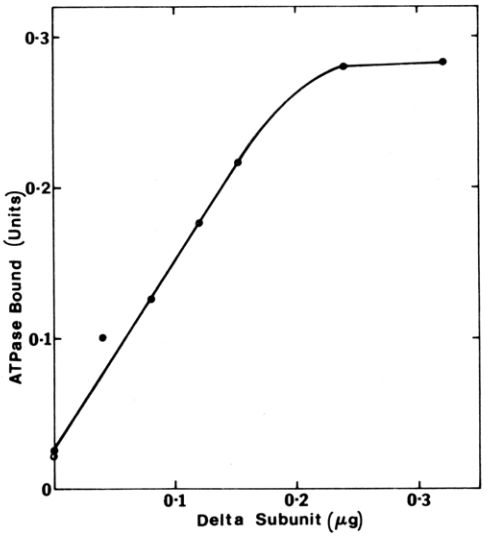

FIGURE 3: Restoration of binding capability to enzyme lacking δ by addition of the purified δ subunit. Depleted membranes (0.16 mg of protein) were mixed witlh a saturating amount of 4-subunit ATPase (~0.6 unit) and increasing amounts of the purified δ subunit and assayed for ATP-coupled transhydrogenase (Figure 4), as described under Methods. After completion of the assay, the membranes were collected by centrifugation at 75 000*g* for 30 min and suspended in TMG. The total amount of ATPase bound to the membranes was determined from the specific activity measured in these samples and the initial amount of membrane protein added to the assay. The open circle represents residual ATPase remaining in the membranes after extraction with EDTA. There was no significant binding of the ECF_1 missing δ in the absence of the added δ.

enzyme which were concentrated in the pyridine supernatant along with the two minor subunits.

δ eluted from the column (Figure 1) in two peaks. The first δ peak which eluted near the void volume is probably δ that is associated with itself or with other subunits of the enzyme. The aggregate appears to be partially dissociable during the δ assay, since we do not believe that aggregated δ would be active. Usually about half of the total δ activity applied to the molecular-seive column elutes at the void volume (Figure 1). The second peak was homogeneous δ, as judged by gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 2). The homogeneous δ eluted in a single peak in the same position after being concentrated and rechromatographed.

ϵ eluted in a single peak after the second peak of δ (Figure 1) and was homogeneous, as judged by gel electrophoresis in sodium dodecyl sulfate. Sodium dodecyl sulfate gels of the δ–ϵ fraction and the purified δ and ϵ subunits are shown in Figure 2.

The results of a typical purification of the δ and ϵ subunits are summarized in Table I. About 100–200 μg of each subunit was obtained from 20 mg of purified enzyme. Since each of the minor subunits represents only about 5% of the total protein of the enzyme, the overall yield of δ and ϵ is about 10–20% with most of the loss occurring in the pyridine precipitate. As shown previously, δ restores coupling-factor activity to ECF_1 missing δ, and ϵ inhibits the hydrolytic activity of the enzyme (Smith and Sternweis, 1975; Smith et al., 1975). The specific activities of the purified δ and ϵ are 20-fold greater than the activities in the dialyzed and concentrated pyridine supernatant (Table I).

Both subunits have also been purified by this procedure from the K12 (λ) strain of *E. coli*. From this strain, the ϵ subunit was

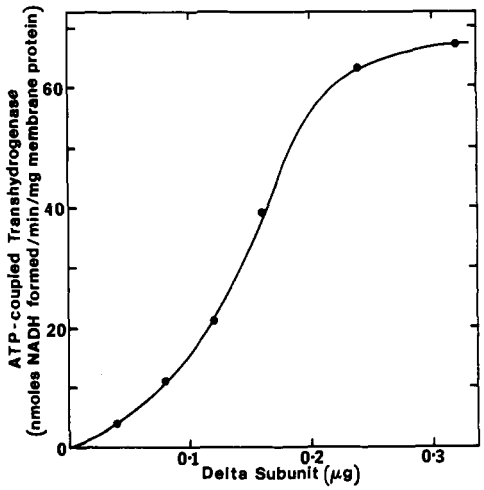

FIGURE 4: Restoration of ATP-dependent transhydrogenase in ECF_1-depleted vesicles with enzyme lacking δ and the purified δ subunit. Assays were performed with a saturating amount of 4-subunit ATPase and increasing amounts of the purified δ subunit as described in Figure 3 and Methods.

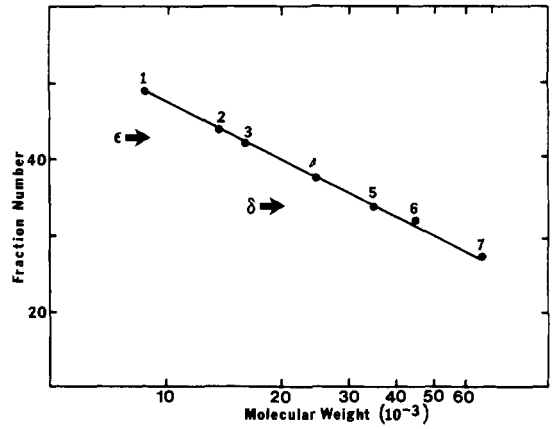

FIGURE 5: Estimation of the molecular weight of δ and ϵ by chromatography on a calibrated Sephadex G-75 column. The conditions are the same as in Figure 1. The column was calibrated with tlhe following proteins: (1) lima bean trypsin inhibitor, (2) ribonuclease, (3) myoglobin, monomer, (4) β-chymotrypsinogen, (5) myoglobin, dimer, (6) ovalbumin, and (7) bovine serum albumin.

again obtained as a single homogeneous peak. The second δ peak, however, contained an impurity which was present in the purified K12 (λ) ATPase. The impurity was removed from the δ subunit by chromatography on a hydroxylapatite column. Partially purified δ fractions from the Sephadex G-75 column were applied to a 1-ml column of hydroxylapatite equilibrated with the Sephadex G-75 column buffer. The elution was carried out in two steps. The first with 2 ml of buffer containing 30 mM potassium phosphate at pH 7.0 eluted the impurity. The second with 2 ml of buffer containing 100 mM potassium phosphate eluted the δ subunit in virtually homogenous form with about a 75% yield. The purified δ and ϵ from the K12 (λ) strain had the same activities as the subunits from the ML308-225 strain.

Restoration of Membrane Binding and Energy Coupling Activity to ECF_1 Missing δ. Previously (Smith and Sternweis, 1975), a minor subunit fraction containing chiefly just the δ and ϵ subunits of ECF_1 was shown to restore the capacity of enzyme missing δ to bind back to membranes depleted of ECF_1. Figure 3 shows that this restoration was achieved with the purified δ subunit. The restoration of the binding capacity of the enzyme was linearly dependent on the amount of δ added until all of the ECF_1 receptors became occupied (Figure 3).

The slope of the binding curve (Figure 3) shows that 1 μg of δ restored binding capacity to 1.2 units of ATPase or about 12 μg based on a measured specific activity of about 100 units/mg for the purified enzyme. Assuming molecular weights of about 20 000 for δ and 300 000 for ECF_1, this result shows that only 1 or 2 δ peptides are needed to reattach a single ECF_1 molecule to the membrane.

The enzyme that became reattached to the inverted membrane vesicles by the added δ was found to function in energy transduction. This is shown in Figure 4 using the ATP-dependent transhydrogenase activity as a measure of energy coupling. The purified δ restored the capacity of the δ-deficient enzyme to couple ATP hydrolysis to the transhydrogenase in membrane vesicles depleted of ECF_1. The relationship between the amount of δ added and the amount of energized transhydrogenase restored was clearly sigmoidal (Figure 4) in contrast to the linear dependence on δ of the binding of the enzyme to the membrane (Figure 3). The sigmoidal nature of the restoration of energy coupling presumably reflects an increased

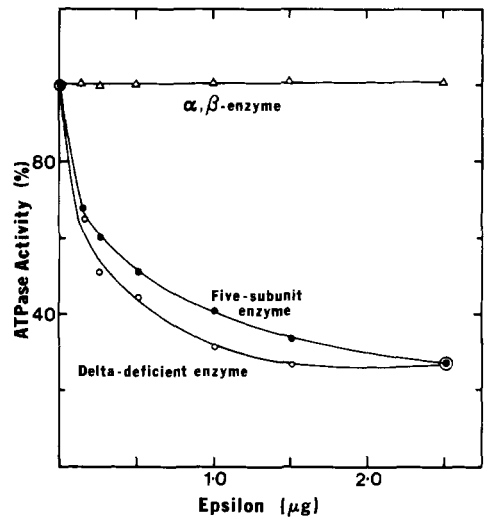

FIGURE 6: Effect of ϵ on the hydrolytic activity of purified five- subunit ECF_1 (●), ECF_1 missing δ (○), and two-subunit ECF_1 (△) consisting of the two larger α and β subunits. The specific activities of enzyme preparations were: 100 units/mg for the five-subunit ECF_1, 113 units/mg for the four-subunit enzyme missing δ, and 80 units/mg, for the α-β enzyme. The α-β enzyme was prepared as previously described (Nelson et al., 1974). The other enzyme preparations are described under Methods.

efficiency of energy coupling due to a reduction in the proton permeability of the vesicles as more F_1 becomes reattached as previously suggested (Smith and Sternweis, 1975). Maximal restoration of ATP-dependent transhydrogenase occurred at the same level of added δ as was required to saturate the binding of δ-deficient ECF_1 to the membrane (Figures 3 and 4).

The complete restoration of the above activities with the virtually homogeneous subunit shows conclusively that the defect in the 4-subunit enzyme resides solely in the lack of the δ peptide. Also, it establishes without doubt the necessary requirement of the δ subunit for a completely functional proton translocating ATPase in *E. coli*.

Figure 5 shows that δ eluted from a calibrated molecular-seive column with an apparent molecular weight of 35 000, which is nearly twice the molecular weight of δ obtained by gel

electrophoresis in the presence of sodium dodecyl sulfate (Bragg and Hou, 1972; Futai et al., 1974).

Inhibition of the ATPase by the ϵ Subunit. Figure 6 shows that ϵ strongly inhibits the hydrolytic activity of purified ECF_1, indicating that ϵ regulates the hydrolytic activity of the enzyme in vivo. It is curious that the ATPase inhibitor was isolated from purified ECF_1, which is highly active hydrolytically. Perhaps the endogenous ϵ in the purified ECF_1 is bound to a noninhibitory site on the molecule. It is known that the chloroplast ATPase inhibitor can be reversibly displaced from its inhibitory site to another site on the molecule without dissociating from the coupling factor (Nelson et al., 1972). The endogenous ϵ may not be sufficient to inhibit the ATPase if the inhibition of the enzyme requires more than one ϵ per ATPase. Accordingly, the endogenous ϵ would be expected to be relatively nonexchangeable but not in itself sufficient for inhibition until another exchangeable site(s) becomes occupied by the added ϵ.

Figure 6 also shows the effect of ϵ on the δ-deficient enzyme containing the α, β, γ, and ϵ subunits and on enzyme containing chiefly just the two larger α and β subunits. The two-subunit enzyme was prepared as previously described (Nelson et al., 1974) by treating the purified five-subunit ECF_1 with TPCK-trypsin, which does not diminish its ATPase activity. The δ-deficient enzyme was just as sensitive to inhibition by ϵ as the enzyme containing δ, indicating that δ is not required for inhibition by ϵ. By contrast, ϵ had no effect on the hydrolytic activity of the α-β enzyme (Figure 6). Since the γ subunit is present in the δ-deficient enzyme which is sensitive to the protein inhibitor but absent from the two subunit enzyme which is insensitive to the inhibitor, an intact γ subunit may be required for inhibitory action. However, since a protease was used in the preparation of the two-subunit enzyme, the possibility cannot be overlooked that the insensitivity of the two-subunit enzyme to the inhibitor may be due to an alteration in the α or β subunits, although no detectable difference in electrophoretic mobility of α or β in sodium dodecyl sulfate gels was observed after the protease treatment (unpublished observations).

ϵ eluted from a calibrated molecular-seive column with an apparent molecular weight of about 16 000 (Figure 5).

Discussion

The two smaller of the five peripheral polypeptides that compose the F_1 portion of the *E. coli* ATPase are called δ and ϵ, and each represents only about 5% of the total enzyme. Here we have described a procedure for purifying active δ and ϵ subunits to homogeneity, as judged by gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 2). Our results with the homogeneous peptides provide conclusive functional evidence that δ and ϵ are authentic subunits of the enzyme. The homogeneous ϵ inhibited the hydrolytic activity of purified ATPase, indicating that ϵ has a role in the regulation of the enzyme in vivo. ϵ maximally inhibits the purified ATPase by 70–80% (Figure 6) and inhibition obeys pure noncompetitive kinetics with respect to substrate (Smith and Sternweis, 1976). ϵ is very potent, since half-maximal inhibition of the purified ATPase was observed when the enzyme and the added inhibitor were present in roughly stoichiometric amounts (Smith and Sternweis, 1976). ϵ has a molecular weight of about 16 000 by molecular-seive chromatography, which is somewhat larger than earlier estimates by gel electrophoresis in the presence of sodium dodecyl sulfate (Bragg and Hou, 1972; Futai et al., 1974). The larger value has also been confirmed by sedimentation equilibrium centrifugation and gel electrophoresis in the presence of sodium dodecyl sulfate and urea (Smith and Sternweis, 1976), indicating that gel electrophoresis in sodium dodecyl sulfate without urea erroneously underestimates the size of ϵ.

It is interesting that Kuriki and Yoshimura (1974) have recently described a protein inhibitor of the elongation factor G-dependent GTPase reaction of ribosomes. The ribosomal GTPase inhibitor protein is about the same size as the ϵ subunit of the proton-pump ATPase and it would be interesting to directly compare the properties of these two proteins which inhibit different purine triphosphatases. Homogeneous δ restored the capacity of enzyme-missing δ to recombine with enzyme-depleted membrane vesicles and to carry out membrane energy transductions (Figures 3 and 4). Since the functional attachment of the purified ECF_1 to the membrane exhibited an absolute dependence upon the presence of an active δ subunit, δ may be a component of the stalk that connects F_1 to the membrane, as seen in electron micrographs (Kagawa and Racker, 1966). δ has an apparent molecular weight of about 20 000 by sodium dodecyl sulfate gel electrophoresis, but elutes from a molecular-seive column at an apparent molecular weight of about 35 000 (Figure 5), suggesting that the active form of δ is either a dimer or rather elongated in shape. It is especially noteworthy that a protein called nectin, which is required for attaching the F_1 of *Streptococcus faecalis* to the membrane, has a molecular weight of 37 000 by molecular-seive chromatography (Baron and Abrams, 1971). It appears likely that nectin is the δ subunit of the streptococcal enzyme (Abrams et al., 1976), although nectin has not yet been purified sufficiently to permit its identification on acrylamide gels so its electrophoretic mobility in the presence of sodium dodecyl sulfate has not yet been compared with the δ polypeptide of the streptococcal enzyme. The interaction of nectin with the streptococcal F_1 apparently requires Mg^{2+} (Abrams and Smith, 1974). By contrast, a complex between δ and the δ-deficient ECF_1 was formed in the presence of EDTA and was sufficiently stable to be isolated by molecular-seive chromatography in the presence of EDTA (Smith and Sternweis, 1976).

The δ subunit of ECF_1 clearly has the structural role of attaching the F_1 headpiece to the membrane. Moreover, if δ is the stalk between the F_1 headpiece and the membrane, then the proton-motive force which is developed across the membrane would have to be transmitted through δ to the F_1 headpiece where ATP is synthesized. According to this view it should be possible to modify δ in such a way that would not impair its capacity to bind F_1 to the membrane, but that would no longer permit coupling between oxidation and phosphorylation.

Acknowledgments

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Mechanism of Pigeon Liver Malic Enzyme. Kinetics, Specificity, and Half-Site Stoichiometry of the Alkylation of a Cysteinyl Residue by the Substrate-Inhibitor Bromopyruvate†

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ABSTRACT: Malic enzyme from pigeon liver is alkylated by the substrate analogue bromopyruvate, resulting in the concomitant loss of its oxidative decarboxylase and oxalacetate decarboxylase activities, but not its ability to reduce α-keto acids. The inactivation of oxidative decarboxylase activity follows saturation kinetics, indicating the formation of an enzyme-bromopyruvate complex ($K \simeq 8$ mM) prior to alkylation. The inactivation is inhibited by metal ions and pyridine nucleotide cofactors. Protection of malic enzyme by the substrates L-malate and pyruvate and the inhibitors tartronate and oxalate requires the presence of the above cofactors, which tighten the binding of these carboxylic acids in accord with the ordered kinetic scheme (Hsu, R. Y., Lardy, H. A., and Cleland, W. W. (1967), *J. Biol. Chem. 242,* 5315-5322). Bromopyruvate is reduced to L-bromolactate by malic enzyme and is an effective inhibitor of L-malate and pyruvate in the overall reaction. The apparent kinetic constants (90 μM-0.8 mM) are one to two orders of magnitude lower than the half-saturation constant (K) of inactivation, indicating a similar tightening of bromopyruvate binding in the E-$NADP^+$ (NADPH)-Mn^{2+} (Mg^{2+})-BP complexes. During alkylation, bromopyruvate interacts initially at the carboxylic acid substrate pocket of the active site, as indicated by the protective effect of substrates and the ability of this compound to form kinetically viable complexes with malic enzyme, particularly as a competitive inhibitor of pyruvate carboxylation with a K_i (90 μM) in the same order as its apparent Michaelis constant of 98 μM. Subsequent alkylation of a cysteinyl residue blocks the C-C bond cleavage step. The incorporation of radioactivity from [^{14}C]bromopyruvate gives a half-site stoichiometry of two carboxyketomethyl residues per tetramer, indicating strong negative cooperativity between the four subunits of equal size, or alternatively the presence of structurally dissimilar active sites.

Pigeon liver malic enzyme (L-malate:$NADP^+$ oxidoreductase; decarboxylating EC 1.1.1.40) plays a major role in lipogenesis by providing NADPH reducing equivalents for the hepatic biosynthesis of fatty acids (Lardy et al., 1964; Young et al., 1964; Wise and Ball, 1964). The oxidative decarboxylation of L-malate (eq 1) catalyzed by this enzyme involves two mechanistically distinct functions: the $NADP^+$-dependent oxidation of L-malate, followed by decarboxylation of the enzyme-bound α-keto acid to produce CO_2 and pyruvate. These reactions are resolvable by the appropriate activity measurements into the decarboxylase, the reductase, and the pyruvate-medium proton-exchange partial reactions shown in eq 1-4 (Ochoa et al., 1948; Salles and Ochoa, 1950; Hsu and Lardy, 1967c; Tang and Hsu, 1973; Bratcher, 1974).

Oxidative Decarboxylation

$$\text{L-malate} + NADP^+ \overset{Me^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + NADPH \quad (1)$$

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