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PARTIAL PURIFICATION OF ACTIVE DELTA AND EPSILON SUBUNITS OF THE MEMBRANE ATPASE FROM ESCHERICHIA COLI

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We have partially purified active delta and epsilon subunits of the E. coli membrane-bound Mg^{2+}-ATPase (ECF_1). Treating purified ECF_1 with 50% pyridine precipitates the major subunits (α, β, and γ) of the enzyme, but the two minor subunits (δ and ϵ), which are present in relatively small amounts, remain in solution. The delta and epsilon subunits were then resolved from one another by anion exchange chromatography. The partially purified epsilon strongly inhibits the hydrolytic activity of ECF_1. The epsilon fraction inhibits both the highly purified five-subunit ATPase and the enzyme deficient in the δ subunit. The latter result indicates that the delta subunit is not required for inhibition by epsilon. By contrast, two-subunit enzyme, consisting chiefly of the α and β subunits, was insensitive to the ATPase inhibitor, suggesting that the γ subunit may be required for inhibition by epsilon.

The partially purified delta subunit restored the capacity of ATPase deficient in delta to recombine with ATPase-depleted membranes and to reconstitute ATP-dependent transhydrogenase. Previously we reported (Biochem. Biophys. Res. Commun. 62:764 [1975]) that a fraction containing both the delta and epsilon subunits of ECF_1 restored the capacity of ATPase missing delta to recombine with depleted membranes and to function as a coupling factor in oxidative phosphorylation and for the energized transhydrogenase. These reconstitution experiments using isolated subunits provide rather substantial evidence that the delta subunit is essential for attaching the ATPase to the membrane and that the epsilon subunit has a regulatory function as an inhibitor of the ATPase activity of ECF_1.

INTRODUCTION

The energy-transducing ATPase present in the inner membrane of E. coli is a large multimeric protein whose subunit composition resembles that of coupling factor 1 from mitochondria and chloroplasts (see reference 1 for review). The highly purified coupling factor from E. coli (ECF_1)* contains five distinct types of polypeptides as seen by SDS gel electrophoresis, and is capable of reconstituting energy coupling in enzyme-depleted membranes (2, 3). By contrast, other preparations of the enzyme which lack the delta polypeptide (MW ~ 20,000) are inactive as coupling factors (3–7), presumably because

*Abbreviations: ECF_1, E. coli Mg^{2+}-ATPase coupling factor 1; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecylsulfate.

they have lost the capacity to recombine with ATPase-depleted membranes (3). Recently (8) we found that a fraction containing chiefly the delta and epsilon subunits of ECF_1 restored the capacity of the enzyme missing delta to bind back to depleted membranes, a result which clearly implicated the delta subunit in the attachment of the ATPase to the membrane. It was also shown that once the delta-deficient enzyme became reattached to the membrane in the presence of the added delta and epsilon subunits, it functioned as a coupling factor in oxidative phosphorylation and for the energized transhydrogenase (8). Since both the delta and epsilon subunits were present in the fraction which restored coupling factor activity to the enzyme missing the delta subunit, the possibility remained that epsilon participated in the conversion of the enzyme lacking the delta subunit to an active coupling factor.

Now we have resolved the delta and epsilon subunits from one another and have found that only the delta subunit fraction is effective in restoring the coupling factor activity to the delta-deficient enzyme, confirming the essentiality of the delta subunit for attaching the coupling factor to the membrane. Possibly, delta provides a structural link between the coupling factor and its membrane receptor. The delta subunit of ECF_1 is functionally similar to nectin from Streptococcus faecalis (9).

The partially purified epsilon subunit, although ineffective in restoring coupling factor activity to enzyme missing delta, potently inhibits the hydrolytic activity of the enzyme. Although others have previously reported suggestive evidence for an ATPase inhibitor in bacteria (1, 10, 11), the present report, to our knowledge, provides the first indication that the epsilon subunit inhibits the ATPase activity of ECF_1 and therefore has a regulatory function. The ECF_1 inhibitor is apparently homolgous to the low molecular weight ATPase inhibitor proteins present in mitochondria (12) and chloroplasts (13). .

METHODS

Enzyme Preparations

Five-subunit ECF_1 having a specific activity of about 100 U/mg was purified from 50 g (wet weight) quantities of frozen K12 (λ) cells by the procedure of Futai, Sternweis, and Heppel (3). The delta subunit was selectively removed from purified five-subunit enzyme by molecular sieve chromatography at pH 9.4. About 2 mg of the purified enzyme was concentrated by precipitation with solid ammonium sulfate to 65% saturation. The protein precipitate was dissolved with about 0.2 ml of 50 mM glycine-NaOH, pH 9.4, containing 2 mM EDTA, 1 mM ATP, and 10% glycerol, and applied to an A-0.5 m Biogel column (1.1 × 90 cm) (BioRad Laboratories) equilibrated with the buffer at room temperature. The specific activity of the delta-deficient enzyme was about 60 U/mg. Figure 1 shows SDS gels of four- and five-subunit ECF_1. Enzyme containing chiefly just the two larger α and β subunits was prepared by treating purified ECF_1 with TPCK-trypsin, according to the procedure of Nelson et al. (6).

Transhydrogenase

The reduction of $NADP^+$ by NADH was assayed in the presence of cyanide which blocks respiration-driven transhydrogenase. ATPase-depleted membranes (0.15 mg pro-

tein) were incubated with about 1 unit of ATPase deficient in the delta subunit and a delta subunit fraction in a volume of 0.05 ml containing 0.1 M TrisCl, pH 8, and 10 mM $MgCl_2$ for 10 min at 38°C. One ml of a buffer containing 50 mM TrisCl, pH 8, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 5 μl/ml ethanol was added, followed by 10 μl 1 M NaCN and 25 μl 3.0 mM NAD^+ dissolved in 0.2 M Na acetate, pH 6, and 50 μl 16.3 mM $NADP^+$ dissolved in 0.2 M Na acetate, pH 6. After an additional 10 min incubation at room temperature, 200 μg of alcohol dehydrogenase was added to start the reaction. After an initial rapid increase in absorbance due to the reduction of NAD^+, the steady state increase in absorbance was recorded at 340 nm by a Gilford 2400 spectrophotometer to obtain the rate of the energy-independent reduction of $NADP^+$. The increase in the rate after adding 10 μl of 0.06 M NaATP, pH 7, represents the ATP-dependent transhydrogenase.

ATPase Inhibitor

A sample of enzyme (0.01–0.02 units) was incubated for 5 min at room temperature in 0.1 ml of 2 mM TrisCl, pH 8, with or without the epsilon subunit fraction as indicated. Then an additional 0.2 ml of 2 mM TrisCl, pH 8, was added. The ATPase reaction was started by adding 0.3 ml of a solution containing 50 mM TrisCl, pH 8, 4 mM $MgCl_2$, and 8 mM NaATP. After 10 min at 38°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 (14).

Protein was determined by a modification of the method of Lowry et al. (15).

RESULTS AND DISCUSSION

Purification of the Subunits

A fraction containing chiefly the delta and epsilon subunits was isolated as previously reported (8) by treating purified ECF_1 with pyridine following the procedure that Nelson et al. (13) used to separate the subunits of the chloroplast ATPase. Then the delta and epsilon subunits were resolved from one another on the basis of their different affinities at pH 6.2 for an anion exchange resin. The delta-epsilon fraction (about 300 μg of protein) was applied to a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) column (0.9 × 4 cm) equilibrated at pH 6.2 with 20 mM sodium succinate containing 1 mM NaEDTA. The column was washed successively with 1 ml of equilibration buffer, 3 ml of buffer plus 25 mM NaCl, 1 ml buffer plus 0.1 M NaCl, and finally 3 ml of buffer plus 0.8 MNaCl. Most of the epsilon subunit was eluted by 25 mM NaCl, whereas the delta subunit was eluted by the 0.8 M salt wash.

Figure 1 shows SDS gels of the delta-epsilon fraction (gel 3), the purified delta subunit (gel 4), and the purified epsilon subunit (gel 5). The purified ATPase from which the δ and ε subunits were isolated contained some proteins, believed to be contaminants, which migrated more slowly in the SDS gels (Fig. 1, gel 1) than the largest ATPase subunit. These proteins were also present in the DEAE-Sephadex purified delta and epsilon subunits (Fig. 1, gels 4 and 5).

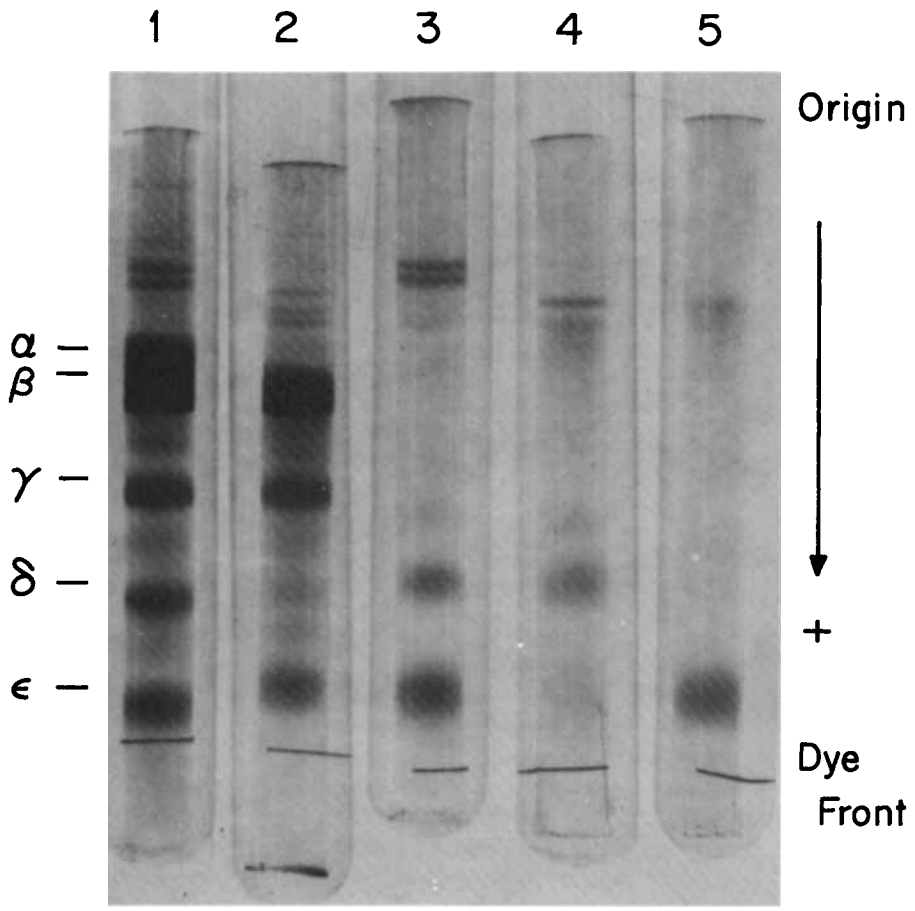

Fig. 1. SDS gel electrophoresis of five-subunit ATPase (1), ATPase deficient in delta (2), the delta-epsilon fraction (3), the delta subunit fraction (4), and the epsilon subunit fraction (5). Protein samples were incubated in 1% SDS and 2% β-mercaptoethanol for 3 min in a boiling waterbath. Electrophoresis was carried out for about 250 volt-hours in 7.6% polyacrylamide gels according to the procedure of Weber, Pringle, and Osborn,(16). The gels were stained with Coomassie blue. About 65 μg, 45 μg, 9 μg, 3 μg, and 4μg of protein were applied to gels 1–5, respectively. The pin near the bottom of each gel indicates the position of the bromphenol blue tracking dye. The α and β subunits in gels 1 and 2 are smeared together due to overloading of the gels with protein so that the delta and epsilon subunits would stand out.

Restoration of ATP-Driven Transhydrogenase

Previously we (8) showed that a fraction containing chiefly the delta and epsilon subunits of ECF_1 restored the capacity of ATPase missing delta to reattach to enzyme-depleted vesicles, to couple energy to the transhydrogenase, and to function as a coupling factor in oxidative phosphorylation. Figure 2 shows that the partially purified delta subunit by itself was effective in restoring the capacity of the delta-deficient enzyme to couple ATP hydrolysis to the transhydrogenase. The epsilon subunit fraction was not by it-

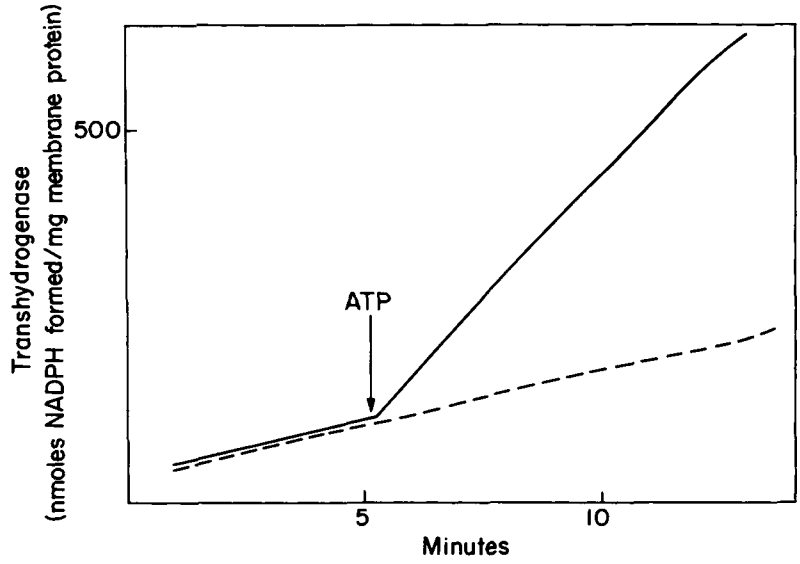

Fig. 2. Restoration of ATP-driven transhydrogenase in ATPase-depleted membrane vesicles by the delta subunit fraction plus delta-deficient enzyme. Dashed line: depleted membrane vesicles plus ATPase deficient in the delta subunit. Solid curve: depleted membrane vesicles plus delta subunit fraction plus ATPase deficient in the delta subunit.

self effective in restoring coupling factor activity to delta-deficient enzyme, nor did it have any detectable effect on the extent of the restoration of ATP-driven transhydrogenase produced by the partially purified delta subunit (data not shown). The extent of the restoration of ATP-dependent transhydrogenase increased with increasing amounts of the partially purified delta (Fig. 3). Not quite enough delta subunit was added to achieve maximal restoration of ATP-driven transhydrogenase (Fig. 3). Under the conditions of this experiment (Fig. 3), the maximal rate of ATP-driven transhydrogenase is about 50 μmoles/min per mg membrane protein when delta is in excess. The observation that the partially purified delta restored the ability of the delta-deficient enzyme to recombine with depleted membrane vesicles, as indicated by the reconstitution of energy coupling to the transhydrogenase in the vesicles, provides clear evidence that the delta subunit is required for attaching ECF_1 to the membrane.

Inhibition of the ATPase by the Epsilon Subunit Fraction

Figure 4 shows that the partially purified epsilon subunit strongly inhibited the hydrolytic activity of purified ECF_1 indicating that the epsilon subunit may have a key role as an inhibitor of the ATPase activity of ECF_1 in intact E. coli. TPCK-trypsin destroyed the activity of the ATPase inhibitor (Table I) indicating that it is a protein. Since the ATPase inhibitor was isolated from purified ECF_1 which is highly active hydrolytically, it

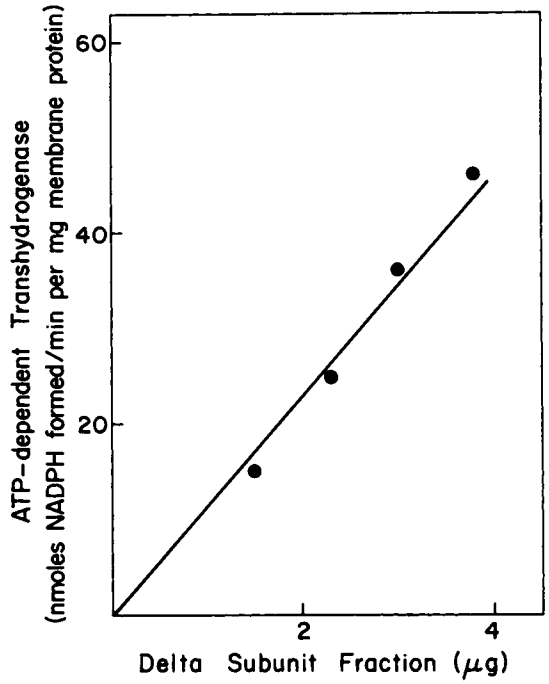

Fig. 3. Effect of the delta subunit fraction on the restoration of ATP-driven transhydrogenase in depleted-membrane vesicles.

TABLE I. Effect of TPCK-Trypsin on the Epsilon Subunit Fraction

Additions to purified ECF_1[1]	ATPase activity (U/mg)
None	77
Epsilon subunit fraction[2]	26
Epsilon subunit fraction treated with TPCK-trypsin[3]	70

[1] Five-subunit ECF_1 (0.25 μg) was incubated with the indicated components for 5 min at room temperature in 0.1 ml of 2 mM TrisCl, pH 8. Then an additional 0.2 ml of buffer was added and the ATPase activity assayed.

[2] 5 μg.

[3] The epsilon subunit fraction (5 μg) was treated with 1 μg of TPCK-trypsin (Worthington) for 25 min at 38°C. Then 2 μg soybean trypsin inhibitor (Worthington) was added to inactivate the TPCK-trypsin before the incubation with ECF_1. The inhibitory activity of the epsilon subunit fraction was not significantly affected by a similar treatment with TPCK-trypsin in the presence of the trypsin inhibitor.

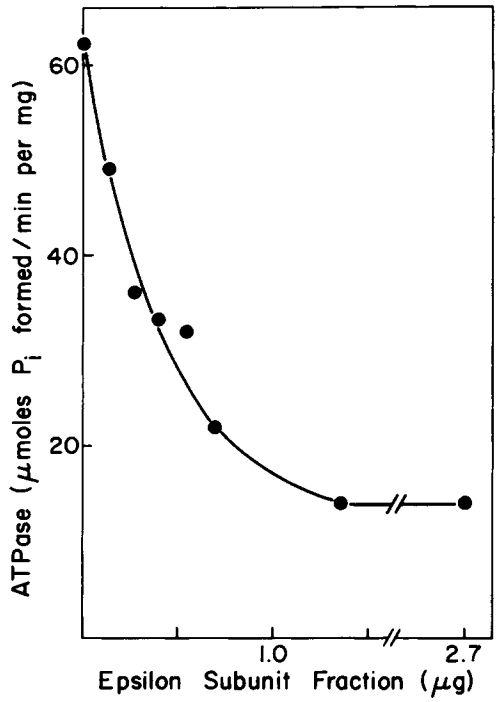

Fig. 4. Inhibition of five-subunit Mg^{2+}-ATPase by partially purified epsilon subunit. Purified ECF_1 (0.25 μg protein) was incubated with the indicated amount of the epsilon subunit fraction in 0.1 ml of 2 mM TrisCl, pH 8, for 5 min at room temperature before assaying for ATPase activity.

would appear that the inhibitor protein that is present in the highly purified ECF_1 is dislodged from its inhibitory site. It is known that chloroplast ATPase inhibitor can be displaced from its inhibitory site without dissociating from the coupling factor (13).

We also tested the effect of the ATPase inhibitor on the delta-deficient enzyme containing 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 by treating purified five-subunit enzyme with TPCK-trypsin as previously described (6). Figure 5 shows that the partially purified epsilon strongly inhibited the delta-deficient enzyme but had no effect on the two-subunit enzyme. Since the γ subunit is present in the delta-deficient enzyme which is sensitive to the inhibitor, but virtually absent from the two-subunit enzyme which is insensitive to the protein inhibitor, an intact γ subunit may be required for inhibitor action. However, since a protease was used in the preparation of the two-subunit enzyme, one cannot overlook the possibility that the insensitivity of the two subunit enzyme to the inhibitor may be due to an alteration in the α or β subunits.

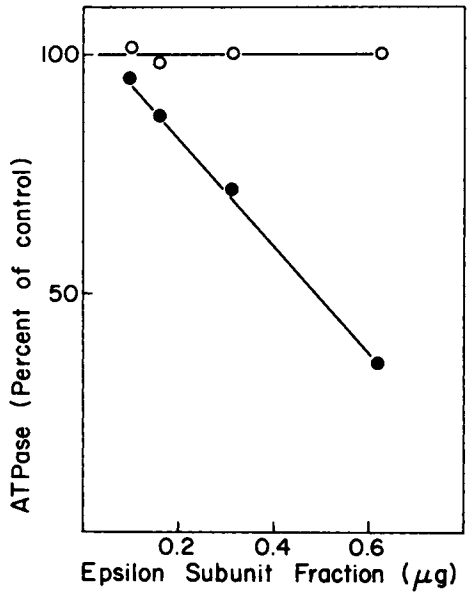

Fig. 5. Effect of the partially purified epsilon subunit on ATPase deficient in the delta subunit (•) and two-subunit ATPase (○) consisting of the two larger α and β subunits. The specific activities of the four-subunit and two-subunit enzymes were 14 and 60 U/mg, respectively.

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