RESTORATION OF COUPLING FACTOR ACTIVITY TO ESCHERICHIA COLI ATPase MISSING THE DELTA SUBUNIT

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SUMMARY

We separated the two minor subunits (δ and ε) of the E. coli ATPase from the major subunits (α, β, and γ). The minor subunit fraction was obtained by treating purified ATPase with pyridine following the procedure that Nelson et al. (J. Biol. Chem. 348, 2049 [1973]) used to separate the subunits of chloroplast ATPase. The minor subunit fraction restored the capacity of ATPase lacking the delta subunit to recombine with ATPase-depleted membrane vesicles and to reconstitute energy coupling to the transhydrogenase and oxidative phosphorylation in the vesicles. These results clearly implicate the delta subunit in the attachment of the ATPase to the membrane.

The coupling factor ATPase in E. coli (EC 3,6,1,4) catalyzes ATP synthesis in oxidative phosphorylation and, by acting in the reverse direction, mediates ATP utilization for the active transport of certain solutes and for the energy-linked reduction of $NADP^+$ by NADH [1]. Highly purified coupling factor (ECF_1) capable of restoring energy coupling to the transhydrogenase in ATPase-depleted membranes contains five subunits (α, β, γ, δ and ε) ranging in size from about 60,000 to about 10,000 by SDS gel electrophoresis [2,3]. Other preparations of ATPase contain only four subunits; the delta subunit, which has a molecular weight of about 20,000 in SDS gels, is virtually absent from the four-subunit enzyme [3-7]. The four subunit enzyme does not restore the coupling of energy to the transhydrogenase [3,4] and does not bind back to depleted membranes [3].

Now we report that a fraction containing chiefly the delta and epsilon subunits of ECF_1 restores the capacity of enzyme lacking the delta subunit to

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Abbreviations used: ECF_1, E. coli Mg^{2+}-ATPase; Tricine-EDTA buffer, 10 mM Tricine-NaOH, pH 8 containing 1 mM sodium ethylenediaminetetracetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide m-chlorophenyhydrazone.

recombine with ATPase-depleted membrane vesicles and to reconstitute oxidative phosphorylation and energy coupling to the transhydrogenase. To our knowledge this is the first reconstitution performed with isolated subunits of a bacterial ATPase. These observations clearly implicate the delta subunit in the attachment of the ATPase to the membrane and suggest that the delta subunit may be the structural connection between the ATPase and the membrane. The delta subunit may be functionally similar to nectin from Streptococcus faecalis [8].

METHODS

E. coli K12(λ) was grown aerobically in a medium containing Bacto-Tryptone (3%), yeast extract (1.5%), and NaCl (1.5%). The cells were harvested during the late log phase and stored at -90°. Five-subunit coupling factor was isolated from 50 g (wet weight) quantities of the frozen K12(λ) cells by the purification procedure of Futai et al. [3].

Four-subunit ATPase was isolated from another K12 strain, Bio A126 kindly supplied by Dr. D. B. Wilson. This strain was grown aerobically in a minimal medium containing 0.2% glucose [9] and supplemented with biotin (2.5 μg/ml) and leucine (40 μg/ml). The cells were harvested from stationary phase and used as a source of four-subunit ATPase which was purified by the method of Nelson et al. [6].

Membranes from E. coli ML308-225 were isolated and at least 80% of the ATPase was removed by extraction with EDTA as previously described [3].

ATPase and transhydrogenase activity were assayed as previously described [3]. One unit of ATPase is defined as the amount of enzyme which liberates one micromole inorganic phosphate per minute at 37°. Protein was determined by a modification of the method of Lowry et al. [10].

RESULTS AND DISCUSSION

Preparation of the minor subunit fraction. A fraction containing chiefly the delta and epsilon subunits was obtained by treating purified ECF_1 with pyridine following the procedure that Nelson et al. [11] used to separate the

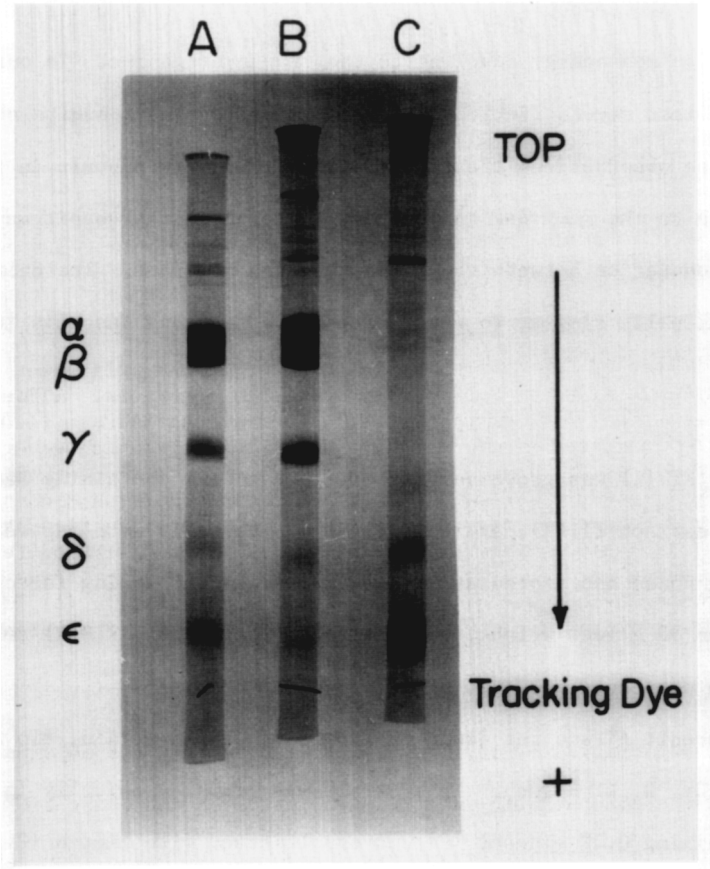

Figure 1. SDS-PAGE of ECF_1 before pyridine treatment (A), the precipitate after treating ECF_1 with pyridine (B), and the minor subunit fraction (C). Samples were incubated in 1% SDS and 2% β-mercapto-ethanol for 3 min in a boiling water bath, electrophoresed for about 250 volt-hours in 7.6% acrylamide gels, and stained with Coomassie blue according to the method of Weber, Pringle and Osborn [11]. About 17 µg, 25 µg, and 4 µg protein were applied to gels A, B and C, respectively. The pins near the bottom of each gel mark the position of the tracking dye.

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subunits of the chloroplast ATPase. About 4 mg (protein) of highly purified ECF_1 (about 100 U/mg) was precipitated by adding solid ammonium sulfate to 65% saturation. The precipitate was dissolved with 1.5 ml Tricine-EDTA buffer. An equal volume of pyridine was added and the mixture was stirred for 10 minutes at room temperature. The pyridine concentration was reduced to 20% by adding water and the mixture allowed to sit for 5 hrs at 4°. The protein

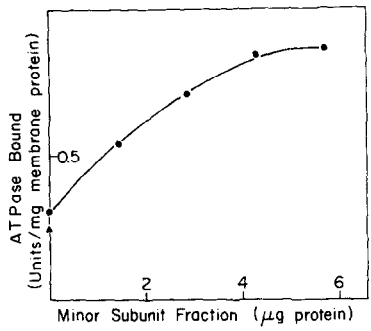

Figure 2. Effect of the minor subunit fraction on the attachment of four-subunit ATPase (lacking δ) to depleted membranes. ATPase-depleted membranes (0.45 mg of protein), four-subunit ATPase (1.0 units) and the indicated amount of the minor subunit fraction were incubated for 10 min at 38° in 0.1 ml of 50 mM Tris-HCl, pH 8 containing 5 mM $MgCl_2$. After adding 3.0 ml of 10 mM Tris-HCl, pH 8 containing 5 mM $MgCl_2$ the membranes were sedimented by centrifugation at 100,000 x g for one hour. The membrane pellet was washed with buffer by suspension and recentrifugation. Then the specific ATPase activity of the membranes was determined. The solid triangle (Δ) indicates the level of residual ATPase present in the depleted membranes.

precipitate was removed by centrifugation at 8,000 x g for 10 minutes. The supernatant was concentrated to 1 ml by ultrafiltration using a UM-10 filter (Amicon Corporation, Lexington, Mass.) and then dialyzed against 1 ℓ of Tricine-EDTA buffer to remove pyridine. SDS gels of ECF_1 before pyridine treatment and the pyridine precipitate and supernatant fractions are shown in Figure 1. The pyridine supernatant (gel C) contained chiefly the delta and epsilon subunits and will be referred to as the minor subunit fraction. There was very little of either the α or β subunits and practically no γ in the minor subunit fraction (gel C). Some proteins migrating slower than the α subunit which were present in the ECF_1 preparation were also present in the minor subunit fraction and the pyridine precipitate (gels A, B, C). Both the pyridine precipitate and the minor subunit fraction were devoid of any detectable ATPase activity.

Attachment of four-subunit ATPase to membranes. We prepared four-subunit ATPase by the procedure of Nelson et al. [6] and confirmed the absence of the delta subunit by SDS-PAGE (result not shown). Enzyme containing only the α,

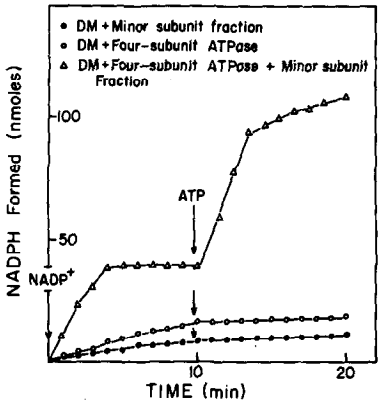

Figure 3. *Restoration of energy coupling to the transhydrogenase in ATPase-depleted membrane vesicles by four-subunit ATPase plus the minor subunit fraction.* Depleted membrane vesicles (0.45 mg of protein) were incubated at 37° for 10 min with four-subunit ATPase (1.0 units) and the minor subunit fraction (6 µg protein) in 0.1 ml of buffer containing 50 mM Tris-HCl, pH 8 and 5 mM $MgCl_2$. Then 1.0 ml buffer (50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 0.1 mM dithiothreitol) was added followed by 25 µl of 3 mM NAD, 5 µl ethanol, and 50 µl of alcohol dehydrogenase (4 mg/ml). The respiration-driven reaction was initiated by adding 50 µl of 16.3 mM $NADP^+$ and the absorbance at 340 nm was recorded. When the oxygen in the reaction cuvette was depleted, NADPH production stopped. Then 10 µl of 60 mM NaATP, pH 7 was added to initiate the ATP-driven reaction. The respiratory-driven rate was corrected for a small amount of non-energy-linked transhydrogenase activity.

β, γ, and ε subunits does not reattach to ATPase-depleted membranes ([3], and Fig. 2). Figure 2 shows that the minor subunit fraction restored the capacity of the four-subunit enzyme to bind to depleted membranes. Increasing the amount of the minor subunit fraction increased the amount of ATPase reattached until a maximal level was obtained (Fig. 2). With an excess of four-subunit enzyme and delta-epsilon fraction the amount of ATPase reattached was about the same as the maximal amount of binding obtainable with five-subunit enzyme. Maximal binding is presumably limited by the number of membrane receptor sites for the ATPase.

Restoration of energized transhydrogenase. Removal of ECF_1 from membrane vesicles virtually abolishes the coupling of energy to the reduction of $NADP^+$ by NADH, and reattachment of ECF_1 restores both respiration-driven and ATP-driven transhydrogenase [2,3]. Four-subunit enzyme has lost the ability to

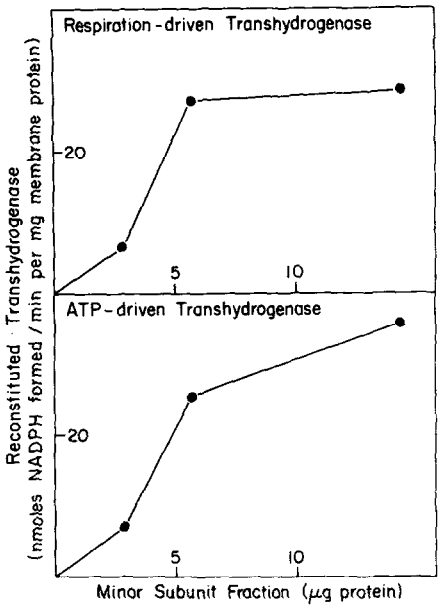

Figure 4. Effect of the minor subunit fraction on energization of the transhydrogenase in depleted-membrane vesicles. The experimental conditions are the same as in the legend to Figure 3 with the indicated amounts of the minor subunit fraction.

restore the coupling of energy to the transhydrogenase in depleted membranes ([3] and Fig. 3) presumably because the four-subunit enzyme cannot recombine with the membranes. By itself the minor subunit fraction was also unable to restore energized transhydrogenase in depleted membranes (Fig. 3). However, combining the minor subunits with four-subunit enzyme restored both respiration-driven and ATP-driven transhydrogenase in depleted membrane vesicles (Fig. 3). The capacity of the four-subunit ATPase to restore energy coupling to the transhydrogenase in depleted membrane vesicles depended on the amount of the minor subunit fraction added until a maximal value was reached (Fig. 4). Maximal restoration of energized transhydrogenase and maximal binding of ATPase were achieved at about the same level of the minor subunit fraction (Fig. 2 and Fig. 4).

A requirement for membrane-bound ECF_1 in the coupling of electron transport to the transhydrogenase has been previously reported [2,3] but remains to be clarified. Perhaps removing ECF_1 from the vesicles uncouples respiration

TABLE I

RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION IN DEPLETED MEMBRANE VESICLES[a]

Additions to depleted vesicles[b]	P/O
None	0.00
Minor subunit fraction	0.00
Four-subunit ATPase	0.06
Four-subunit ATPase + Minor subunit fraction	0.31
Four-subunit ATPase + Minor subunit fraction + CCCP	0.06

[a] Inverted membrane vesicles for oxidative phosphorylation were prepared and assayed by the method of Hertzberg and Hinkle [15]; these vesicles were depleted of ATPase as described [3]. For reconstitution, depleted membranes were mixed with ATPase and minor subunits plus $MgCl_2$ to 10 mM and incubated for 20 minutes at room temperature prior to addition to the assay mix.

[b] About 0.80 mg membrane protein was used for each assay. Additions consisted of: minor subunit fraction, 9 µg protein; four-subunit enzyme, 3.7 units; CCCP, 83 µM.

from the transhydrogenase by rendering the vesicles leaky to protons [13]. According to this view adding the ATPase back would restore the coupling between respiration and the transhydrogenase by resealing the proton leak. The sigmoidal character of the relationship between the amount of the minor subunits added and the amount of energized transhydrogenase restored (Fig. 4) might reflect changes in the efficiency of energy coupling possibly due to changes in the permeability of the vesicles to protons as more ATPase becomes reattached.

Restoration of oxidative phosphorylation. Hertzberg and Hinkle [14] recently described a preparation of inverted vesicles capable of highly efficient oxidative phosphorylation which is sensitive to uncouplers. Our preparation of inverted vesicles had a P/O value of about 0.4 before ECF_1 was removed by extraction with EDTA. After removing most of the ECF_1 oxidative phosphorylation was virtually absent. Table I shows that the combination of four-subunit ATPase with the minor subunit fraction restored oxidative phos-

phorylation in the vesicles to almost the level observed before ECF_1 was removed. Neither the four-subunit ATPase nor the minor subunit fraction by itself was able to restore significant oxidative phosphorylation in the vesicles (Table I). The restored activity achieved by the combination of four-subunit enzyme with the minor subunits was sensitive to the uncoupler CCCP (Table I).

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