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Mutants of Escherichia coli H^+-ATPase defective in the δ subunit of F_1 and the b subunit of F_0

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Summary: Complete nucleotide sequence of the genes for subunits of the H^+ ATPase of E.coli has been determined and several hybrid plasmids carrying various portions of these genes have been constructed. Genetic complementation and recombination tests of about forty mutants of E.coli defective in the ATPase were performed using these plasmids for identifying the locations of the mutations. Two mutants defective in the δ subunit and a novel type of mutant defective in the b subunit of F_0 were identified. The δ subunit mutants showed no proton conduction, suggesting that this subunit has an important role for the proton conduction. The ATPase of the b subunit mutant has a normal activity of proton channel portion, which phenotype is clearly different from that of mutants of the b subunit reported previously.

Proton-translocating ATPase (H^+-ATPase) is a ubiquitous enzyme for the synthesis of ATP in living cells (1). This enzyme also catalyzes the hydrolysis of ATP and functions as a proton pump. The enzyme is composed of two distinct portions, F_1 and F_0. F_1 is an extrinsic membrane portion and has five different subunits, α, β, γ, δ and ε. F_0 is an intrinsic membrane portion and has three different subunits, a, b and c (2). The entire complex of F_1F_0 has been purified from E.coli (2) and its function has been studied in detail (1). However, the precise molecular mechanism of the synthesis of ATP by the enzyme is still unknown.

Genetic and biochemical approaches are useful in studies on this question. Recently the complete nucleotide sequence of the gene cluster for the ATPase of E.coli was determined (3-11). Several hybrid plasmids carrying various portions of the gene cluster are now available, and the portion of genomic DNA in the plasmids can be described exactly in terms of the nucleotide sequence. We isolated about forty mutants of the ATPase by localized mutagenesis and analyzed them with the hybrid plasmids described above. In this way, we found two novel mutants defective in the δ subunit

Abbreviations used: SDS, sodium dodecyl sulfate ; EDTA, ethylene diamine tetraacetate ; DCCD, dicyclohexylcarbodiimide.

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of F_1 ATPase and a mutant defective in the b subunit of F_0. To our knowledge, no mutants defective in the δ subunit have previously been reported. This paper describes the biochemical properties of these mutants.

MATERIALS AND METHODS

The mutants used in the present study were selected from KM230 (also named KY7230) (14) after localized mutagenesis as described by Hong and Ames (13). The mutants cannot utilize succinate as a sole carbon source. KF5 and KF8 were isolated previously (14) and KF22 was obtained in the present study. recA derivatives of these mutants were selected after conjugation (14). Tanaka medium and L broth (12) were used in this study.

Hybrid plasmids pMCR533 (14), pKY159-16 (6), pFT302 (15) and pFT1502 (7) were described previously. pNR1 was constructed in this study by inserting the genomic DNA segment shown in Fig. 1 into pBR322 (14). pRPG45, pRPG56 and pRPG57 (16) were kindly provided by Dr. R.P.Gunsalus, Stanford University. The plasmids used in the present study were prepared by the published procedure (14).

Cells were disrupted in a French press and membranes were prepared as described previously (12).

Formation of a proton gradient in the membrane vesicles was measured by monitoring quenching of fluorecence (quinacrine) after addition of ATP or succinate (12).

Electrophoresis on polyacrylamide gel containing SDS was performed as described previously (12).

RESULTS AND DISCUSSION

Genetic analysis of mutants: KF5, KF8 and KF22 are mutants with low H^+ ATPase activities of 0.22, 0.30 and 0.09 units per mg of membrane protein, respectively, while the wild type has an activity of 1.50 units per mg of protein. These mutations were located by genetic recombination experiments using plasmids carrying various portions of the gene cluster (Fig. 1).

KF5 and KF22 were complemented by plasmids carrying the papE (uncH, δ subunit) gene (pMCR533, pRPG45 and pRPG57)(Table 1). pRPG57 carries a portion of papA (uncA, α subunit) and papF (uncF, b subunit) and the entire cistron of papE (Fig. 1). recA derivatives of both mutants were complemented by this plasmid (pRPG57), indicating that both mutants are defective in the δ subunit. As we described previously, no typical sequence for a promoter was found between the papF and papE genes (5). Therefore, the papE gene is possibly transcribed using a promoter in the vector plasmid.

KF8 ($recA^+$) was complemented by plasmids pKY159-16 and pRPG56. However, a $recA^-$ derivative of the strain was not complemented by these plasmids. These findings suggest that KF8 is defective in the b subunit of F_0 and the mutation takes place within 66 amino acid residues of the proximal portion to the amino terminal. Another mutant AN1419 (uncF469) defective in the b subunit, which was isolated and described by Downie et al. (16), gave the same results in the complementation test.

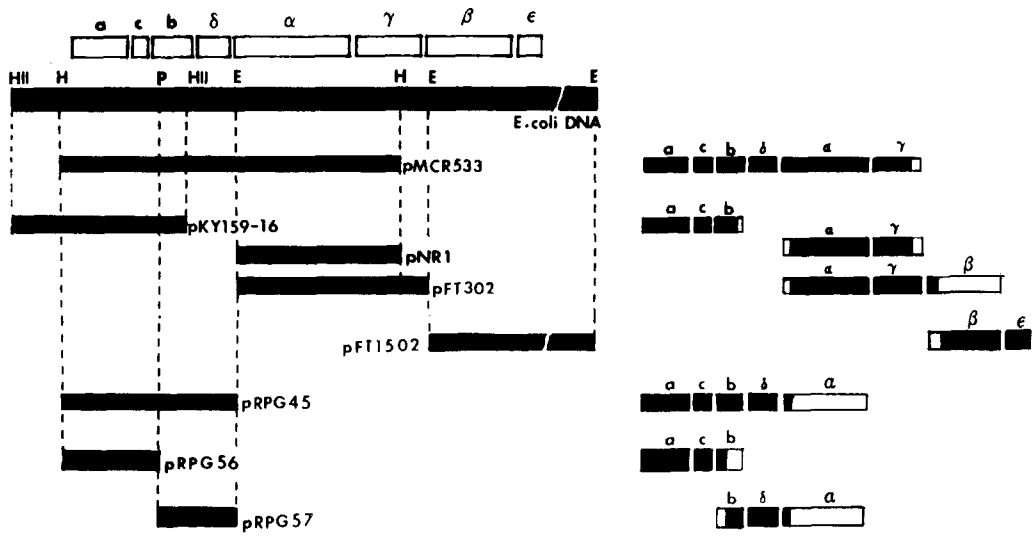

Fig. 1 Hybrid plasmids carrying various portions of the gene cluster for the H^+-ATPase. In the left column, solid lines indicate genomic DNA carried on various plasmids. The coding frame of each subunit gene is shown above the E.coli DNA. The sites of cleavage by restriction endonuleases are abbreviated as follows: HII, HaeII; H, HindIII; P, PstI; E, EcoRI. Solid lines on the right indicate portions of the gene cluster carried on the corresponding hybrid plasmid shown on the left. For instance, pFT302 carries the entire cistron of the γ subunit gene and a part of the cistron for the α and β subunits. From the results shown in Table 3, mutations of KF5 and KF22 were located within the δ subunit gene. Mutation site of KF8 was estimated within 66 amino acid residues from the amino terminal of the b subunit.

Phenotype of the mutants defective in the δ subunit: Formation of a proton gradient in membrane vesicles of the mutants was analyzed using fluorescent dye. Quenching of fluorescence of quinacrine was measured as an index of the proton gradient formed by respiration or the hydrolysis of ATP. It is known that the magnitude of quenching is qualitatively proportional to the proton gradient (12). In both mutants (KF5 and KF22), the magnitude of quenching of quinacrine-fluorescence depending on succinate did not differ before and after washing of the membrane vesicles with dilute buffer (Fig. 2, C and D). This result suggested that proton-pathway in the ATPase of mutants is blocked by the mutations (KF5 and KF22), because protons did not permeate the membrane vesicles of mutants washed with dilute buffer (washed membranes) (Fig. 2, D), conditions in which the membrane vesicles of the wild type are permeable to protons through the F_0 portion (Fig. 2, B).

When purified F_1 from the wild type was added exogenously to the washed membranes (KF5 and KF22), the ATPase did not restore proton conduction driven by ATP (Fig. 2, C). The F_1 also did not bind to the washed membranes (data not shown). The washing fluid of membranes (EDTA extract)

Table 1
Genetic complementation of various H^+-ATPase mutants by plasmids

mutant / plasmid	KF5		KF22		KF8		AN120	
	recA$^+$	*recA*$^-$	*recA*$^+$	*recA*$^-$	*recA*$^+$	*recA*$^-$	*recA*$^+$	*recA*$^-$
pMCR533	+	n.t.	n.t.	n.t.	+	n.t.	+	+
pKY159-16	-	-	-	-	+	-	-	-
pNR1	-	-	-	-	-	-	+	-
pFT302	-	n.t.	-	n.t.	-	n.t.	+	-
pFT1502	-	n.t.	-	n.t.	-	n.t.	-	-
pRPG45	+	+	+	+	+	+	-	-
pRPG56	-	-	-	-	+	-	-	-
pRPG57	+	+	+	+	-	-	-	-

For genetic complementation and recombination tests, competent cells of each mutant were mixed with plasmids and spreaded over a plate of L broth medium containing ampicillin (20 μg/ml). Plates were incubated for 20 hr at 37 C and then ampicillin resistant transformants were transferred on both plates of Tanaka medium supplemented with succinate and glucose, respectively, as the sole carbon source. '+' indicates that Suc$^+$ transformants were observed. 'n.t.' indicates that experiments were not performed. Genetic complementation in this experiment includes genetic recombination between plasmid and host DNA. A strain AN120, which is defective in the α subunit (23, 24), was tested as a control. The gene for the α subunit (papA, uncA) is located next to the gene for the δ subunit (papE, uncH). AN1419(uncF469) gave the same results as KF8.

was subjected to electrophoresis on polyacrylamide gel containing SDS (Fig. 3). Results showed that the extract contained less of the α, β and γ subunits than EDTA extract of the wild type (Fig. 3). Preliminary studies by SDS-polyacrylamide gel electrophoresis with silver staining (22) of the total membrane proteins indicated that the quantities of the α and β subunits in the mutants and the wild type were similar (data not shown).

From all the results described above, it was suggested that assembly of the F_1 components in the mutants is defective and that some portion of the F_1 complex including at least the α and β subunits could not be detached from the membranes even under conditions that removed all F_1 in the wild type. This altered assembly possibly results in formation of incorrect pathway for proton conduction. It should be noted that the δ subunit is postulated to form a connecting bridge between the F_0 portion and the catalytic complex, αβγ (1). However, the results suggest that the δ subunit has an important role in proton conduction, since mutations in the δ subunit could block proton conduction (Fig. 2, C, D). Recently an assembly process of F_1F_0 in E.coli cells was postulated (18). It will be of interest to investigate the structure of F_1F_0 in the two mutants more precisely to determine the intermediate stage of the assembly process, because no other mutants defective in the δ subunit has yet been reported. Studies on this subject are in progress in our laboratory.

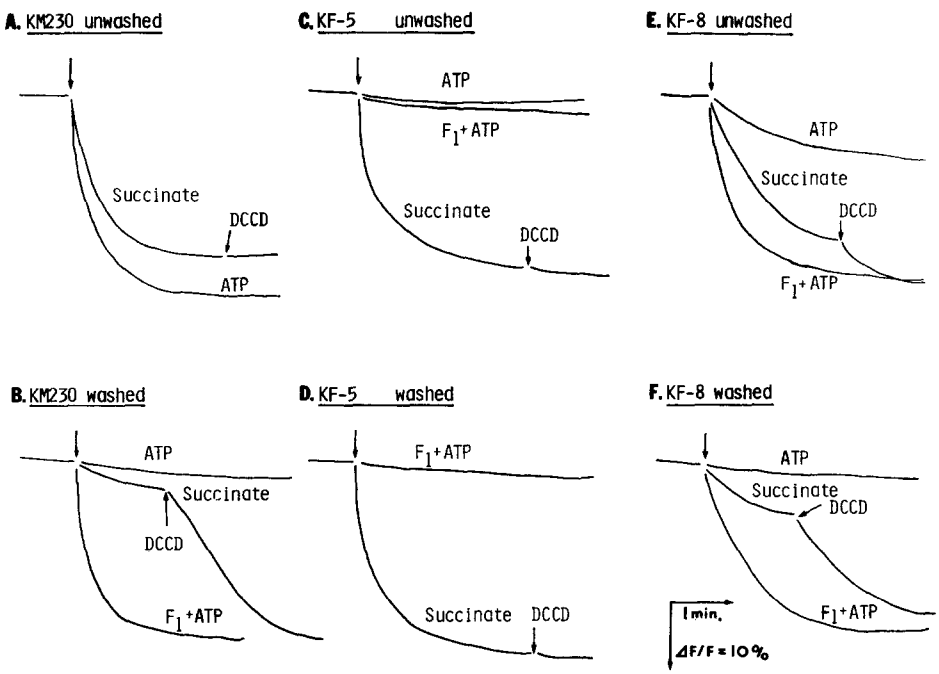

Fig. 2 Formation of a proton gradient in membrane vesicles of mutants measured by quenching of quinacrine fluorescence. Membrane vesicles from the wild type and mutants (200 μg) in 2.0 ml of 10mM tricine buffer, pH 8.0, containing 140 mM choline chloride and 1 uM quinacrine were mixed with 20 μl of 1 M $MgCl_2$ and fluorescence (emission, 500nm; excitation, 420nm) was monitored in a Hitachi 650-10S fluorophotometer. In experiments B, D and F, membranes were washed with 1.0 mM Tris-HCl (pH 7.0) containing 0.5 mM EDTA. At the indicated point, 10 μl of 0.2 M ATP, 10 μl of 1.0 M tris succinate, or 2 μl of 20 mM DCCD was added. For 'F_1+ATP', 25 μg of purified F_1 ATPase from the wild type strain ML308-225 (21) was added to the reaction mixture containing membranes and the mixture was incubated for 10 min before adding ATP. The amount of ATPase used (25 μg) is excess of that required to recover full proton conduction driven by ATP. Basically similar results were obtained in the case of KF22 as KF5.

Phenotype of the mutants defective in the b subunit of F_0: As shown in Fig. 2, E, proton transport driven by ATP is much lower in KF8 than in the wild type. However, the level in KF8 is higher than that in the mutants, KF5 and KF22, in which proton conduction is totally blocked by the mutations. Moreover, purified F_1 ATPase added exogenously could bind to washed membranes of the mutants and subsequent proton conduction driven by ATP was as great as that in the wild type (Fig. 2, F). These results are not necessarily consistent with the phenotype of an ATPase mutant expected if proton flux coupled with the synthesis and hydrolysis of ATP is completely reversible. One possible explanation for the phenotype of KF8 is that the synthesis of ATP and the coupled proton flux in the mutant are defective, because the mutant could not grow in minimal medium (Tanaka medium) supplemented with succinate as the sole carbon source.

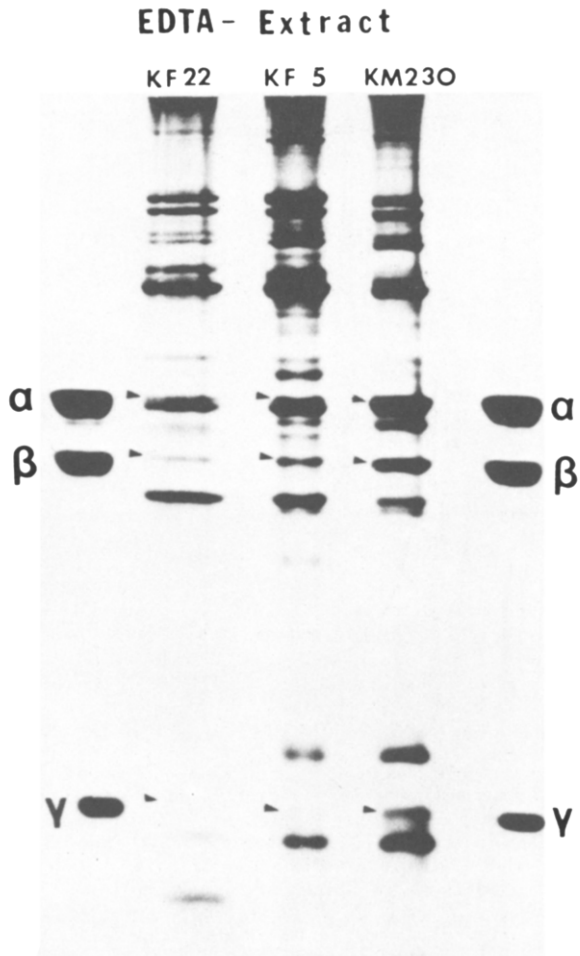

Fig. 3 Polyacrylamide gel electrophoresis of EDTA extracts. Samples were incubated in 1.0 % SDS and 2.0 % β-mercaptoethanol for 3 min in a boiling water bath. 25 ug of EDTA extract was applied to polyacrylamide gel (12 % (w/v) acrylamide). As a control, 3 μg of purified ATPase from ML308-225 was applied. For good resolution of protein bands, relatively small amounts of proteins were applied to the gels and the silver staining method (22) was used. Protein bands of the α, β and γ subunits, respectively, are marked from the top to the bottom of the gels by an arrow (►).

Previously we suggested that the b subunit is integrated into the membranes in regions close to its two terminals. It was also suggested that most of the protein is extruded from the membranes and possibly has a role in binding F_1(19). Membrane vesicles of KF8 are partly leaky to protons (Fig. 2, E), because the magnitude of fluorescence-quenching increased after addition of DCCD, which is known to block the proton flux through the F_0 portion (1). This result suggests that the binding function of the b subunit is altered in this mutant. The phenotype of KF8 is totally different from those of mutants defective in the b subunit reported previously (AN1419 and AS12) (17, 20), in neither of which the subunit is present in the membranes and which have membranes completely impermeable to protons. In this

connection, it may be possible to obtain a defective b subunit from KF8 and if so, it will be interesting to analyze the protein chemical properties of this subunit for understanding its function. In AN1419 the altered base(s) might be located within a domain corresponding to the hydrophobic amino acid cluster (11-33) close to the amino terminal in the gene.

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REFERENCES

(1) Futai, M. and Kanazawa, H. (1980) Current Top. Bioenerg. 10, 181-215.
(2) Foster, D.L. and Fillingame, R.H. (1979) J. Biol. Chem. 254, 3230-3236
(3) Kanazawa, H., Mabuchi, K., Kayano, T., Tamura, F. and Futai, M. (1981) Biochem. Biophys. Res. Commun. 100, 219-225.
(4) Mabuchi, K., Kanazawa, H., Kayano, T. and Futai, M. (1981) Biochem. Biophys. Res. Commun. 102, 172-179
(5) Kanazawa, H., Kayano, T., Mabuchi, K. and Futai, M. (1981) Biochem. Biophys. Res. Commun. 103, 604-612
(6) Kanazawa, H., Mabuchi, K., Kayano, T., Noumi, T., Sekiya, T. and Futai, M. (1981) Biochem. Biophys. Res. Commun. 103, 613-620
(7) Kanazawa, H., Kayano, T., Kiyasu, T. and Futai, M.(1982) Biochem. Biophys. Res. Commun. 105, 1257-1264
(8) Kanazawa, H., Mabuchi, K. and Futai, M. (1982) Biochem. Biophys. Res. Commun. 107, 568-575
(9) Gay, N.J., Walker, J.E. (1981) Nucleic Acid Res. 9 2187-2194.
(10) Gay, N.J., Walker, J.E. (1981) Nucleic Acid Res. 9 3919-3926.
(11) Saraste, M., Gay, N.J., Eberle, A., Runswick, M.J., Walker, J.E. (1981) Nucleic Acid Res. 9 5287-5296.
(12) Kanazawa, H., Miki, T., Tamura, F., Yura, T. and Futai, M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1126-1130
(13) Hong, J.S. and Ames, B.N. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 3158-3162
(14) Kanazawa, H., Tamura, F., Mabuchi, K., Miki, T. and Futai, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7005-7009
(15) Tamura, F., Kanazawa, H., Tsuchiya, T. and Futai, M. (1981) FEBS. Lett., 127, 48-52
(16) Gunsalus, R.P., Bruislow, W.S.A. and Simoni, R.D. (1982) Proc. Natl. Acad. Sci. U.S.A., 79, 320-324
(17) Downie, J.A., Cox, G.B. Langman, L., Senior, A.E., Ash, G. and Gibson, F. (1981) J. Bacteriol. 145, 200-210
(18) Cox, G.B., Downie, J.A., Langman, L., Senior, A.E., Ash, G. and Fayle, D.R.H. and Gibson, F. (1982) J. Bacteriol. 148, 30-42
(19) Kanazawa, H. and Futai, M. (1982) Ann. New York Acad. Sci. in the press.
(20) Friedl, P., Bienhaus, G., Hoppe, J. and Schairer, H.U. (1981) Proc. Natl. Acad. Sci. U.S.A., 78, 6643-6646
(21) Futai, M., Sternweis, P.C. and Heppel, L.A. (1974) Proc. Natl. Acad. Sci. U.S.A., 71, 2725-2729
(22) Oakly, B.R., Kirsh, D.R. and Morris, N.R. (1980) Anal. Biochem. 105, 361-363
(23) Kanazawa, H., Saito, S. and Futai, M. (1978) J. Biochem. 84, 1513-1517
(24) Dunn, S.D. (1978) Biochem. Biophys. Res. Commun. 82, 596-602