Subunits of the H⁺-ATPase of Escherichia coli

OVERPRODUCTION OF AN EIGHT-SUBUNIT F,Fo-ATPase FOLLOWING INDUCTION OF A λ-TRANSUDUCING PHAGE CARRYING THE unc OPERON

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The proton-translocating ATPase complex (F₁,Fₒ) of Escherichia coli was purified after induction of a λ-transducing phage (λasn5) carrying the ATPase genes of the unc operon. ATPase activity of membranes prepared from the induced λ-unc lysogen was 6-fold greater than the activity of membranes prepared from strains lacking the unc-transducing phage, confirming the report of Kanazawa et al. ((1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1126–1130). The F₁,Fₒ-ATPase complex was purified in comparable yield from either enriched membranes or control membranes using a modification of the procedure reported by Foster and Fillingame ((1979) J. Biol. Chem. 254, 8230–8236). Each of the eight subunits that had been reported as components of the F₁,Fₒ complex from wild type E. coli was overproduced in the λ-unc lysogen. All eight subunits co-purified in the same stoichiometric proportion as in the complex purified from wild type E. coli. We conclude that all eight subunits are likely coded by the small segment of chromosomal DNA carried by the λ-transducing phage. These experiments provide the first evidence that all eight polypeptides are authentic subunits of the ATPase complex rather than contaminants that fortuitously co-purify.

The synthesis of ATP during oxidative or photophosphorylation is catalyzed by enzyme complexes in mitochondria, chloroplasts, and bacteria that share common structural features (1–3). These complexes are composed of two distinct sectors: the Fₒ-ATPase, an extrinsic membrane protein which catalyzes the hydrolysis of ATP when removed from the membrane, and the F₁ sector, a component intrinsic to the membrane which functions as a proton conductor on removal of F₁ (1, 2). The F₁,Fₒ complex reversibly couples the translocation of H⁺ across the membrane to the synthesis or hydrolysis of ATP (2, 4). The F₁,Fₒ-ATPase has been purified from several species of bacteria, mitochondria, and chloroplasts (1, 3). It is an extremely complex enzyme, composed of at least five nonidentical subunits in most species and perhaps six subunits in mammalian mitochondria (1, 3). Fragmentary information on the function of different subunits of F₁ has been obtained by biochemical reconstitution experiments with purified subunits from two species of bacteria (2, 5). The F₁ sector of the complex has been less thoroughly analyzed than Fₒ and the subunits only tentatively identified. The F₁,Fₒ-ATPase complexes purified from the thermophilic bacterium PS3 and Escherichia coli contained three polypeptides in addition to the five which compose F₁ (6, 7). On the other hand, the F₁,Fₒ complex purified from chloroplasts contained four polypeptides in addition to those of F₁ (8). Mitochondrial F₁,Fₒ preparations are considerably more complicated (9–12). A common subunit found in the F₁ of all species studied to date is a hydrophobic “proteolipid” protein which is the site of covalent reaction with dicyclohexylcarbodiimide (DCCD), an inhibitor of the proton-translocating activity of F₁ (4). A second subunit from the F₁ of the thermophilic bacterium PS3 has been implicated in the binding of the F₁-ATPase (13). It remains uncertain whether there are other subunits that are authentic components of F₁ and what function these subunits may perform.

The F₁,Fₒ-ATPase of E. coli has been studied extensively in recent years because questions of function are subject to genetic as well as biochemical analysis in this organism (14, 15). All mutations affecting the ATPase complex have mapped at a single locus termed unc, and these genes seem to be organized in an operon (15). Biochemical analysis of mutants altered in different subunits should provide definitive information on the function of each subunit. For example, it has been shown by this approach that both the α and β subunit of F₁ play some role in catalytic activity, since genetic alteration of either of these subunits abolishes ATPase activity (5, 15, 16–20). Similarly, it was through the use of DCCD-resistant mutants that the proteolipid protein of F₁ was most clearly shown to be the site of specific DCCD reactivity, the reaction leading to inhibition of both proton translocation and ATPase activity (4, 21, 22). Genetic techniques also provide a means of amplifying the genes coding for the complex. Overproduction of the complex would facilitate its preparation in large quantity. This approach was used by Young et al. (23) to obtain a substantial increase in the level of membrane-associated NADH dehydrogenase.

Miki et al. (24) have described the isolation of a specialized transducing phage, λasn5, which carries chromosomal DNA including the unc operon. Induction of this phage was shown by Kanazawa et al. (25) to result in increased levels of mem-
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braze-associated ATPase activity and evidence was presented indicating that the five subunits of F₁ were overproduced. Since the ATPase activity was membrane-bound and DCCD-sensitive, it seems likely that all three components of F₀ were overproduced as well as F₁. We have extended this work and demonstrate here that the eight subunits found in the purified F₁,F₆-ATPase of E. coli are overproduced during induction of λααααα.

EXPERIMENTAL PROCEDURES

Bacterial and Viral Strains—The following derivatives of strain KH716 [as-n3, thi, and rif] (24) were used. Strain MM585 was derived by lysogenicizing strain KH716 with λ158757. This phage is thermosensitive due to the c557 mutation and is unable to lyse cells due to the S7 mutation (24). Strain KY7485 (24) was derived from strain KH716 by lysogenicity with λ158757 and the transducing phage λααααα (λc158757[bgIR-C⁺, glms⁻, uncA⁻, asn⁻]), which carries a segment of DNA including the unc operon. In the text, strain KY7485 will be referred to as the λ-unc lysogen and strain MM585 as the λ-ligogen control. Strains AN180 and ML308-229 are nonslyogenic unc⁺ strains which were utilized as sources of F₁,F₆, and F₀, respectively, as described previously (7).

Growth of Cells and Induction of λ Phages—Cells were grown on minimal medium containing 0.1 M potassium phosphate (pH 7.5), 53 mM NH₄Cl, 0.8 mM NaNO₃, 16 mM MgCl₂, and 3.6 mM FeSO₄ supplemented with 78 mM glucose, 14.8 mM thiamin, and 1.8 mM L-asparagine. Asparagine was omitted for growth of strain KY7485. To avoid formation of a precipitate, the concentration of minerals in the medium was initially one-half that stated; the remainder was added 2 h before phage induction. Cells were grown in 10 liters of medium in a 1-liter New Brunswick fermentor, with aeration at 8.5 liters/min and stirring at 400 rpm. Under these conditions, saturation was reached at an OD of 9 units at 550 nm (2.4 × 10¹⁰ cells/ml). Cells were grown at 32°C to an optical density of 3 units (8 × 10¹⁰ cells/ml). λ phage production was then induced by raising the temperature of the medium to 42°C over a period of 17 min. After 30 min, the temperature was reduced to 37°C (over a period of 3 min) and aeration and stirring were continued for 3 h. Membrane-associated ATPase activity was maximal at this time. Cells were harvested, washed, and stored as described previously (7).

Preparation of the ATPase Complex—Membranes were prepared as described (7), except that 6 mM p-amino-benzenesulfonyl fluoride was included in all buffers. In order to purify the ATPase complex in high yield from membranes of the induced λ-unc lysogen, the procedure of Foster and Fillingame (7) (described under "Experimental Procedures") was used without modification. The polyacrylamide slab gels used contained 13% (w/v) acrylamide, 0.2% (w/v) sodium dodecyl sulfate and had dimensions 9 cm × 14 cm. Electrophoresis was carried out with the buffer described previously (21) for 6 h at 20 mA/slab gel.

Analyshe Procedures and Assays—The procedures described (7) were used without modification. The polyacrylamide slab gels used contained 15% (w/v) acrylamide, 0.4% (w/v) N,N,N',N'-tetramethylethylenediamine-bis-acrylamide, and 0.2% (w/v) sodium dodecyl sulfate and had dimensions of 1.5 mm × 9 cm × 14 cm. Electrophoresis was carried out with the buffer described previously (21) for 6 h at 20 mA/slab gel.

Chemicals—Sodium deoxycholate was obtained from CalbiochemBehring (La Jolla, CA). Phenylmethylsulfonyl fluoride was obtained from Sigma (St. Louis, MO). Cholic acid (Sigma) was recrystallized twice from 70% ethanol and neutralized with NaOH. p-Aminobenzenesulfonyl fluoride was obtained from Aldrich (Milwaukee, WI).

| Strain     | Characteristics | Growth conditions | Specific ATPase activity μmol/min/mg protein |
|------------|-----------------|-------------------|--------------------------------------------|
| KH716      | Nonlysogenic unc⁺ | 32°C              | 0.39                                       |
| KY7485 λ-unc lysogen |              | Noninduced        | 0.90                                       |
| KY7485 λ-unc lysogen | Thermally induced |                  | 2.69                                       |
| MM585      | Control λ-lysogen | Noninduced        | 0.40                                       |
| MM585 λ-lysogen | Thermally induced |                  | 0.37                                       |

* Cells were grown on small scale on the minimal glucose medium described under "Experimental Procedures."

| Purification step | Induced λ-unc | Induced λ-lysogen control | Nonlysogenic control |
|-------------------|---------------|----------------------------|---------------------|
| Membrane          | 3.0           | 100%                       | 0.48                |
| Solubilized membra extract* | 57 | 61                          | 100%                |
| Particulate dialyzed extract | 1.55 | 49                          | 0.95                |
| Resolubilized particulate extract* | 35 | Omitted                     | 83*                 |
| (NH₄)₂SO₄ cut (25 to 35%) | Omitted | 2.6                         | 28                  |
| Pooled sucrose gradient fraction | 18 | 12.9                        | 9                   |

* Approximately 20% of the total ATPase activity was inactivated during the extraction step, i.e. it could not be accounted for when the activities of the soluble extract and membrane residue were added.
* The yield at this point was more typically 60%.
* Material applied to sucrose gradient.
* Includes resolubilization of particulate dialyzed extract, ammonium sulfate fractionation, and resolubilization for application to sucrose gradients.
* Material was not pure.
RESULTS

Gene Dosage-dependent Increase in Membrane ATPase—Kanazawa et al. (25) indicated that lysogeny with the \( \lambda \)-unc-transducing phage resulted in an increase in membrane ATPase activity consistent with that expected due to gene dosage. Under the conditions used here, lysogeny with \( \lambda \)-unc resulted in a 2-fold increase in ATPase activity relative to either a nonlysogenic or nontransducing \( \lambda \)-lysogen control (Table I). Thermal induction of the \( \lambda \)-unc lysogen resulted in a further 3-fold increase in ATPase activity, whereas no change in membrane ATPase was observed on induction of the nontransducing \( \lambda \)-lysogen control (Table I). These results are consistent with those reported previously (25), but apply to cells grown on glucose minimal medium and on a large scale (Table II). The level of membrane ATPase activity seems to correlate well with the number of copies of the \( unc \) operon present per cell.

Purification of \( \text{F}_1\text{F}_0 \) from Induced \( \lambda \)-unc Membranes—The \( \text{F}_1\text{F}_0 \)-ATPase was purified from the induced \( \lambda \)-unc membranes by a modification of the procedure of Foster and Fillingame (7). In order to minimize any effects arising from phase induction alone, the complex was also purified by this procedure from strain MM95, which contained a heat-inducible \( \lambda \) prophage identical with the helper phage in strain KY7485. The complex was purified several times from both types of cells and the results of a typical purification are summarized in Table II. In order to efficiently solubilize the ATPase complex from induced \( \lambda \)-unc membranes, the detergent concentration used for extraction was increased relative to that described (7). Similarly, the detergent concentration had to be increased to resolubilize the particulate ATPase formed after dialysis in reasonable yield. Methanol rather than glycerol was used to stabilize the ATPase during resolubilization since it diminished formation of a precipitate that occasionally floated during centrifugation. The ammonium sulfate fractionation procedure was not used in the modified procedure since it proved possible to obtain high purity \( \text{F}_1\text{F}_0 \) from membranes of induced \( \lambda \)-unc without it, and the yield was poor due to problems in resolubilization. Inclusion of the ammonium sulfate fractionation step was necessary in order to obtain high purity \( \text{F}_1\text{F}_0 \) from membranes of the induced, nontransducing \( \lambda \) control. This is indicated by the lower specific activity in Table II and by analysis on acrylamide gels as discussed below.

Subunits Overproduced in Induced \( \lambda \)-unc—The subunit compositions of the purified ATPase preparations described in Table II were compared by SDS-polyacrylamide gel electrophoresis (Fig. 1). The \( \text{F}_1\text{F}_0 \) complex prepared from induced \( \lambda \)-unc by the abbreviated procedure contained eight subunits which migrated identically with those found in the complex purified from a nonlysogenic strain by the original procedure of Foster and Fillingame (7). Furthermore, the relative proportions of each subunit, as judged by the staining intensity, were very nearly equal in the \( \lambda \)-unc and wild type preparations, suggesting a constant stoichiometric relationship. When the complex was prepared from the induced, nontransducing \( \lambda \) control by the abbreviated procedure (Table II), these eight subunits were found as major components in the same relative proportion (Fig. 1). However, other contaminants were also observed, the most prominent being polypeptides with apparent molecular weights of 76,000, 26,000, and 15,000. The relative amount of these contaminants in the induced \( \lambda \)-unc preparation was negligible because of the 6-fold increase in \( \text{F}_1\text{F}_0 \) subunits relative to general membrane proteins.

The results described above indicate that the eight subunits found in \( \text{F}_1\text{F}_0 \) preparations of high purity are all overproduced in equal proportion in the induced \( \lambda \)-unc strain. This was directly demonstrated for most of the subunits by analysis of membranes and partially purified fractions (Fig. 2). Membranes from the induced \( \lambda \)-unc strain show an obvious enrichment in polypeptides migrating at the position of \( \alpha, \beta, \) and the 24,000, 19,000, and 8,400 molecular weight subunits relative to membranes of the induced \( \lambda \)-lysogen control. Increase in the
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MEMBRANE EXTRACT PURIFIED

$F_1 F_0$

FIG. 2. SDS slab gel electrophoresis of fractions from induced λ-unc (+) and induced λ-lysogen control (−) at various stages of purification. From left to right: lanes 1 and 10, $F_1$, 3 μg; lanes 2 and 9, $F_1 F_0$ purified by complete procedure, 5 μg; lanes 3 and 4, membranes of λ-unc or control, 40 μg; lanes 5 and 6, particulate dialyzed extract of detergent-solubilized ATPase from λ-unc or control, 25 μg; lanes 7 and 8, sucrose gradient pool of $F_1 F_0$ from λ-unc or control, 5 μg. Greek letters refer to $F_1$ subunits; 24K, 19K, and 8K refer to $F_0$ subunits; arrows point to induced proteins of unknown identity in λ-unc membranes with apparent molecular weights of 31,000, 20,000, 15,000, and 11,000.

In our earlier report (7), a polypeptide with an apparent molecular weight of 14,000 co-purified with the $F_1 F_0$-ATPase complex prepared from cells grown on succinate/acetate/malate and could not be ruled out as a possible component of the $F_1 F_0$ complex in cells grown under these conditions. When $F_1 F_0$ was purified from the λ-unc lysogen grown on these carbon sources, overproduction of a polypeptide of this molecular weight was not detected.

DISCUSSION

Purification of an $F_1 F_0$-ATPase from *E. coli* was previously reported to yield a complex composed of eight nonidentical polypeptides. The question remained as to whether all eight components were authentic subunits of the complex. Here we have shown that all eight components co-purify in constant stoichiometric proportion from membranes enriched 6-fold for the ATPase complex. This result by itself strongly suggests...
that each of the eight subunits is an unc gene product. However, one could envision such a result if one of these polypeptides was a contaminant that was coded for elsewhere in the chromosome if this contaminant was normally produced in a 6-fold or greater excess over the ATPase complex. This possibility can be dismissed since on comparing membranes from the induced \( \lambda \text{unc} \) strain to control membranes we observed an obvious increase in intensity of polypeptides corresponding in molecular weight to \( \alpha, \beta, \gamma, 24,000, 19,000, \) and \( 8,400 \) (DCCD-binding protein). Subunits \( \delta \) and \( \epsilon \) also appeared to be overproduced in the membrane although identification was less certain. An unlikely possibility that cannot be ruled out is that the 18-megadalton segment of DNA (0.65\% of the E. coli chromosome) carried on the \( \lambda \) phase codes not only for the ATPase subunits but also for a contaminant that fortuitously co-purifies with the complex.

These experiments suggest but do not prove that all eight subunits are coded by genes at the \( \text{unc} \) locus. Conceivably, a regulator produced at the \( \text{unc} \) locus could promote overproduction by genes at another chromosomal location. However, in considering this possibility, it should be noted that mutations affecting the ATPase complex have never been mapped in chromosomal locations other than \( \text{unc} \) (15).

The eight-subunit ATPase complex has been demonstrated to be active in several properties indicative of its function in oxidative phosphorylation. These include \( ^{32}\text{P}-\text{ATP} \) exchange (7), ATP-driven proton pumping as judged by quinacrine quenching (7), and F-\text{mediated} proton translocation (22). Despite these demonstrations, it is possible that the complex is composed of more than eight subunits in \text{vitro}, some of which are lost during purification. Several other membrane proteins were induced during \text{in vivo}, some of which are lost during purification. Several other membrane proteins were induced during \text{in vivo}, some of which are lost during purification.

Other workers have recently reported DCCD-sensitive ATPase preparations of \( E. \) \text{coli}. The preparation of Friedl \text{et al.} (29) is very similar to that discussed here, but does contain components which we think correspond to the \( M_r = 76,000 \) and 26,000 contaminants discussed in Fig. 1 and the original purification paper (7). This preparation exhibited energy-transducing activities similar to those described above (29). Friedl \text{et al.} (29) have cited preliminary findings indicating that the major subunits in their preparation can be synthesized \text{in vitro} from the DNA of an independently constructed \( \lambda -\text{unc} \)-transducing phage, but the data supporting this claim have yet to be published. The composition of the preparation reported by Rosen and Hasan (30) differs significantly from that discussed here and that reported by Friedl \text{et al.} (29). Their preparation seemed to lack not only the \( M_r = 24,000 \) and 19,000 subunits of \( \text{F}_0 \), but also the \( \delta \) subunit of \( \text{F}_0 \). The sensitivity of this ATPase preparation to inhibition by DCCD was greater than that of \( \text{F}_0 \), but less than that of membranes. However, due to its wide reactivity, DCCD is not an entirely specific inhibitor (4), and it will under appropriate conditions inhibit the activity of the \( \text{F}_1\)-ATPase (31). Energy-transducing activities were not demonstrated with this unusual preparation.

Prior to this work, there was little question that the \( \alpha-\epsilon \) subunits of \( \text{F}_1 \) and the \( M_r = 8,400 \) subunit (DCCD-reactive proteolipid) were true components of \( \text{F}_1\text{F}_0 \). The results presented here provide strong \text{prima facie} evidence that the \( M_r = 24,000 \) and 19,000 polypeptides are also true subunits. It is of interest that Kagawa and co-workers (6, 32) observed two subunits other than the proteolipid and those of \( \text{F}_0 \); in their \( \text{F}_1\text{F}_0 \) and \( \text{F}_0 \) preparations from thermophilic bacterium PS3.

However, one of these subunits was not required in what appeared to be a fully reconstituted \( \text{F}_1\text{F}_0 \) complex (13). Unequivocal proof that both the \( M_r = 24,000 \) and 19,000 subunits are essential to the function of \( \text{F}_0 \) will require more refined genetic and/or reconstitution experiments analogous to those reported for the \( \alpha \) and \( \beta \) subunits (\text{unc}A and \text{unc}D genes) (5, 15, 16-20).

REFERENCES

1. Senior, A. E. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R. A., ed), pp. 233-278, Marcel Dekker, New York.
2. Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45-83.
3. Panet, R., and Sanadi, D. R. (1976) Curr. Top. Membr. Transport 8, 99-160.
4. Fillingame, R. H. (1980) Annu. Rev. Biochem. 49, 1079-1113.
5. Futai, M., and Kanazawa, R. (1980) Curr. Top. Bioenerg. 10, in press.
6. Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1979) J. Biol. Chem. 250, 7917-7923.
7. Foster, D. L., and Fillingame, R. H. (1979) J. Biol. Chem. 254, 8220-8236.
8. Pick, U., and Racker, E. (1979) J. Biol. Chem. 254, 2793-2799.
9. Serrano, R., Kanner, B. L., and Racker, E. (1975) J. Biol. Chem. 251, 2453-2461.
10. Stigall, D. L., Galante, Y. M., and Hatzis, Y. (1978) J. Biol. Chem. 253, 966-964.
11. Soper, J. W., Decker, G. L., and Pedersen, P. L. (1979) J. Biol. Chem. 254, 11170-11176.
12. Ryrie, J. L., and Gallagher, A. (1979) Biochim. Biophys. Acta 545, 1-14.
13. Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4219-4222.
14. Haddock, B. A., and Jones, C. W. (1977) Bacteriol. Rev. 41, 47-99.
15. Downie, J. A., Gibson, F., and Cox, G. B. (1979) Annu. Rev. Biochem. 48, 103-131.
16. Fayle, D. R. H., Downie, J. A., Cox, G. B., Gibson, F., and Radik, J. (1978) Biochem. J. 172, 523-531.
17. Dunn, S. D. (1978) Biochem. Biophys. Res. Commun. 82, 596-602.
18. Kanazawa, H., Saito, S., and Futai, M. (1978) J. Biochem. (Tokyo) 83, 1513-1517.
19. Sekior, A. E., Downie, J. A., Cox, G. B., Gibson, F., Langman, I., and Fayle, D. R. H. (1979) Biochem. J. 180, 103-109.
20. Senior, A. E., Fayle, D. R. H., Downie, J. A., Gibson, F., and Cox, G. B. (1979) Biochem. J. 180, 111-118.
21. Fillingame, R. H. (1973) J. Bacteriol. 124, 870-883.
22. Negrin, R. S., Foster, D. L., and Fillingame, R. H. (1980) J. Biol. Chem. 255, 5643-5648.
23. Young, I. J., Jaworowski, A., and Poulis, M. I. (1978) Gene 4, 25-36.
24. Miki, T., Hiraga, S., Nagata, T., and Yura, T. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5089-5103.
25. Kanazawa, H., Miki, T., Tanura, F., Yura, T., and Futai, M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1126-1130.
26. Mizushima, S. (1974) Biochim. Biophys. Res. Commun. 61, 1221-1226.
27. Wolf-Watz, H., and Matters, M. (1979) J. Bacteriol. 140, 50-58.
28. Prasad, L., and Schaefer, S. (1974) J. Bacteriol. 120, 628-650.
29. Friedl, P., Friedl, C., and Schairer, H. U. (1979) Eur. J. Biochem. 100, 175-180.
30. Rosen, B. P., and Hasan, S. M. (1979) FEBS Lett. 104, 339-342.
31. Saito, M., Lunardi, J., Pougeois, R., and Vignais, P. V. (1979) Biochemistry 18, 3134-3140.
32. Okamoto, H., Sone, N., Hirata, H., Yoshida, M., and Kagawa, Y. (1977) J. Biol. Chem. 252, 6125-6131.