RECONSTITUTION OF THE PURIFIED PROTON CONDUCTOR (F_0) OF THE ADENOSINE TRIPHOSPHATASE COMPLEX FROM *ESCHERICHIA COLI*

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1. Introduction

The ATP synthetase (F_0F_1) from bacteria, as well as from mitochondria and chloroplasts, plays a crucial role in energy transducing reactions (reviewed [1–3]). In *Escherichia coli*, as well as in other organisms, the enzyme complex was shown to be composed of a soluble portion (F_1) and of a membrane integral part (F_0), which renders F_1 sensitive to DCCD. Since the hydrolysis of ATP by the bacterial and mitochondrial ATP synthetase complexes is coupled to the translocation of protons across the membrane, it was reasonable to assign the F_0 component, especially the DCCD-reactive protein, a role in the translocation of protons. This view was supported by several lines of experimental evidence [4–6].

For the elucidation of the H^+ translocating mechanism at the molecular level it is necessary to purify the subunits of the F_0 part and to reconstitute them in a defined system. As a first step in this direction the DCCD-reactive protein from *E. coli* has been purified and the amino acid sequences of wild-type and mutant proteolipids have been established [7–10]. Since the F_0 part from *E. coli* is probably composed of three nonidentical subunits, it is quite conceivable that not only the DCCD-reactive protein, but also one or both of the other two subunits may play a role in the H^+ translocating mechanism. Therefore, it is also necessary to characterize the other two polypeptides of the F_0 part. Since both polypeptides lack enzymatic activity and specific labeling procedures are also not available, a purified ATP synthetase complex, active in energy-transducing functions, is the most suitable starting material for the characterization of the F_0 subunits.

Here we describe the preparation of a functional F_0 part from *E. coli*. This F_0 preparation is composed of three polypeptides with app. mol. wt 19 000, 14 000 and 8300.

2. Materials and methods

Escherichia coli K12 was grown in the minimal medium of [11], with either 1% ammonium succinate or 0.2% glucose as the carbon source.

ATP synthetase complex (F_0F_1) was prepared as in [12] with a minor modification: The active ATPase fractions from DEAE–Sepharose CL-6B-column were collected and centrifuged for 15 h at 220 000 × *g*. The precipitated enzyme was resuspended in a small volume of 50 mM Tris–HCl (pH 8) containing 10 mM taurodeoxycholate, 1 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM phenylmethylsulfonylfluoride, 20% (v/v) methanol and 50 μg/ml soybean phospholipids.

For the preparation of F_0, purified ATP synthetase (45 mg) was first dialyzed for 18 h at room temperature against 2 × 1 l of a solution containing 50 mM Tris–SO_4 (pH 8), 5 mM EDTA and 5 mM dithiothreitol. Subsequently, F_0 (2.5 mg) was obtained by treatment of the preparation with 7 M urea [13].

Liposomes containing F_0 protein were prepared from partially purified soybean phospholipids using the cholate dialysis method, and loaded with potassium

Abbreviations: F_0F_1, ATP synthetase complex; F_1, ATP hydrolyzing moiety of F_0F_1; F_0, hydrophobic moiety of F_0F_1; DCCD, *N,N'*-dicyclohexylcarbodiimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; SDS, sodium dodecyl sulfate as in [14]. Fluorescence of ACMA [12] was measured with an Aminco fluorometer SPF 500 using the wavelength 410 nm for excitation and 490 nm for emission. The assay medium in the quartz cuvette (1 X 1 cm) was kept at a constant 26°C.

SDS gel electrophoresis was done by the method in [15] using 13% acrylamide gels. The following proteins were used as molecular weight markers: bovine serum albumin (68 000); ovalbumin (45 000); chymotrypsinogen (25 000); trypsin inhibitor (21 500); cytochrome *c* (12 500). Soybean phospholipids were partially purified as in [16]. Protein was determined by the method in [17] with the modification in [18].

ACMA and TTFB were generous gifts from Dr P. Friedl (Braunschweig).

3. Results

Two different procedures have been published for the purification of the ATP synthetase complex from *E. coli* K12 [12,19].

Deoxycholate was used for the solubilization of the enzyme, followed by fractionation with $(NH_4)_2SO_4$ and further purification on sucrose gradients [19].

ATP synthetase was extracted from thoroughly prewashed membranes using Aminoxid WS 35 and purified the enzyme by ion exchange chromatography [12].

For the dissociation of the enzyme complex into the hydrophilic F_1 part and the membrane-integrated F_0 moiety, we have used a purified ATP synthetase prepared as in [12]. Compared to the procedure in [19], the method used yields a much higher amount of purified enzyme.

In agreement with [12], SDS gel electrophoresis of the purified ATP synthetase complex from *E. coli* revealed 11 polypeptides (fig.1A). In addition to the 5 subunits of F_1 ($\alpha, \beta, \gamma, \delta, \epsilon$) three major protein bands could be observed with the mol. wt 28 000, 19 000 and 8300. According to Friedl [12] the latter three proteins are likely to be constituents of the F_0 part. In addition, polypeptides were observed with app. mol. wt 69 000, 24 000 and 14 000 [12].

Treatment of the F_0F_1 preparation with 7 M urea precipitated a protein fraction (F_0), the subunit composition of which is shown in fig.1B. Predominant polypeptides with app. mol. wt 19 000, 14 000 and 8300 can be observed clearly. The smallest subunit is identical with the DCCD-reactive protein, the molecular weight given being that determined by amino acid sequence analysis [9]. Only traces of polypeptides belonging to the F_1 part are present. Also the contaminating proteins still present in the F_0F_1 preparation are missing. Identical results were obtained with F_0F_1 preparations derived from cells grown either on succinate or on glucose.

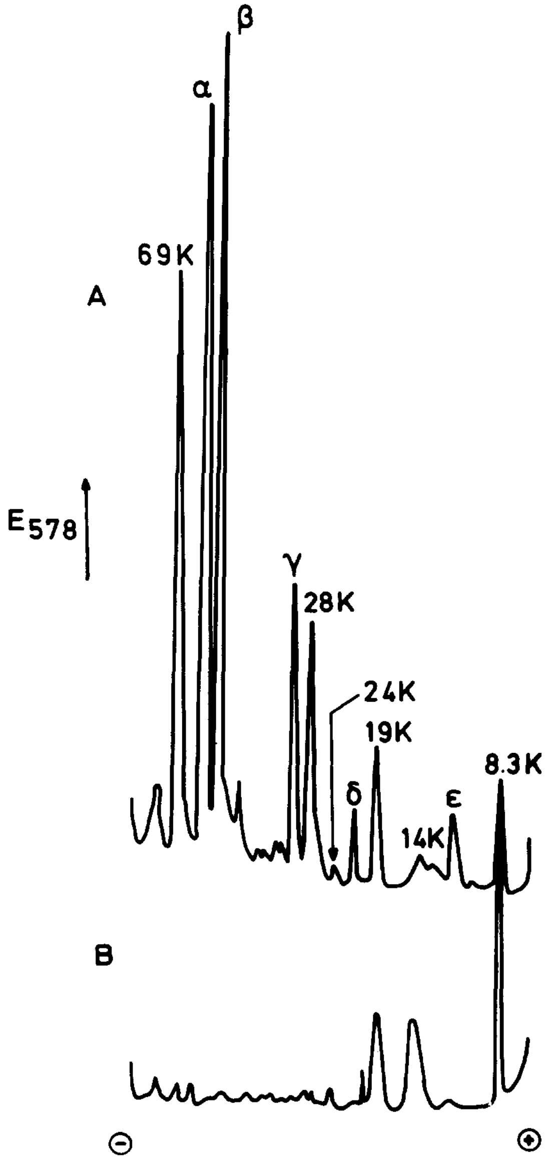

Fig.1. SDS-gel electrophoresis of F_0F_1. (A) F_0F_1 preparation (80 μg); (B) F_0 (80 μg) prepared as in section 2. Gels were stained with Coomassie blue R-250 and the A_{578} was recorded densitometrically. Greek letters denote subunits derived from F_1.

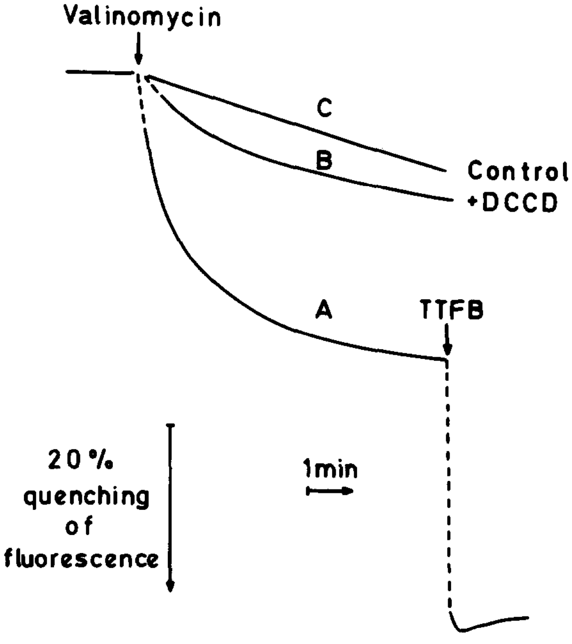

Fig.2. Proton conduction driven by an artificially-imposed membrane potential in F_0 vesicles. The vesicles were prepared as in section 2. K^+-loaded F_0 vesicles (50 µl) were suspended in 1980 µl final vol. in a solution containing 0.5 M sucrose, 2.5 mM $MgSO_4$ and 10 mM Tricine–NaOH (pH 8.0). After the addition of 20 µl ACMA (20 µM) the reaction was started by the addition of 3.6 µl valinomycin (50 µM). TTFB was added at 1 µM final conc. In the control experiment 50 µl K^+-loaded liposomes (without F_0) were used. DCCD (100 nmol) was added 5 min before addition of valinomycin.

To probe that the F_0 component had been purified in a functional state, the F_0 fraction was incorporated into liposomes which were subsequently loaded with K^+. Addition of valinomycin induced a K^+ diffusion potential across the membrane, internally negative. This in its turn provides a driving force for H^+ uptake. As can be seen from fig.2, influx of H^+, as monitored by the fluorescence quenching of ACMA, is very rapid (fig.2A). Further uptake of H^+ could be induced by the addition of the uncoupler TTFB. The latter phenomenon is probably due to the fact that the liposome preparation is composed of F_0-rich and F_0-deficient vesicles [14]. Treatment of the F_0-liposomes with DCCD inhibited H^+ influx to a large extent (fig.2B). As a control, K^+-loaded liposomes lacking the F_0 component were treated in the same manner (fig.2C). The extent of the fluorescence quenching is very similar as in fig.2B.

4. Discussion

A method for dissociating the ATP synthetase complex from a thermophilic bacterium was described in [14]. The F_0 part obtained was still active in proton translocation. Applying a slightly modified procedure for the dissociation of the F_0F_1 complex we were also able to obtain a functional F_0 component from *E. coli*. This preparation contains three polypeptides (fig.1B) with mol. wt 19 000, 14 000 and 8300. This result is somewhat surprising, since SDS gels of the purified F_0F_1 complex prepared by the method in [12] assigned the F_0 subunits mol. wt 28 000, 19 000 and 8300. The 14 000 mol. wt component is also present in the F_0F_1 preparation (fig.1A). Since it was always found in very small amounts it was designated as an impurity [12]. At least two plausible explanations are at hand to explain this puzzling result:

(i) The protein with mol. wt 28 000 is not a constituent of the F_0 part, it disappears by extracting the enzyme with urea.

(ii) This protein is present in the F_0F_1 complex as a dimer and it appears as a monomer after treatment with urea.

Although we cannot exclude either of these two possibilities at the moment, it seems to us that the latter explanation is the most likely one. The question of the subunit composition of the F_0 part is even more complicated by indication of a 24 000 mol. wt protein instead of that of 28 000 being a constituent of the F_0 component [19]. However, the 24 000 mol. wt protein is present in our F_0F_1 preparations only in minute amounts and, even more important, it is not enriched upon treatment of the enzyme complex with urea.

These results are in line with in [13]. The F_0 preparation [13] was initially composed of three polypeptides with the mol. wt of 19 000, 13 500 and 5400. Removal of the 19 000 mol. wt protein still resulted in a functional F_0 component. On the other hand, we have observed that F_0 preparations lacking the 14 000 mol. wt polypeptide are no longer active in H^+ translocation (unpublished). Despite the current discrepancy about the molecular weights of the F_0 subunits there is general agreement that the F_0 part is composed of three nonidentical subunits. Since it is now possible to separate the F_1 from the F_0 component, the elucidation of the function of each subunit is feasible. Together with the analysis of F_0 components defective in H^+ translocation, these studies should lead to a better understanding of the H^+ translocating mechanism.

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References

[1] Bragg, P. D. (1979) in: Microbiology (Schlesinger D. ed) pp. 54–57, Am Soc. Microbiol.
[2] Downie, J. A., Gibson, F. and Cox, G. B. (1979) Ann. Rev. Biochem. 48, 103–131.
[3] Baird, B. A. and Hammes, G. G. (1979) Biochim. Biophys. Acta 549, 31–53.
[4] Altendorf, K., Harold, F. M. and Simoni, R. D. (1974) J. Biol. Chem. 249, 4587–4593.
[5] Nelson, N., Eytan, E., Notsani, B., Sigrist, H., Sigrist-Nelson, K. and Gitler, C. (1977) Proc. Natl. Acad. Sci. USA 74, 2375–2378.
[6] Criddle, R. S., Packer, L. and Shieh, P. (1977) Proc. Natl. Acad. Sci. USA 74, 4306–4310.
[7] Fillingame, R. H. (1976) J. Biol. Chem. 251, 6630–6637.
[8] Altendorf, K. (1977) FEBS Lett. 73, 271–275.
[9] Wachter, E., Schmid, R., Deckers, G. and Altendorf, K. (1980) FEBS Lett. 113, 265–270.
[10] Hoppe, J., Schairer, H. U. and Sebald, W. (1980) FEBS Lett. 109, 107–111.
[11] Davis, B. D. and Mingioli, G. S. (1950) J. Bacteriol. 60, 17–28.
[12] Friedl, P., Friedl, C. and Schairer, H. U. (1979) Eur. J. Biochem. 100, 175–180.
[13] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1978) Proc. Natl. Acad. Sci. USA 75, 4219–4223.
[14] Okamoto, H., Sone, N., Hirata, H., Yoshida, M. and Kagawa, Y. (1977) J. Biol. Chem. 252, 6125–6131.
[15] Laemmli, U. K. (1970) Nature 227, 680–685.
[16] Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1977) J. Biochem. (Tokyo) 81, 519–528.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Dulley, J. R. and Grieve, P. A. (1975) Anal. Biochem. 64, 136–141.
[19] Foster, D. L. and Fillingame, R. H. (1979) J. Biol. Chem. 254, 8230–8236.