PURIFICATION OF THE DCCD-REACTIVE PROTEIN OF THE ENERGY-TRANSDUCING ADENOSINE TRIPHOSPHATASE COMPLEX FROM *ESCHERICHIA COLI*

Karlheinz ALTENDORF
Lehrstuhl Mikrobiologie II, Universität Tübingen, D 7400 Tübingen, FRG

Received 24 December 1976

1. Introduction

The membrane-bound adenosine triphosphatase (EC 3.6.1.3.) from bacteria plays a crucial role in energy-conserving reactions: It catalyzes ATP synthesis proper during oxidative phosphorylation. Furthermore, the hydrolysis of cytoplasmic ATP by the same enzyme is coupled to the translocation of H^+ into the medium, thereby generating an electrical potential and a pH gradient across the membrane which in turn provides energy for many work functions, including active transport of various nutrients and reduction of NADP by NADH [1].

It has been demonstrated that the energy transducing unit, the ATPase complex of *Escherichia coli*, is composed of two structurally distinct entities: One component is the ATPase (BF_1) which actually catalyzes hydrolysis of ATP and which belongs to the category of peripheral proteins. In the case of *E. coli*, the BF_1 component is composed of five different kinds of subunits [2]. The other component (BF_0) is buried within the cytoplasmic membrane and belongs to the category of integral proteins. The BF_1 component of *E. coli* is probably bound to the BF_0 part via the δ-subunit of BF_1 [3]. The energy transformation reactions of the ATPase complex are only observed when the BF_1 is associated with the BF_0 component. The isolation of numerous mutants of *E. coli* defective in oxidative phosphorylation have demonstrated that not only the BF_1 but also the BF_0 component is necessary for energy-transducing functions [4]. Therefore, it seemed desirable to characterize components of the BF_0 part and to ascertain their functional intraplay and interplay with the BF_1 component.

It is now well established that hydrolysis of ATP by the bacterial ATPase complex is coupled to the translocation of protons [1,5,6]. Therefore, it was reasonable to assign the BF_0 component, especially the DCCD-reactive protein, a role in the translocation of protons. This view was supported recently by several lines of experimental evidence [7–11].

Energy-transducing reactions carried out by the ATPase complex are inhibited by dicyclohexylcarbodiimide (DCCD). It is now established that the inhibitor irreversibly exerts its effect on the BF_0 component [12,13], presumably by forming covalent bonds with one of the BF_0 subunits. The DCCD-reactive protein of the BF_0 component has been conclusively identified [12,13]. Further, using mutants whose ATPase activity is no longer inhibited by DCCD, it was possible to correlate directly binding of [^{14}C]DCCD to the protein with inhibition of the ATPase activity [12,13].

It is the aim of this article to describe purification of the DCCD-reactive protein from *E. coli* in DCCD-labeled and unlabeled form

2. Materials and methods

2.1. *Organisms and growth conditions*

E. coli K-12 was grown in the minimal medium of Davis and Mingioli [14], with 0.5% ammonium succinate as the energy source.

2.2. *Chemicals*

Unlabeled dicyclohexylcarbodiimide (DCCD) was obtained from Sigma. [^{14}C]DCCD (25.3 mCi/mmol) was synthesized from [^{14}C]urea by the procedure of Beechey (personal communication) almost identical to that described by Fillingame [12].

2.3. *Preparations of membranes*

Cells were grown to early stationary phase. After harvesting, cells were washed once with 50 mM Tris (hydroxymethyl)aminomethan Tris–SO_4 (pH 7.8), 10 mM $MgSO_4$ and resuspended in that buffer containing 1 mM dithiothreitol (DTT), DNAase and RNAase (about 0.1 mg/ml suspension). Cells were broken by passage through a Ribi press, 20 000 lb . in^{-2}, at 15°C. After 15 min at room temperature, cell debris was removed by centrifugation for 15 min at 10 000 × g. Membranes were pelleted by centrifugation at 35 000 rev./min (142 000 × g_{max}) for 90 min, resuspended in the same buffer and centrifuged again.

2.4. *Labeling of membranes*

The washed membranes were resuspended (20 mg protein/ml) in a medium containing (final concentrations) 0.20 M sucrose, 10 mM Tris–SO_4 (pH 7.5), 5 mM $MgSO_4$ and 0.1 mM DTT. The suspension was made 0.1 mM in [^{14}C]DCCD and stirred for 18–20 h at 4°C. Control assays revealed that this treatment inhibited the ATPase activity up to 80%. The membranes were then washed four times with that buffer and resuspended in water.

2.5. *Crude proteolipid fraction*

[^{14}C]DCCD-labeled membranes were treated with 25 vol. chloroform/methanol (C/M, 2:1) as described [15] and modified [12]. The chloroform/methanol (C/M) extract was carefully washed with water by the procedure [16]. The lower phase and remaining rinsing fluid were made one phase by addition of methanol. One volume of chloroform was then added slowly with stirring and the requisite amount of methanol required to keep the solution clear. That solution could then be taken to dryness on a roto-evaporator at 25–30°C without phase separation and frothing. The residue was dissolved in a small volume of C/M (2:1) and 4 vol. diethylether added slowly at −10°C with stirring. After 24 h at −20°C, the precipitate was removed by centrifugation at 2000 × g for 1 h at −20°C. The precipitate was then dissolved in C/M (2:1, v/v) and precipitation of proteolipids with diethylether repeated. Preparation of proteolipids from unlabeled membranes was done by essentially the procedure above.

2.6. *DEAE-cellulose chromatography*

DEAE-cellulose (Serva, DEAE 52) was prepared as described [17] and the column carefully prepared and washed as outlined [17]. The proteolipid sample was applied in C/M (2:1) and the column washed with 5 vol. C/M (2:1), 5 vol. of C/M (1:1) and 2 vol. of C/M/H_2O (3:3:1). For elution of DCCD-reactive protein an ammonium acetate gradient was applied using C/M/H_2O (3:3:1) as solvent. The pooled fractions were concentrated using a C/M/H_2O mixture resulting in a biphasic system as above [16]. For this purpose, water and chloroform were added to give the following proportions by vol.: C/M/H_2O, 8:4:3. The solvents were mixed and, after phase separation had occurred, the upper phase removed and the lower phase washed with pure solvents upper phase [16]. The lower phase was then taken to dryness and proteolipids precipitated as above.

2.7. *Hydroxypropyl-Sephadex G-50 chromatography*

Hydroxypropyl-Sephadex G-50 was prepared as described [18]. Columns were run in C/M (2:1) containing 24 mM ammonium acetate.

2.8. *Analytical methods*

Polyacrylamide gel electrophoresis was carried out as described [19] with the following modifications: 13% acrylamide, 0.65% *N,N'*-diallyltartardiamide (Serva) as cross-linking agent [20], 0.2% sodium dodecyl sulfate and 0.35 M Tris–HCl (pH 8.8). Since it was found for other purposes that the presence of urea in separating gel leads to better resolution of low molecular weight proteins, 2,4,6 and 8 M urea was included in the gels. The samples were dissolved as described [12]. The gels were stained as outlined [12]. Phosphorus was determined as described [21]. The protein assays were carried out as described [12].

3. Results

In many cases purification of proteins can easily be followed by measuring enzymatic activities. The DCCD-reactive protein, however, lacks such activities. In that case binding of [^{14}C]DCCD to the protein might be a suitable marker. It was found, however, that 80–85% of radioactivity from [^{14}C]DCCD incorporated into membranes was due to unspecific labeling. Since the DCCD-reactive protein was the only

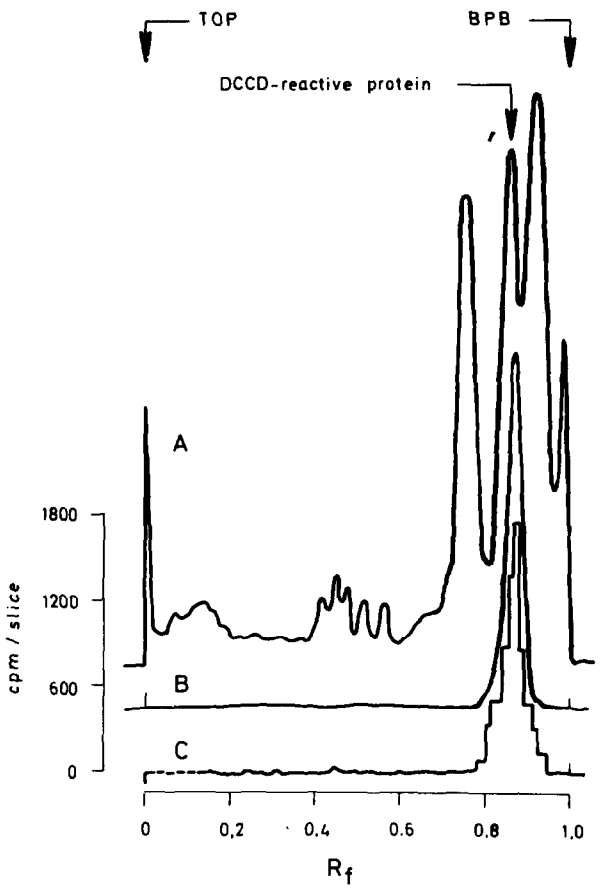

Fig.1. SDS–gel electrophoresis of crude proteolipids (A,C) derived from [^{14}C]DCCD-treated membranes and of purified, [^{14}C]DCCD-labeled carbodiimide-reactive protein (B). The gels contained 13% acrylamide, 0.65% *N,N'*-diallyl-tartardiamide and 0.2% SDS. (A) and (B): Scans of gels stained with Coomassie brilliant Blue. (C): Gel identical to that in (A), cut into 1 mm slices and radioactivity determined. BPB: Bromophenol Blue, tracking dye.

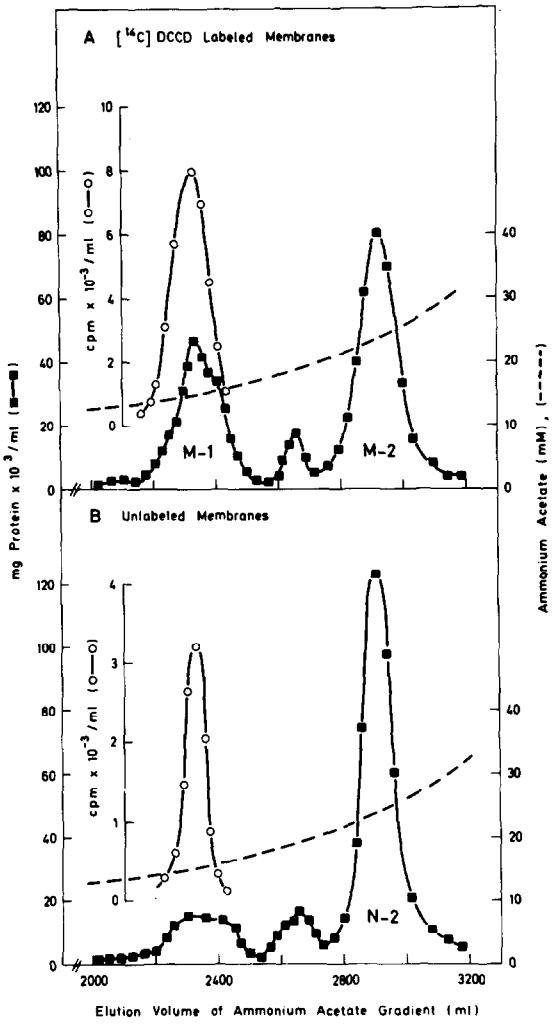

Fig.2. DEAE-cellulose chromatography of crude proteolipids from (A) [^{14}C]DCCD-labeled membranes and (B) unlabeled membranes. The crude proteolipids were applied to the column (340 ml) in C/M (2:1) and the column washed with 5 vol. C/M (2:1), 5 vol. C/M (1:1) and 2 vol. C/M/H_2O (3:3:1). This part of the chromatography was omitted from the figure; for convenience, only part of the ammonium acetate gradient running in C/M/H_2O (3:3:1) is shown: (A) membranes had been treated with sufficient [^{14}C]DCCD (0.5 mCi/mmol) to inhibit ATPase activity maximally (80%). About 50 mg of protein was applied to the column. M-1 refers to the labeled, M-2 to the unlabeled, DCCD-reactive protein; (B) about 46 mg of unlabeled protein, with 1 mg of protein treated with [^{14}C]DCCD (25.3 mCi/mmol) was applied to the column. N-2 refers to the unlabeled DCCD-reactive protein. Symbols: (■—■) indicates protein, (o—o) indicates radioactivity, (- - - -) ammonium acetate gradient.

radioactive component extracted into chloroform/methanol and subsequently precipitated with diethylether [12,13], the purification could be followed easily by monitoring radioactivity.

Since the crude proteolipid fraction still contained several protein components (fig.1) and a considerable amount of phospholipids, indicated by the phosphorus content (10.8 μmol/mg protein), DEAE-cellulose column chromatography, used for the separation of proteins and lipids [17,18], was applied. After trying a series of elution sequences, we arrived at a sequence

that separated well the DCCD-reactive protein from contaminating proteins and phospholipids. The radioactively labeled DCCD-reactive protein was eluted from the DEAE-cellulose column with an ammonium acetate gradient (fig.2). Two major protein peaks were observed, eluting at a salt concentration of 15 mM (M-1) and 24 mM (M-2). The ratio of protein represented by the two peaks was constantly found to be around 1 (M-1): 2 (M-2). The radioactivity peak co-chromatographed with peak M-1. SDS–gel electrophoresis revealed that both proteins had the same relative migration value (data not shown). For further characterization of the labeled DCCD-reactive protein, only peak fractions with constant specific activity were used. In most cases the labeled DCCD-reactive protein was homogeneous after DEAE-cellulose chromatography as judged by SDS-gel electrophoresis in the presence of urea (data not shown).

In some experiments it was observed by SDS–gel electrophoresis that pooled fractions from the DEAE-cellulose column containing [^{14}C]DCCD-labeled protein were contaminated with protein(s) of lower molecular weight. They were, however, successfully removed by chromatography on hydroxypropyl–Sephadex G-50 [22] (data not shown). Also in this case, only fractions with constant specific activity were pooled. SDS–gel electrophoresis revealed that labeled DCCD-reactive protein, purified by this method, was homogeneous (fig.1). Specific activity was about 680-times that of starting material (0.33 nmol DCCD/mg protein). Determination of phosphorus revealed that the so purified, labeled DCCD-reactive, protein contained 0.11 μmol phosphorus/mg protein.

When the proteolipid fraction from membranes, not treated with [^{14}C]DCCD, was applied to a DEAE-cellulose column, only one major protein peak was observed during the ammonium acetate gradient (fig.2). The protein peak appeared at a salt concentration of around 24 mM (N-2). Addition of a small amount of labeled DCCD-reactive protein to the unlabeled proteolipid fraction revealed that the radioactivity, and therefore labeled DCCD-reactive protein, did not co-chromatograph with protein peak N-2. The radioactivity peak eluted at a salt concentration of about 15 mM. SDS–gel electrophoresis in the presence of various concentrations of urea strongly suggests that all three protein peaks (M-1,M-2,N-2) represent the same protein (data not shown).

4. Discussion

The purification of the DCCD-reactive protein of *E. coli* has been achieved using ion-exchange and molecular-sieve chromatography in organic solvents. As judged by gel electrophoresis under different conditions, the protein appeared homogeneous with apparent mol. wt 8000–9000, not 12 000–13 000 as reported earlier [13]. This value agrees well with that found by Fillingame [12]. Purification of DCCD-reactive protein from washed membrane fraction was almost 700-fold.

The behaviour of DCCD-labeled and DCCD-unlabeled protein on DEAE-cellulose was somewhat surprising. DCCD-reactive protein from unlabeled membranes eluted at a higher salt concentration than DCCD-labeled protein. Since both preparations differ only in DCCD-modification, it is tempting to speculate that loss of negative charge may occur by DCCD-reaction with a carboxyl group forming an *N*-acyl urea derivative [13,23].

Another surprising phenomenon was observed upon purification of the [^{14}C]DCCD-labeled protein. When membranes were treated with sufficient [^{14}C]DCCD for maximal inhibition (about 80%) of ATPase activity, roughly one third of total protein represented by peaks M-1 and M-2 (fig.2) was labeled with [^{14}C]DCCD. Since both proteins seem identical, as judged by SDS–gel electrophoresis, at least two explanations are possible. It is conceivable that the DCCD-reactive protein is organized as an oligomer, i.e., a trimer or even hexamer, within the BF_0 component. The reaction of DCCD with one part of the oligomer might lead to reorganization so that the other parts of the oligomer cannot be attacked by DCCD. The other possibility could be that part of the oligomer is associated with other subunits of the BF_0 component in such a way that it is inherently inaccessible to DCCD.

It has been firmly established that the DCCD-reactive protein plays an important role in proton translocation [7–11], in which the whole BF_0 component has been implicated [5,6]. However, the exact role of the BF_0 part in the translocation process is still unknown. Conceivably, the DCCD-reactive protein alone or with other proteins of the BF_0 component might bring about proton translocation. Since the DCCD-reactive protein is the only one so far directly implicated in this process, this protein alone might be required for proton translocation. The first reconstitu-

tion experiments with purified, unlabeled DCCD-reactive protein seem to support this view. Incorporation of that protein into liposomes makes the membrane specifically permeable for protons and treatment with DCCD reduces the high proton permeability. This indicates that the so purified DCCD-reactive protein maintains at least part of its biological activity. However, many more experiments have to be done to ascertain the exact role of DCCD-reactive protein in the proton translocation process and the functional interplay of that protein with other subunits of the BF_0 component.

Acknowledgements

I wish to thank Professor Dr V. Braun for generous financial support, Professor Dr H. Sandermann for suggesting the use of hydroxypropyl–Sephadex G-50, Professor Dr P. Overath for using the Ribi press and Dr E. Oldmixon for a critical revision of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76).

References

[1] Harold, F. M. (1976) Curr. Top. Bioenerg. in press.
[2] Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208–216.
[3] Smith, J. B. and Sternweis, P. C. (1975) Biochem. Biophys. Res. Commun. 62, 764–771.
[4] Schairer, H. U., Friedl, P., Schmid, B. J. and Vogel, G. (1976) Eur. J. Biochem. 66, 257–268.
[5] Mitchell, P. (1973) FEBS Lett. 33, 267–274.
[6] Mitchell, P. (1975) FEBS Lett. 50, 95–97.
[7] Altendorf, K., Harold, F. M. and Simoni, R. D. (1974) J. Biol. Chem. 249, 4587–4593.
[8] Rosen, B. P. (1973) Biochem. Biophys. Res. Commun. 53, 1289–1296.
[9] Rosen, B. P. and Adler, L. W. (1975) Biochim. Biophys. Acta 387, 23–36.
[10] Patel, L., Schuldiner, S. and Kaback, H. R. (1975) Proc. Natl. Acad. Sci. USA 72, 3387–3391.
[11] Patel, L. and Kaback, H. R. (1976) Biochemistry 15, 2741–2746.
[12] Fillingame, R. H. (1975) J. Bacteriol. 124, 870–883.
[13] Altendorf, K. and Zitzmann, W. (1975) FEBS Lett. 59, 268–272.
[14] Davis, B. D. and Mingioli, G. S. (1950) J. Bacteriol. 60, 17–28.
[15] Cattell, K. J., Lindop, C. R., Knight, I. G. and Beechey, R. B. (1971) Biochem. J. 125, 169–177.
[16] Folch, J., Lees, M. and Stanley, G. H. S. (1975) J. Biol. Chem. 226, 497–509.
[17] Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C. and Baumann, A. J. (1969) Meth. Enzymol. 14, 272–317.
[18] Ellingboe, J., Nyström, E. and Sjövall, J. (1969) Meth. Enzymol. 14, 317–329.
[19] Laemmli, U. K. (1970) Nature (London) 227, 680–685.
[20] Anker, H. S. (1970) FEBS Lett. 7, 293.
[21] Ames, B. N. (1966) Meth. Enzymol. 8, 115–118.
[22] Sandermann, H., Jr. and Strominger, J. L. (1972) J. Biol. Chem. 247, 5123–5131.
[23] Khorana, H. G. (1953) Chem. Rev. 53, 145–166.