IDENTIFICATION OF THE DCCD-REACTIVE PROTEIN OF THE ENERGY TRANSDUCING ADENOSINETRIPHOSPHATASE COMPLEX FROM *ESCHERICHIA COLI*

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1. Introduction

Convincing evidence exists that the membrane-bound adenosinetriphosphatase (EC 3.6.1.3.) from *Escherichia coli* is involved in energy transformation. The energy transducing unit, ATPase complex, consists of two components: One is the ATPase (BF_1) itself which can easily be stripped off the membrane; the other component (BF_0) is tightly bound to the cytoplasmic membrane. This ATPase complex catalyzes ATP synthesis proper during oxidative phosphorylation and also makes available cytoplasmic ATP for many work functions, including active transport of various nutrients. Consequently, under anaerobic conditions these work functions are strongly inhibited by dicyclohexylcarbodiimide (DCCD) and are deficient in mutants that have a defective ATPase complex (reviews: refs. [1–5]). The observation that DCCD inhibits only the membrane-bound ATPase and not the solubilized one lent support to the notion that the inhibitor exerts its effect on the BF_0 component of the ATPase complex [6].

There is considerable evidence that the hydrolysis of ATP by the bacterial ATPase complex is coupled to the translocation of protons [7,8]. If that complex itself directly translocates the protons across the cytoplasmic membrane, the BF_0 component, or at least part of it, should meet the requirement of a channel. Evidence for such a channel, specific for protons only, was provided with mutants of *E. coli*, DL-54 [9] and NR70 [10,11], lacking ATPase activity. Both, whole cells (NR70) and vesicles from DL-54 were deficient in respiration-driven transport but could be recoupled following treatment with DCCD. It was demonstrated convincingly that the impaired capacity of whole cells and vesicles to establish an energized state could be attributed to the high permeability of the membrane to protons. This is probably due to an exposed proton-conducting channel (ATPase is either missing, readily lost or altered in some other way) which the reaction with DCCD can seal [9–11], (see also [12,13]). This, indeed, may be the mechanism by which DCCD inhibits the ATPase of the wild type, rather than by the reaction of DCCD in the BF_0 component initiating conformational changes which are transmitted to the attached ATPase [14]. Recently, Mitchell has formulated a mechanism which explicitly assigns the DCCD-reactive protein a role in translocation of the protons ([15,16] for an opposite view see [17]).

It is the aim of this article to describe the identification of the DCCD-reactive protein from *E.coli* membranes by use of the radioactively labeled inhibitor. This approach, as already described for mitochondrial membranes [18,19], is feasible since the carbodiimide is irreversibly bound to the site of action within the membrane.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; ATPase, adenosine triphosphatase (E C 3.6.1.3.); BF_1, soluble ATPase factor from bacteria; BF_0, membrane-located site of DCCD inhibition from bacteria.

2. Materials and methods

2.1. *Organisms and growth conditions*

E.coli strain ML 308–225 (wild type) was grown in the minimal medium of Davis and Mingioli [20],

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with 0.5% glucose as the energy source. *E.coli* strain K12 DG 7/1 (uncB mutant, kindly provided by H.U. Schairer, Max-Planck-Institute, Tübingen) was grown in the same medium supplemented with isoleucine, valine and methionine (100 μg/ml each).

2.2. *Chemicals*

All chemicals used were analytical grade from Merck (Darmstadt) or Serva (Heidelberg). Unlabeled dicyclohexylcarbodiimide (DCCD) was obtained from Sigma. [^{14}C] DCCD (3.1 mCi/mmol) was a generous gift from R. B. Beechey (Shell Research Limited, Sittingbourne, Kent). Radiochemical purity was tested by thin-layer chromatography on pre-coated basic alumina plates (aluminium oxide F_{254}, type E; Merck) with benzene as the developing solvent. A radiochromatogram scanner (Berthold Dünnschicht-Scanner II) was used to detect radioactivity on the plates. The [^{14}C] DCCD was stored at −20°C as a solution in dry methanol.

2.3. *Preparation of membrane vesicles and [^{14}C]DCCD-treated membranes*

Cells were taken from the exponential phase of growth and membrane vesicles were prepared as described by Kaback [21]. The vesicles were suspended at a protein concentration of 20 mg/ml in a medium containing (final concentrations) 50 mM potassium phosphate, pH 7.0 and 1 mM $MgSO_4$, and incubated for 18–20 h at 4°C with the required amount of [^{14}C] DCCD (1 μg/mg protein). The vesicles were then sedimented by centrifuging at 40 000 *g* for 30 min and were washed three times with the same medium. The DCCD-reactive protein was extracted from the [^{14}C] DCCD-labeled membranes by the method of Cattell et al. [18].

2.4. *Assays and analytical methods*

The ATPase assay was performed according to Hanson and Kennedy ([22], method D). Inhibitor studies were done with unlabeled DCCD present during a 30 min preincubation period at 30°C. DCCD was added from a solution in ethanol. Controls received an equivalent amount of ethanol only. Protein concentrations were determined by a modification [23] of the standard Lowry method, with bovine serum albumin as the standard. In samples from organic solvents the protein was determined according to Hess and Lewin [24]. Polyacrylamide gel electrophoresis and staining of the gels was carried out as described by Cattell et al. [18], with the following modifications: The final concentration of acrylamide was 12% (w/v) and the gels were only 60–65 mm long. The distribution of radioactivity in the gels was determined by slicing them into 1 mm discs with a gel slicer (Joyce Loebl and Co. Ltd.), dissolving each disc in 1 ml of 30% (w/v) hydrogen peroxide by heating at 60°C for 12–14 h and measuring the radioactivity in a liquid scintillation counter with 10 ml of Aquasol (New England Nuclear). The molecular weight of the DCCD-reactive protein was determined by sodium dodecyl sulfate electrophoresis as described by Weber and Osborn [25].

3. Results

The membrane-bound ATPase from *E.coli* split ATP with a specific activity ranging between 200–300 nmol·min^{-1}·mg of protein^{-1}, at pH 7.5 and 30°C. Maximal inhibition by DCCD, amounting to 82%, was obtained at concentrations in the incubation medium of 10–20 nmol/mg protein. Inhibitor blanks containing ethanol in equivalent amounts did not show any inhibition. Due to about 10–15% of [^{14}C] dicyclohexyurea in the [^{14}C] DCCD sample, the inhibition curve (data not shown) for commercial non-radioactive DCCD did not quite coincide with that for the [^{14}C] DCCD.

The carbodiimide-reactive protein was identified by labeling membrane vesicles of ML 308–225 with [^{14}C] DCCD. The concentration of [^{14}C] DCCD was kept low (1 μg/mg protein) to avoid unspecific labeling of other membrane proteins. To achieve maximal inhibition (82–85%) with this concentration, membrane vesicles were incubated with [^{14}C] DCCD about 18–20 h at 4°C. The treatment with chloroform–methanol [18] dissolves about 72% of the radioactivity, of which roughly 35–40% resided in a protein fraction that sedimented out of the water-washed chloroform–methanol phase after addition of ether. The precipitated proteins were separated via gel electrophoresis and the gels were analyzed for radioactivity. The resulting radioactivity pattern is shown in fig.1. Under the conditions given above there is one sharp radioactivity peak coinciding with

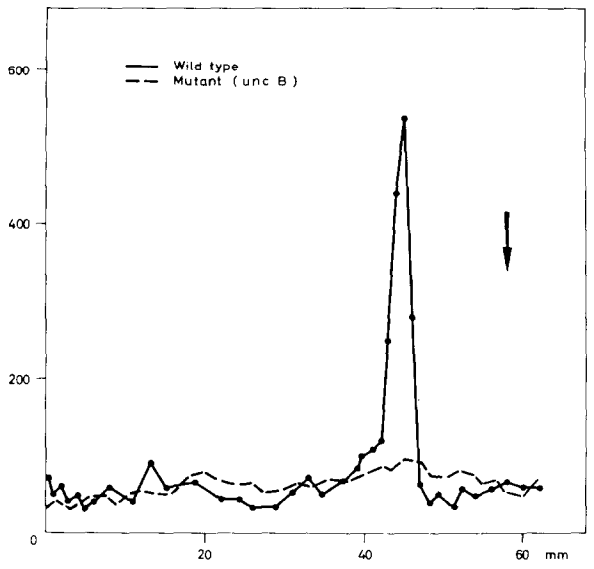

Fig.1 Distribution of radioactivity in polyacrylamide gels from [^{14}C] DCCD-labeled membranes. Membranes (20 mg protein/ml) were treated for 18–20 h with [^{14}C] DCCD (1 μg/mg protein; 3.1 mCi/mmol). Proteins were isolated by chloroform-methanol extraction, gel electrophoresis, fractionation of the gels, and radioactivity analysis as indicated in the Materials and methods section. (——) Membranes from *E. coli* ML 308–225 (wild type); (- - -) membranes from *E. coli* K12 DG 7/1 (uncB mutant). The arrow indicates the tracking dye travel (Bromophenol blue). Ordinate: Radioactivity (d.p.m./slice).

a low mol. wt protein (data not shown) with a mobility of about 0.78, relative to tracking dye travel. Several protein bands on the gel demonstrate that the DCCD-reactive protein is still in an impure form. In some experiments there was also radioactivity at the bottom of the gel (data not shown). This is probably due to radioactively labeled lipids.

Among the mutants with a defective ATPase complex there are strains (uncB mutants) in which the membrane-bound ATPase is no longer inhibitable by DCCD [26,27; H.U. Schairer, personal communication]. Membrane vesicles from such a mutant (K12 DG 7/1) were treated with [^{14}C] DCCD and the proteins were isolated as described above. As shown in fig.1, the sharp radioactivity peak with a mobility of about 0.78 is completely missing, although the protein band is still present on the gel (data not shown).

The mol. wt of the carbodiimide-reactive protein was determined from its electrophoretic mobility, using calibration proteins in the mol. wt range from 11 000 to 60 000. The protein showed electrophoretic mobilities ranging from 0.77 to 0.79. By interpolation, a mol. wt of 12 000–13 000 was determined from the calibration graph in three different experiments.

4. Discussion

The specific inhibition of the membrane-bound ATPase by DCCD is an interesting phenomenon, since the specificity of the inhibitor is not easy to reconcile with the chemistry of such compounds. It is well known that water-soluble carbodiimides react covalently with protein functional groups [28–32]. The potencies, however, of various carbodiimides as inhibitors for the membrane-bound ATPase are related to their non-polar character [33], suggesting that the DCCD-reactive protein is situated within the lipid phase of the cytoplasmic membrane. As already mentioned, low levels of DCCD (less than 10 nmol/mg protein) are necessary to inhibit effectively the membrane-bound ATPase [6]. This is in contrast to the concentrations of water-soluble carbodiimides (100–1000 times more), usually required for covalent reactions with proteins in aqueous solution. It is therefore conceivable that by dissolving in the lipid phase of the membrane the lipophilic DCCD achieves an effective concentration sufficiently high to accomplish a covalent reaction with a protein functional group. As a result of treating membrane vesicles with [^{14}C] DCCD, one protein on the polyacrylamide gel appeared to be radioactively labeled. To account for that specificity it is quite possible that a proton-conducting channel (or a proton carrier) would expose functional groups which might react with the carbodiimide preferentially. Which of the possible functional groups in that membrane protein might be attacked by the carbodiimide is an open question. Some possible stable products formed by the reaction of DCCD with protein functional groups are given in fig.2 [28–32].

The existence of uncB mutants from *E.coli*, where the ATPase is no longer inhibited by DCCD, is of great importance for the argument that the DCCD binds preferentially to one protein. By analyzing the

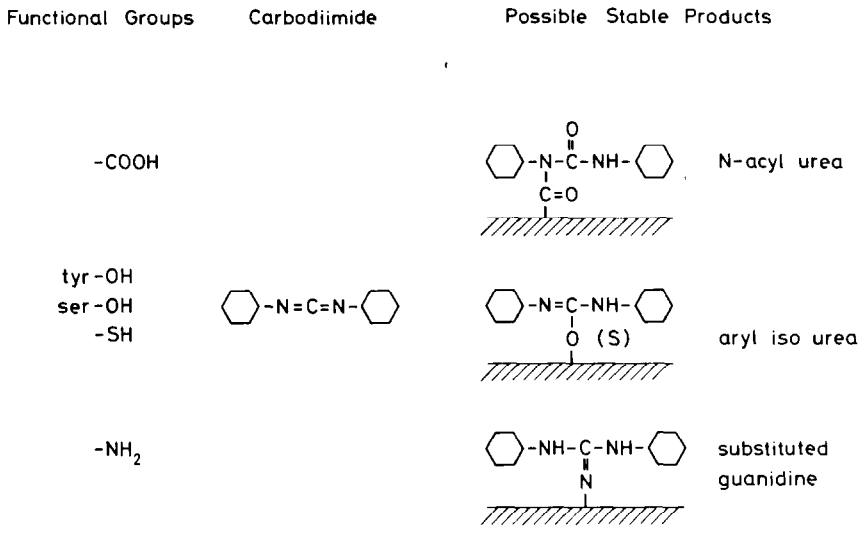

Fig.2 Some possible stable products formed by the reaction of DCCD with protein functional groups.

membrane proteins of such a mutant we found that the radioactivity peak present in the wild type, is completely missing in the mutant. The protein, however, coinciding with the radioactivity peak in the wild type, is still present in the mutant. It is therefore conceivable, that the mutation leads to an altered protein, in which the exposed functional groups are no longer accessible for the DCCD to react. Assuming that the DCCD-reactive protein is a proton-conducting channel, this protein should have a length sufficient to span the lipid portion of the membrane. To evaluate whether the mol. wt of about 12 000–13 000 for this protein can meet the above-mentioned requirements, a comparison with gramicidin A seems indicated. Conductance measurements on the ion flux across lipid bilayer membranes mediated by this antibiotic suggested that transmembrane channels are formed. Furthermore, there was a strong indication that two molecules of gramicidin A are required per channel [34]. Since this would amount to a mol. wt of about 2000, the value found for the DCCD-reactive protein should be sufficient to form a transmembrane channel specific for protons only.

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