Detection of two DCCD-binding components in the envelope membrane of *H. halobium*

Tetsuya Konishi and Naoyuki Murakami

Department of Radiochemistry-Biology, Niigata College of Pharmacy, 5829 Kamishin'ei, Niigata 950-21, Japan

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From labeling studies using [^{14}C]DCCD, the presence of two distinct DCCD-binding sites was proved in halobacterial membrane, whose binding constants are 0.03 and 0.08 nmol DCCD/mg envelope protein, respectively. HPLC and SDS–PAGE analysis revealed that the radioactivity bound to these sites migrated with apparent molecular masses of 45 and 10.2 kDa protein components, respectively.

DCCD-binding protein H. halobium *envelope membrane* *H^+ transport* *[^{14}C]DCCD*

1. INTRODUCTION

Halobacterium halobium which lives in a highly salinated environment has developed a unique photosynthetic system [1], where two retinoid proteins, bacteriorhodopsin [2] and halorhodopsin [3], act as primary light-driven ion pumps. Recently this bacterium was classified into the Archaebacteriaceae together with *Methanobacterium* and *Bacteium thermoacidophilus* based on the unique characteristics of the cell components [4]. The mechanism of ATP synthesis of this bacterium remains as yet unclear. It is therefore of interest whether the mechanism is the same as those of other cell systems.

We have been studying the H^+-coupled membrane potential generation in halobacterial envelope vesicle under illumination and have found [5] that there are at least two DCCD-sensitive H^+-influx process in halobacterial membrane one of which is a hypothetical Na^+/H^+-antiporter [6].

We report here evidence for the presence of two DCCD-binding components in halobacterial membrane.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography; SDS–PAGE, SDS-polyacrylamide gel electrophoresis; $\Delta\psi$, membrane electrical potential; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); V_t, bed volume

2. MATERIALS AND METHODS

[^{14}C]DCCD was obtained from CEA, France (spec. act. 54 Ci/mol). DCCD and SDS were purchased from Nakarai, Pipes from Dojin Kagaku and all other reagents from Wako. All were special reagent grade.

The growth conditions of halobacterial cells (R_1) and preparation of envelope vesicles were done as in [5].

2.1. Determination of bound DCCD

One ml of envelope vesicle suspension in 2.9 M NaCl, 0.1 M KCl, 1 mM Pipes (pH 6.8) containing 1 mg protein was incubated with increasing amounts of [^{14}C]DCCD (spec. act. 2 μCi/mol) for 12 h in the dark at 4°C. Then the labeled envelope vesicles were centrifuged down at 100000 × *g* for 20 min and the pellets purified by repeating resuspension in 1 ml ethanol and centrifugation at 3000 × *g* for 10 min. The pellets were solubilized with 10% (w/v) SDS before radioactivity determination.

2.2. HPLC determination of DCCD-binding components

Each 1 ml envelope suspension was incubated with 1 μl ethanolic solution of [^{14}C]DCCD (spec. act. 54 Ci/mol) with or without 100 nmol unlabeled DCCD overnight at 4°C in the dark, and

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then was centrifuged down. After washing the surface of the pellet with approx. 1 μM aqueous DCCD, the pellet was solubilized with 0.5 ml of 10% SDS without ethanol washing. Ten μl of the solubilized membrane was subjected to HPLC. HPLC conditions were as in [7]. Each portion of 50 drops of column eluents was collected by a fraction collector and the radioactivity determined.

2.3. SDS–PAGE of labeled envelope membrane

SDS–PAGE on gradient gel (7–20% acrylamide) was carried out as in [8]. Protein distribution was determined with a Shimazu DW TLC scanner (at 500–560 nm) after the gel was stained with Coomassie blue. Radioactivity distribution was determined for each 2 mm gel slice.

Radioactivity was measured using an Aloka model LSC-700 liquid scintillation counter after addition of 10 ml universal scintillater (Sintisol EX-H, Dotite).

Protein was determined as in [9] using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Our previous studies showed that the effect of DCCD on the H^+-transport properties in *H. halobium* envelope vesicles was concentration dependent, so the envelope vesicles were titrated by increasing concentrations of [^{14}C]DCCD to determine whether the DCCD binding is also concentration dependent.

As shown in fig.1, a two-step binding profile was obtained, where the first phase was saturated at approx. 30 nmol DCCD/mg protein (30 μM) and the second at above 100 nmol DCCD/mg protein (100 μM), respectively. Scatchard analysis of the above data clearly indicated the presence of two different DCCD-binding sites, whose binding constants were 0.03 (2.9×10^{-5} M) and 0.08 nmol/mg envelope protein (1.6×10^{-4} M), respectively. It was also apparent that the number of sites was 2 for the high-affinity and 3 for the low-affinity binding site, respectively. This DCCD-binding property is quite consistent with the effect of DCCD on light-driven H^+-transport properties reported previously [5], in which an electrically silent H^+-influx process was initially inhibited by approx. 30 nmol DCCD/mg protein, and then inhibition of H^+ influx coupled to $\Delta\psi$ was followed above this concentration.

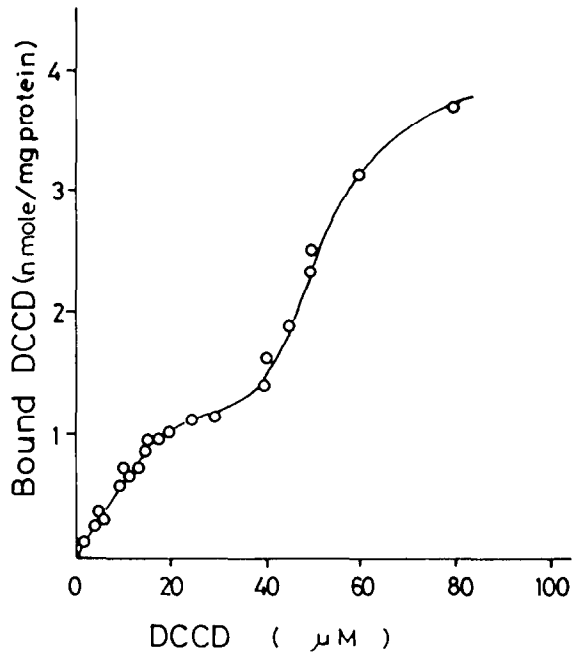

Fig. 1. DCCD titration of membrane vesicles of *H. halobium*. Experimental details are given in section 2.

To identify the membrane components to which DCCD bound, [^{14}C]DCCD-labeled envelope membrane was separated by gel permeation HPLC

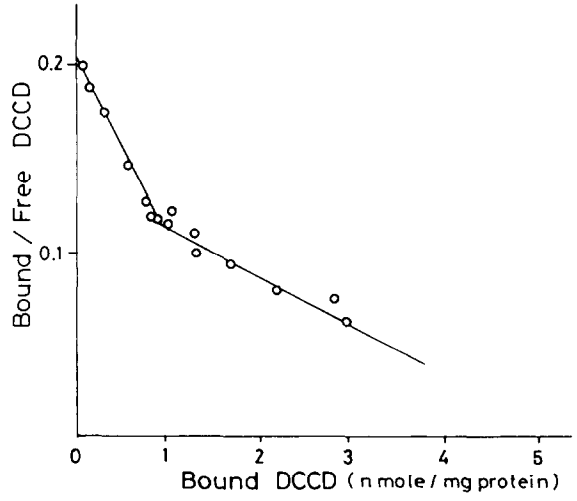

Fig.2. Scatchard analysis of DCCD-binding data of fig.1. Binding constants: 0.03 nmol/mg protein, first component; 0.08 nmol/mg protein, second component.

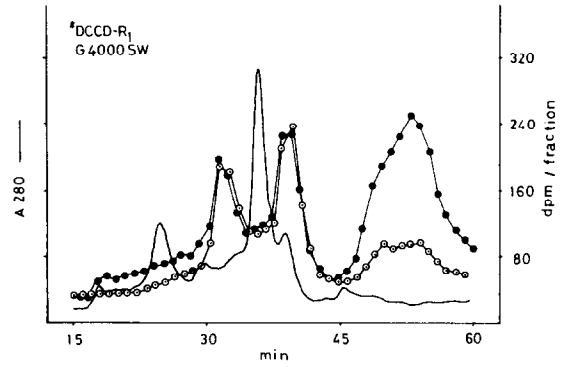

Fig.3. HPLC analysis of DCCD-binding components using a TSK G-4000 SW column. Membrane vesicles were labeled with a low (○) and a high (●) concentration of DCCD.

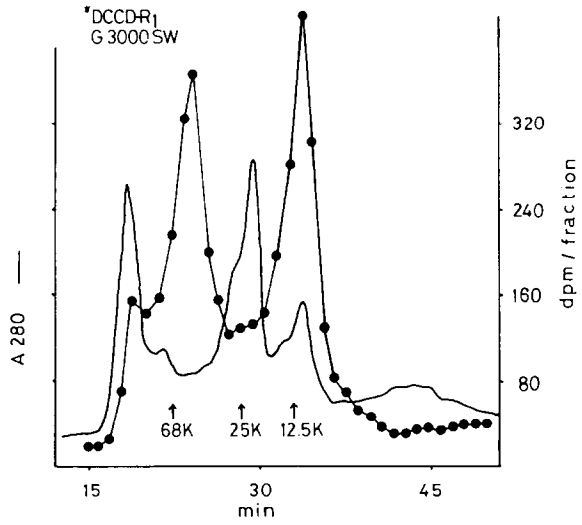

Fig.4. HPLC analysis of DCCD-binding components using a TSK G-3000 SW column. Radioactivity (●).

using a TSK-G4000 SW column (fig.3). Of the three radioactive peaks that appeared, the last one was considered to be due to free DCCD or degradation product, because the peak eluted slower than V_t of the column. Thus the first and the second peaks are protein bound. Apparent molecular masses calibrated for these peaks were 52 and 9.8 kDa, respectively. It was concluded that the first peak corresponds to the high-affinity DCCD-binding component and the second to the low-affinity one from the observation that the radioactivity in the first peak was relatively greater when the membrane was labelled by a smaller amount of [^{14}C]DCCD (this is also apparent in fig.5).

The result obtained with a G3000 SW column, which has a lower exclusion limit than G4000 SW, also indicated the presence of two DCCD-binding components and the second radioactive peak appeared on 10.5 kDa protein peak (fig.4). The molecular mass of the first peak was calibrated as 45 kDa in this case, although the component could not be identified as an apparent protein peak because of small protein contents in the vesicle membrane.

To determine the protein component corresponding to the high-affinity DCCD-binding site, the membrane labeled at a very low DCCD concentration (1 nmol/5 mg protein) was fractionated by Percoll gradient centrifugation and the red membrane fraction thus obtained was further separated on SDS–PAGE because approx. 80% of the radioactivity was appeared in the red membrane fraction. As shown in fig.5, two radioactive peaks appeared exclusively on 45 and 10.2 kDa protein peaks as obtained with the G-3000 SW column. However, the apparent molecular mass for high-affinity component varied from 62 to 45 kDa and for the low-affinity one from 12 to 10.2 kDa depending on PAGE conditions. Thus the actual molecular mass could not be determined until the components were purified.

DCCD is a well known F_0,F_1-type ATPase inhibitor. This binds specifically to the DCCD-binding proteolipid component of F_0 [10] and binds to the β-subunit of F_1 at acidic pH [11], which in combination inhibits H^+ translocation through the H^+-ATPase complex and the catalytic activity of F_1 in mitochondria [12], chloroplasts [13] and bacteria such as *E.coli* [14]. Although the molecular masses obtained for high- and low-affinity DCCD-binding components in *H. halobium* be similar to those of the β-subunit and DCCD-binding peptide of the other system mentioned above, the DCCD-sensitive H^+-transport characteristics which we reported previously cannot be explained solely by these components. Our previous study showed that the electrically silent H^+-influx process was initially inhibited at lower DCCD concentrations and then the electrogenic Na^+/H^+ antiporter activity was inhibited at much higher DCCD concentrations where DCCD began to bind to the low-affinity site (cf. fig.1). Thus, the low-affinity binding component (say 10.2 kDa component) is related to Na^+/H^+-antiporter

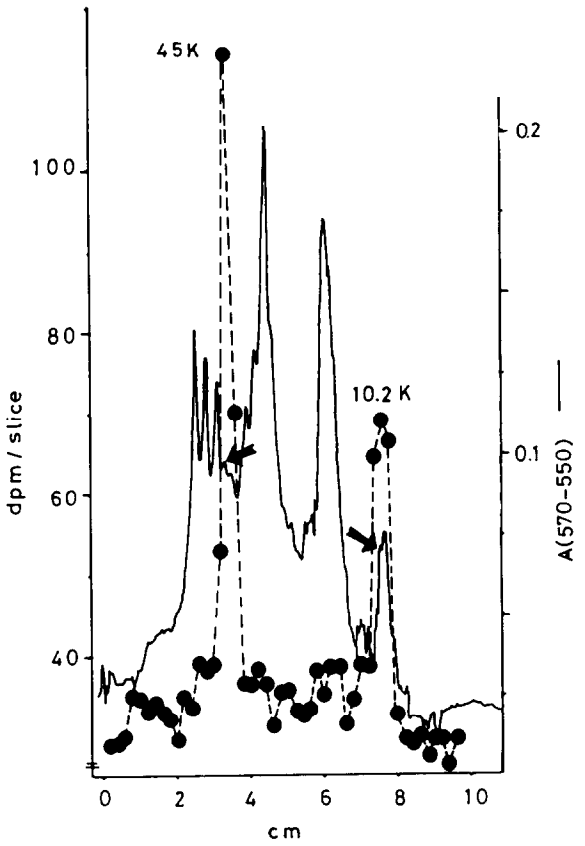

Fig.5. SDS–PAGE separation of [^{14}C]DCCD-labeled red membrane fraction. Envelope vesicles labeled with a low DCCD concentration (1 nmol/5 mg protein) were separated on a Percoll gradient. The red membrane thus obtained was solubilized with 10% SDS, then applied to SDS–PAGE (gradient gel 7–20%). Experimental details are given in section 2. Radioactivity: (●).

activity rather than to F_0. Besides this, our preliminary observation that $CHCl_3$-CH_3OH-extracted proteolipid from ^{14}C-labeled R mR vesicle did not contain any radioactivity also suggests a defect of the conventional DCCD-binding proteolipid in *H. halobium*.

On the other hand, the physiological role of high-affinity DCCD-binding 45 kDa components remains unclear as yet. It is difficult to conclude that this is the same β-subunit of F_1 as that of the other system, because of the above-mentioned role in H^+ transport. However, our preliminary studies strongly suggested that this component is responsible for light-dependent ATP synthesis, because the inhibition constant DCCD (2.2×10^{-5} M) for light-dependent ATP synthesis determined in live cells (unpublished) was almost the same as that of the binding constant for the high-affinity site. It is quite interesting in relation to the unique physiological nature of these organisms that the two DCCD-binding components found here in *H. halobium* could not be determined as conventional F_0,F_1 components. This suggests some uniqueness of the energy-transduction mechanism of *H. halobium*. To approach this, the purification and characterization of these DCCD-binding components are in progress.

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