Biochimica et Biophysica Acta, 723 (1983) 150–159
Elsevier

BBA 41286

PURIFICATION AND FUNCTIONAL PROPERTIES OF THE DCCD-REACTIVE PROTEOLIPID SUBUNIT OF THE H^+-TRANSLOCATING ATPase FROM *MYCOBACTERIUM PHLEI*

NEERAJ AGARWAL and VIJAY K. KALRA *

Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, CA 90033 (U.S.A.)

(Received January 7th, 1983)

Key words: Dicyclohexylcarbodiimide; Proteolipid; ATPase; Vanadate; Proton translocation; (M. phlei)

Interaction of *N,N'*-dicyclohexylcarbodiimide (DCCD) with ATPase of *Mycobacterium phlei* membranes results in inactivation of ATPase activity. The rate of inactivation of ATPase was pseudo-first order for the initial 30–65% inactivation over a concentration range of 5–50 μM DCCD. The second-order rate constant of the DCCD-ATPase interaction was $k = 8.5 \cdot 10^5\ M^{-1} \cdot min^{-1}$. The correlation between the initial binding of [^{14}C]DCCD and 100% inactivation of ATPase activity shows 1.57 nmol DCCD bound per mg membrane protein. The proteolipid subunit of the F_0F_1-ATPase complex in membranes of *M. phlei* with which DCCD covalently reacts to inhibit ATPase was isolated by labeling with [^{14}C]DCCD. The proteolipid was purified from the membrane in free and DCCD-modified form by extraction with chloroform/methanol and subsequent chromatography on Sephadex LH-20. The polypeptide was homogeneous on SDS-acrylamide gel electrophoresis and has an apparent molecular weight of 8000. The purified proteolipid contains phosphatidylinositol (67%), phosphatidylethanolamine (18%) and cardiolipin (8%). Amino acid analysis indicates that glycine, alanine and leucine were present in elevated amounts, resulting in a polarity of 27%. Cysteine and tryptophan were lacking. Butanol-extracted proteolipid mediated the translocation of protons across the bilayer, in K^+-loaded reconstituted liposomes, in response to a membrane potential difference induced by valinomycin. The proton translocation was inhibited by DCCD, as measured by the quenching of fluorescence of 9-aminoacridine. Studies show that vanadate inhibits the proton gradient driven by ATP hydrolysis in membrane vesicles of *M. phlei* by interacting with the proteolipid subunit sector of the F_0F_1-ATPase complex.

Introduction

The membrane-associated ATPase of *Mycobacterium phlei* has been previously shown [1] to be inhibited by DCCD as are other energy-transducing reactions, e.g., oxidative phosphorylation and ATP-driven transport of amino acids and Ca^{2+} [2,3]. The ATPase activity is not inhibited by DCCD when ATPase is solubilized from the membrane [1,4]. Similar effects of DCCD have been observed in mitochondria, chloroplasts and other bacteria [5–10]. Studies carried out in our laboratory [2] demonstrated that the energy-transducing ATPase complex of *M. phlei* is composed of two basic components; one component is the ATPase (F_1), which catalyzes hydrolysis of ATP as well binding ADP and ATP [11], and is localized on the external surface of inside-out membrane vesicles, while the other component F_0 is buried within the cytoplasmic membrane. The energy-transducing reactions of the ATPase synthase are

* To whom requests for reprints should be addressed.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine.

observed only when F_1 is bound to F_0 and these reactions are inhibited by DCCD, presumably by binding irreversibly to the F_0 component as has been demonstrated in other bacteria (for a review, see Ref. 8). Similarly, vanadate has been shown to inhibit membrane-bound ATPase activity of *M. phlei* but does not affect solubilized ATPase activity [12]. However, inhibition by vanadate was reversible, and was additive to irreversible inhibition by DCCD. Moreover, it has been observed that both DCCD and vanadate inhibit formation of a proton gradient in membrane vesicles, although the molecular mechanism of inhibition of ATPase activity and proton conductivity by these agents is not known in *M. phlei*.

The results presented in this study show the stoichiometry of the DCCD interaction with ATPase in membranes of *M. phlei*, as determined by kinetic analysis. We have purified the DCCD-reactive proteolipid component of the ATPase complex to homogeneity. Amino acid analysis indicates that the content of hydrophobic residues is high, as has been observed for the proteolipid component from other sources. Proteolipid component when incorporated into liposomes exhibits H^+ conductivity which is inhibited by DCCD. Our results also show that vanadate, which inhibits membrane-bound ATPase activity, does not affect the binding of DCCD to membranes, but does affect proton translocation.

Materials and Methods

Preparation of membrane vesicles. M. phlei ATCC 354 was grown and harvested as described by Brodie and Gray [13]. Membrane vesicles were prepared by sonication of the cells followed by centrifugation at $144000 \times g$ for 60 min as described by Brodie [14]. The pellet (membrane vesicles) thus obtained was suspended in 50 mM Tris-acetate buffer, pH 8.0, containing 0.15 M KCl and 4 mM $MgCl_2$.

Assay of latent ATPase activity. The ATPase activity in membrane vesicles was determined after unmasking of the latent ATPase by trypsin treatment. The membrane vesicles (2 mg/ml) in 50 mM Tris-acetate buffer, pH, 8.0, containing 0.15 M KCl and 4 mM $MgCl_2$ were treated with bovine pancreas trypsin (50 μg/mg membrane protein) for 10 min at 30°C. After 10 min the reaction was terminated by the addition of soybean trypsin inhibitor (100 μg/mg protein). ATPase activity of the sample was determined [4] in the presence of 4 mM $MgCl_2$ and 10 mM ATP at 37°C by estimating the P_i released from ATP [1].

The inhibition of membrane-bound ATPase activity by DCCD was assayed by the procedure of Kalra and Brodie [1]. Membranes were preincubated with DCCD for the indicated time intervals prior to unmasking of latent ATPase with trypsin.

Estimation of [^{14}C]DCCD binding. Membrane vesicles (1.2 mg protein) suspended in 50 mM Tris-acetate buffer, pH 8.0, containing 0.15 M KCl and 4 mM $MgCl_2$ were incubated with [^{14}C]DCCD (spec. act. 54.6 Ci/mol) for the indicated time intervals at 37°C in a total volume of 0.5 ml. The reaction was terminated with an equal volume of acetone and centrifuged in microcentrifuge for 5 min at $10000 \times g$. The pellet thus obtained was washed twice with 2.5% trichloroacetic acid. The pellet was solubilized in 1 M NaOH, mixed with 5 ml of Aquasol scintillation fluid and the radioactivity counter after neutralization with HCl.

Isolation of [^{14}C]DCCD reactive proteolipid. Membrane vesicles of *M. phlei* at a protein concentration of 15 mg protein/ml suspended in 50 mM Tris-acetate buffer, pH 8.0, containing 0.15 M KCl and 4 mM $MgCl_2$ were incubated with [^{14}C]DCCD (100 μM) for 16 h at 4°C. The suspension was diluted (10 ml) with the above buffer and the contents centrifuged at $144000 \times g$ for 45 min. The pellet was washed twice with the above buffer and proteolipid extracted essentially according to the method of Sone et al. [15]. The labeled pellet (2–5 mg) was mixed with 5 ml of 50% methanol containing 10 mM $MgSO_4$ and incubated at room temperature for 10 min. The suspension was centrifuged at $10000 \times g$ for 10 min. The pellet obtained was homogenized in 2.5 ml of $CHCl_3/CH_3OH$ (3:2, v/v) using a glass homogenizer and stirred for 30 min at room temperature and again centrifuged at $15000 \times g$ for 15 min. The supernatant was carefully removed from the fluffy layered pellet, cooled to 0°C and mixed with 5 vol. of precooled (−20°C) diethyl ether and the mixture kept at −20°C for 16 h. The precipitated proteolipid was collected by centrifugation

and dissolved in $CHCl_3/CH_3OH$ (3:2, v/v) for application to a Sephadex LH-20 column for further purification as described by Soto et al. [16]. The column (1.8 × 30 cm) was eluted with following solvents: (i) 80 ml $CHCl_3$, (ii) 20 ml $CHCl_3/CH_3OH$ (15:1, v/v), (iii) 20 ml $CHCl_3/CH_3OH$ (10:1, v/v), (iv) 20 ml $CHCl_3/CH_3OH$ (6:1, v/v), and (v) 20 ml $CHCl_3/CH_3OH$ (4:1, v/v). Fractions of 2 ml were collected at a flow rate of 0.5 ml/min. Radioactivity in each fraction was monitored in Beckman LS-8000 scintillation counter.

Reconstitution of F_0 in liposomes. The proteolipid F_0 required for reconstitution studies was extracted by butanol as described by Sigrist et al. [17]. The proteolipid was solubilized from membrane vesicles with either butanol alone [18] or butanol followed by precipitation with diethyl ether. The proteolipid was collected by centrifugation and suspended in a small volume of *n*-butanol. The proteolipid was incorporated into liposomes by a modified procedure of that of Schindler and Nelson [18]. The proteolipid (50–100 μg protein) in butanol was mixed with 18 mg acetone-purified asolectin as a dry film in a round-bottom flask (Associated Concentrates Inc., NY). The contents were dried under N_2 at room temperature and suspended in 1 ml of 10 mM Tricine-NaOH buffer, pH 8.0, 0.25 M sucrose, 0.4 M KCl containing 0.65 mM dithiothreitol. The contents were sonicated in a Corex tube for 3 min in a Branson 220 sonifier bath at 25°C under an N_2 atmosphere. The proteoliposomes thus obtained were dialyzed for 18 h in the above buffer except that KCl was omitted.

Assay of H^+ movement in proteoliposomes. The quenching of 9-aminoacridine was used to monitor H^+ movements in K^+-loaded proteoliposomes as described by Celis [19]. The fluorescence measurement was carried out in a Perkin-Elmer MPF-4 spectrofluorometer equipped with a thermostatically regulated cuvette holder. Unless otherwise mentioned, the temperature was maintained at 37°C.

Phospholipid analysis of proteolipid. Purified proteolipid (500 μg protein) was suspended in 5 ml $CHCl_3/CH_3OH/H_2O$ (64:32:4, v/v) and dried under reduced pressure in a rotary evaporator as described previously [20]. This procedure was repeated twice. The residue was dissolved in chloroform and filtered through Whatman No. 1 filter paper. Individual phospholipids were separated on silica gel G plates using the solvent system $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v). The resolved phospholipids were visualized by exposure to iodine vapor and identified by comparison with authentic standard phospholipids [21]. The phospholipids identified were scraped from the plate and their phosphorus contents were determined according to the method of Marinetti [22].

Estimation of molecular weight by polyacrylamide gel electrophoresis. Gel electrophoresis of the purified proteolipid fraction was carried out in 13% (w/v) acrylamide containing 8 M urea and 0.5% SDS as described by Fillingame [23]. Standard, low molecular weight markers (M_r range 2512–16 949) obtained from BDH Chemicals, U.K., were used for estimation of the R_m value and molecular weight. The gels were cut into 1-mm slices by a Bio-Rad electric gel slicer. After digestion with 200 μl of 30% H_2O_2 at 37°C for 48 h, the slices gels were counted for radioactivity in Aquamix (West-Chem Products, San Diego, CA) using a Beckman LS-8000 scintillation counter. A Hewlett-Packard programmable computer with on-line display was used for plotting the data and obtaining correlation coefficient values.

Protein determination. Protein in proteolipid was determined by the method of Lowry et al. [24] as modified by Bailey [25] essentially according to the procedure of Fillingame [7]. Proteolipid sample was solubilized by adding 2.5% SDS in 0.5 M NaOH followed by incubation at 37°C for 2 h. The 2% Na_2CO_3 (reagent A [24]) contained 1% SDS for these assays.

Analytical methods. The sample protein was hydrolyzed in 6 M HCl at 110°C for 24 h and its amino acid composition was analyzed using a Durrum D-500 amino acid analyzer.

Materials. [^{14}C]DCCD (54.6 Ci/mol) was purchased from Research Products International Corp., IL; asolectin from Associated Concentrates, NY; vanadate (orthovanadate) from Fisher Scientific Co.; and ATP (vanadate-free) and 9-aminoacridine from Sigma Chemical Co. All other reagents were obtained commercially and were of reagent grade. Low molecular weight protein standards, Product No. 44272 (M_r range

2512–16949) were purchased from BDH, Poole, U.K.

Results

Relationship between [^{14}C]DCCD binding and inhibition of ATPase activity in membrane vesicles of M. phlei

The time course of both inhibition of membrane-bound ATPase activity and [^{14}C]DCCD binding was measured in *M. phlei* membrane vesicles at 37°C. As shown in Fig. 1A, the inactivation of ATPase activity was both time and concentration dependent and at higher DCCD concentrations (50 μM) approx. 80% of the membrane-bound ATPase activity was inhibited [1]. Similarly, the binding of [^{14}C]DCCD to *M. phlei* membranes was dependent upon both concentration of DCCD and time of incubation, being completed within 2 h of incubation (Fig. 1B). Fig. 1C shows the relationship between binding of [^{14}C]DCCD and inhibition of ATPase activity. Extrapolation of the initial linear portion of the curve to 100% inactivated ATPase activity reveals that under these conditions 1.57 nmol [^{14}C]DCCD bind per mg membrane protein of *M. phlei* membranes, whereas the value for the second portion of the curve was approx. 6.82 nmol [^{14}C]DCCD bound per mg protein. The latter value presumably involves the interaction of [^{14}C]DCCD with protein sites in the membrane which are not part of the F_0-F_1 complex. Vanadate, which inhibits membrane-bound ATPase activity and formation of proton gradient [12], in *M. phlei* did not significantly affect the binding of [^{14}C]DCCD to membranes as 1.67 nmol [^{14}C]DCCD were bound in the presence of vanadate (100 μM).

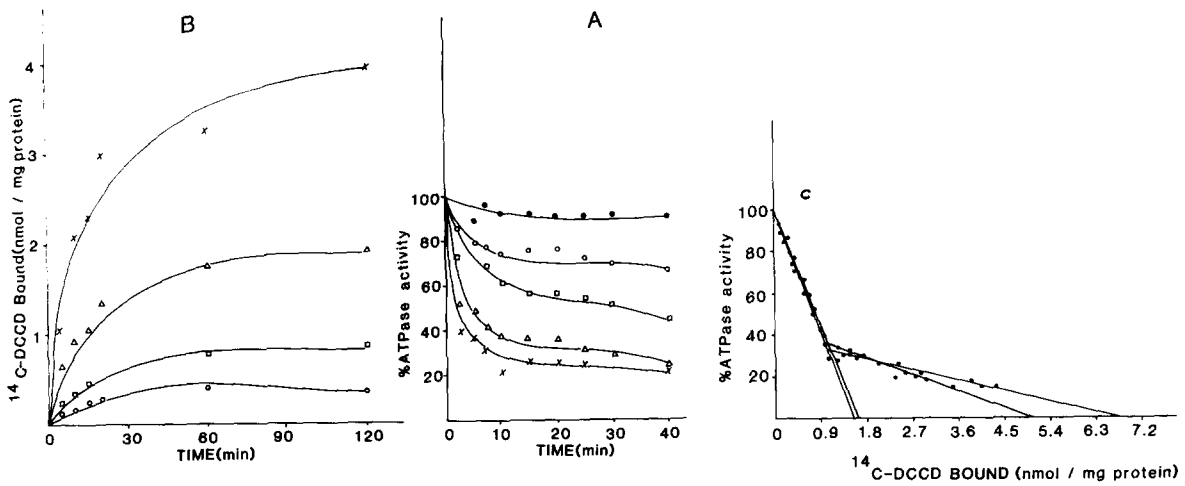

Fig. 1. (A) Time course of inhibition of ATPase activity in membrane vesicles of *M. phlei*. Membrane vesicles (2 mg/ml) were treated with trypsin prior to incubation with various concentrations of DCCD for the indicated time intervals. The ATPase activity was analysed by determining the released P_i as described in Materials and Methods. DCCD: 2 μM (●———●), 5 μM (○———○), 10 μM (□———□), 25 μM (△———△), and 50 μM (×———×). The results are shown for a typical experiment. Similar results were obtained in five separate experiments. (B) Time course of binding of [^{14}C]DCCD to membrane vesicles. Membrane vesicles (1.2 mg protein) were incubated with various concentrations of [^{14}C]DCCD for the indicated time periods at 37°C. After the indicated time interval, the reaction was terminated by the addition of acetone and 2.5% trichloroacetic acid as described in Materials and Methods. The radioactivity associated with the precipitated protein was dissolved in 1 M NaOH and counted in a liquid scintillation counter, after neutralization. [^{14}C]DCCD: 5 μM (○———○), 10 μM (□———□), 25 μM (△———△), and 50 μM (×———×). (C) Relationships between [^{14}C]DCCD binding and inhibition of ATPase activity. The data in B of [^{14}C]DCCD binding and the corresponding inhibition of ATPase activity in A were replotted to determine the relationship between the binding of [^{14}C]DCCD and inhibition of ATPase activity. The data in the plot of % ATPase activity were corrected for activity insensitive to DCCD (13%). Linear regression analysis was performed on a programmable Hewlett-Packard computer attached to a plotter to extrapolate all the points within the range 0–1.8 nmol [^{14}C]DCCD bound/mg protein to zero activity. [^{14}C]DCCD bound (●———●), [^{14}C]DCCD bound in the presence of vanadate (100 μM) (○———○).

Kinetics of the DCCD-ATPase interaction

Since the activity-binding correlation involves the determination of the covalently bound inhibitor, the kinetics of the inhibitory effect were analysed to define the DCCD-ATPase interaction. The kinetic analysis approach, i.e., evaluation of pseudo-first-order rate constants (k') of the reaction as a function of the inhibitor concentration, has been used by Levy et al. [26] for irreversible-inhibitor-enzyme interaction. As shown in Fig. 2A, the rates of inactivation of the latent ATPase of *M. phlei* membranes were pseudo-first order for the initial 30–65% inactivation over a concentration range of 5–50 μM DCCD. The inactivation half-time ($t_{1/2}$) was calculated by determining the time required by DCCD to cause 50% loss of the ATPase activity. As shown in Fig. 2B, a plot of t vs. 1/DCCD gave a straight line with the intercept at t, the minimum inactivation half-time of 1 min.

The plot (Fig. 2C) of pseudo-first order rate constants (k') vs. DCCD concentration shows that the reaction with respect to DCCD is of about first order. The second-order rate constant of DCCD interaction with membranes is $k = 8.5 \cdot 10^5\ M^{-1} \cdot min^{-1}$. However, the experimental points in Fig. 2A deviate from linearity, indicating that the simple pseudo-first-order kinetics may not suffice to give an absolute value for the time course of inhibition. This may be due to the pH-dependent breakdown of DCCD which exists in aqueous solution. As a consequence, the actual concentration of added DCCD which remains bound at the inhibitory site afer completion of the reaction will be less than that actually added.

Purification of the [^{14}C]DCCD proteolipid

The DCCD reactive binding protein of *M. phlei* was extracted with $CHCl_3/CH_3OH$ from [^{14}C] DCCD-treated membranes by a procedure similar to that described for *Escherichia coli* protein [7,23]. The DCCD-reactive proteolipid was precipitated from the $CHCl_3/CH_3OH$ extract by addition of diethyl ether as described previously [23]. The DCCD-reactive protein was purified from the crude proteolipid fraction on Sephadex LH-20. As shown in Fig. 3 the DCCD-reactive protein was eluted with $CHCl_3$ only. SDS gel electrophoresis revealed that this fraction was homogeneous, since a single Coomassie blue-stained band which coelectrophoresed with the radioactivity due to [^{14}C]DCCD was observed (Fig. 4). The molecular weight of the proteolipid was estimated to be 8000 by comparison of the mobility of the known standards. The amino acid analysis of the purified

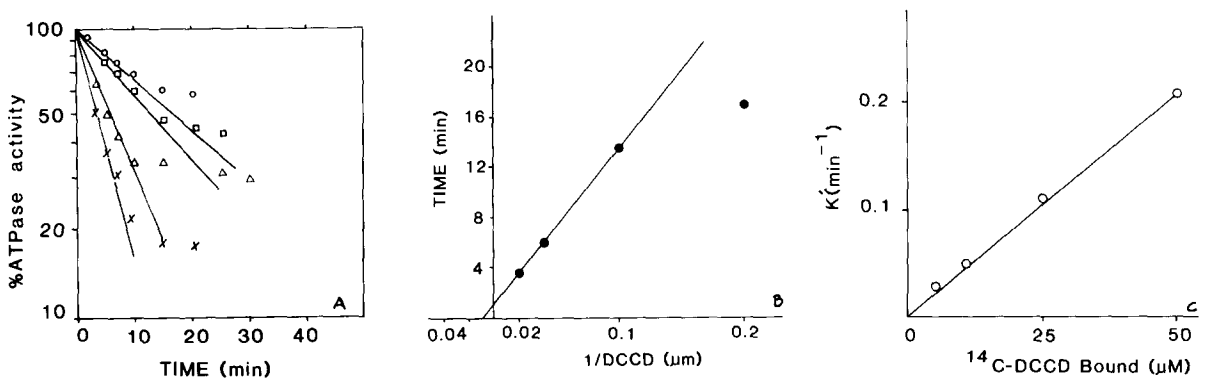

Fig. 2. (A) Semilogarithmic plot of the inhibition of ATPase activity by various concentrations of [^{14}C]DCCD. The data in Fig. 1A were replotted on a semilogarithmic scale. DCCD: 5 μM (○——○), 10 μM (□——□), 25 μM (△——△), and 50 μM (×——×). (B) Inactivation half-time ($t_{1/2}$) for inhibition of ATPase activity as a function of the reciprocal of the DCCD concentration. ATPase activity was determined as described in Materials and Methods. The inactivation half-time ($t_{1/2}$), i.e., the time to obtain 50% inactivation of the enzyme activity, was taken from A and plotted against the reciprocal of DCCD concentration. The least-squares fit was obtained using a programmable computer. (C) Plot of the pseudo-first-order rate constants of the DCCD-ATPase interaction (k') in membrane vesicles of *M. phlei* as a function of DCCD concentration. Values of $t_{1/2}$, i.e., the time to obtain 50% inactivation of ATPase, were derived from A. The k' values were calculated from $k' = 0.693/t_{1/2}$. The second order rate constant of the DCCD-ATPase interaction (k) was $8.5 \cdot 10^5\ M^{-1} \cdot min^{-1}$.

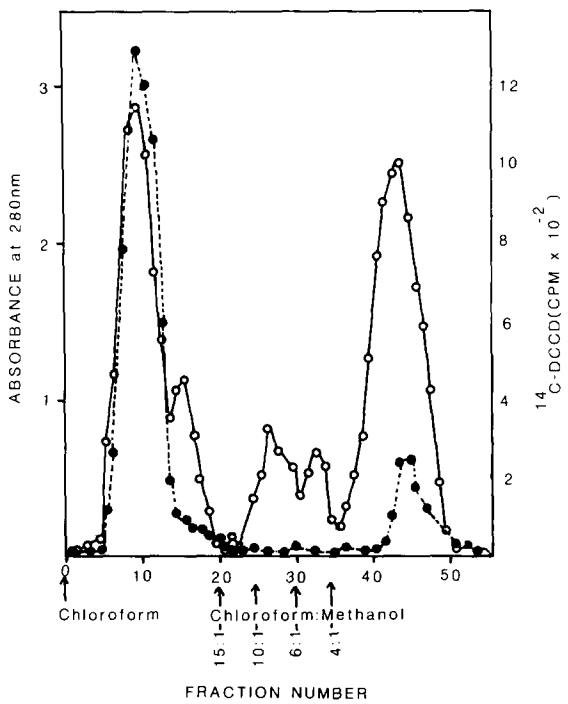

Fig. 3. Sephadex LH-20 chromatography of DCCD-reactive proteolipid. Diethyl ether-precipitated proteolipid (1 mg protein) was loaded on a Sephadex LH-20 column preequilibrated with $CHCl_3$. Elution was carried out with $CHCl_3$ and mixtures of $CHCl_3/CH_3OH$ as described in Materials and Methods. Absorbance at 280 nm (O——O) and [^{14}C]DCCD radioactivity (●——●) in each fraction were monitored.

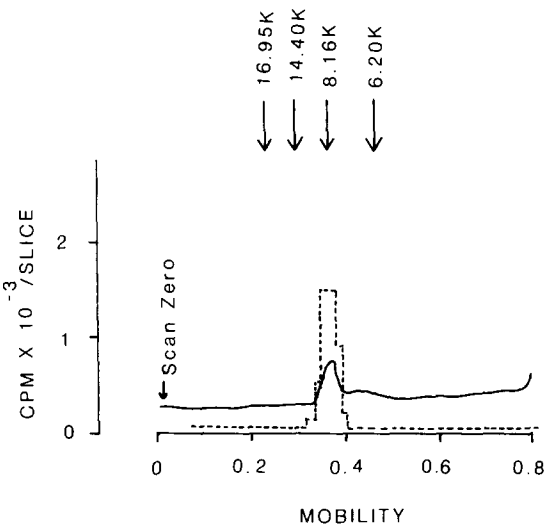

Fig. 4. Polyacrylamide gel electrophoresis of [^{14}C]DCCD-bound proteolipid. [^{14}C]DCCD-bound F_0 proteolipid obtained by Sephadex LH-20 chromatography was electrophoresed on a 13% polyacrylamide gel containing 8 M urea in the presence of 0.5% SDS as described in Materials and Methods. (——) Densitometric scan of the Coomassie blue-stained gel was carried out on a Zeineh soft laser scanning densitometer at 555 nm. (------) Profile of [^{14}C]DCCD radioactivity on an identical gel that was cut into 1-mm slices. The arrows indicate the relative migration of the molecular weight standard (M_r range 2512–16 949: myoglobin III, 2512 polymerized yielding; myoglobin II, M_r 6214; myoglobin I, M_r 8519; myoglobin I and II, M_r 14 404; and myoglobin 16 949) obtained from BDH, Poole, U.K. The molecular weight of proteolipid was determined by plotting R_m values versus log M_r of standards utilizing a linear regression analysis program on a computer plotter. K, kDa.

proteolipid is shown in Table 1. Cysteine and tryptophan were not observed, as has been observed for ATPase synthase proteolipid from mitochondria and other bacterial species [8]. Of the nonpolar amino acids that predominate, abnormally high contents of glycine (15%), alanine (13%) and leucine (13%) were present. The tyrosine content (1%) was low. The polarity calculated according to the method of Capaldi and Vanderkooi [27] was 27%.

Phospholipid composition of proteolipid

The purified proteolipid from *M. phlei* was analyzed for its lipid composition. As shown in Table II, proteolipid contained phosphatidylethanolamine (18%), phosphatidylinositol (67%) and cardiolipin (8%). The nature of phospholipids present in the proteolipid was similar to that observed in membranes from which it was isolated, although the percentage of various phospholipids present in proteolipid was different from the present in native membranes (Table II).

Effect of DCCD on H^+ translocation in F_0 proteoliposomes

As shown in Fig. 5, addition of valinomycin to proteolipid-incorporated liposomes loaded with KCl caused an influx of H^+, as indicated by the quenching of the fluorescence of 9-aminoacridine. Addition of DCCD resulted in an increase in the fluorescence, indicating that DCCD inhibits H^+ transfer. Preincubation of proteoliposomes with DCCD (50 μM) for 10 min prevented the quenching of fluorescence induced by valinomycin, indicating that H^+ migrated through proteolipid rather

TABLE I

AMINO ACID COMPOSITION OF THE PURIFIED DCCD-BINDING PROTEOLIPID FROM *M. PHLEI*

The proteolipid samples were dried under argon and hydrolysed for 24 h in 6 M HCl as described in Materials and Methods. The closest integral number of each residue is shown in parentheses.

Amino acid	Amount in proteolipid (mol/mol)
Aspartic acid	4.37 (4)
Threonine	4.96 (5)
Serine	3.33 (3)
Glutamic acid	5.03 (5)
Proline	5.74 (6)
Glycine	12.00 (12)
Alanine	11.38 (11)
Cysteine [a]	0 (0)
Valine	2.96 (3)
Methionine	2.17 (2)
Isoleucine	6.69 (7)
Leucine	11.06 (11)
Tyrosine	1.43 (1)
Phenylalanine	4.80 (5)
Histidine	0.54 (1)
Lysine	1.24 (1)
Arginine	3.22 (3)
Tryptophan	0 (0)
Cysteic acid	0 (0)
Total residues	81
Polarity [b]	27%

[a] The lack of cysteine was confirmed by analysis for cysteic acid after performic acid oxidation.
[b] Calculated according to Ref. 27.

TABLE II

PHOSPHOLIPID COMPOSITION OF PURIFIED DCCD-REACTIVE PROTEOLIPID FROM MEMBRANE VESICLES OF *M. PHLEI*

The lipids were extracted and analyzed from 'F_0' proteolipid as described in Materials and Methods. The data of membrane vesicle phospholipid composition were taken from our earlier publication [39]. Values given as % total phospholipid.

	Individual phospholipids			
	Phosphatidylethanolamine	Cardiolipin	Phosphatidylinositol	Uncharacterized lipids
Membrane vesicles	29.40	15.80	47.00	7.80
DCCD proteolipid	18.50	8.75	67.09	5.64

than being adsorbed to it. These results indicate that the proteolipid fraction of the F_0-F_1 complex from *M. phlei* is functional in mediating the translocation of H^+, as has been observed for proteolipids extracted from mitochondria [18] and thermophilic bacterium PS-3 [15].

Effect of vanadate on H^+ translocation in proteoliposomes

Previous studies [12] from our laboratory have shown that vanadate (52 μM) inhibits membrane-bound ATPase activity and the ATP-driven H^+ gradient. Studies were undertaken to delineate whether vanadate inhibited the formation of an H^+ gradient by interacting with the proteolipid sector of the F_0-F_1 complex. As shown in Fig. 5, addition of vanadate (50–100 μM) caused reversal of quenching of the fluorescence of 9-aminoacridine in proteolipid-reconstituted K^+-loaded liposomes, induced by valinomycin.

Addition of DCCD (100 μM) caused further

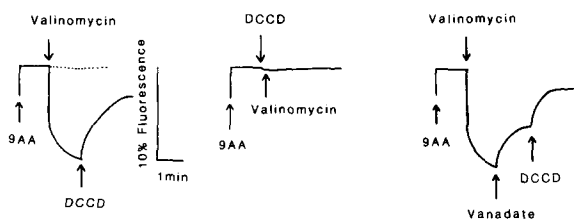

Fig. 5. Effect of DCCD and vanadate on the quenching of fluorescence of 9-aminoacridine (9AA) in proteoliposomes. Butanol-extracted proteolipid from *M. phlei* membranes was incorporated into K^+-loaded liposomes as described in Materials and Methods. Liposomes containing proteolipid (25 μg protein/ml) were suspended in a total volume of 2.5 ml containing 10 mM Tricine-NaOH buffer, pH 6.5, 0.3 M sucrose and 4 μM 9-aminoacridine. The reaction was started by the addition of valinomycin (1 μg/ml) dissolved in ethanol. Excitation was carried out at 365 and emission was recorded at 451 nm. (- - - - -) Control liposomes without proteolipid. (———) Liposomes reconstituted with proteolipid. DCCD (100 μM) and vanadate (50–100 μM) were added to the reaction mixture at the time intervals as indicated by the arrows.

reversal of the quenching of fluorescence, indicating additive effects of inhibition in H^+ translocation by these agents.

Discussion

The results presented in this study show that binding of DCCD to *M. phlei* membranes results in the inactivation of ATPase activity. The inactivation of membrane-bound ATPase activity was dependent upon the concentration of DCCD and incubation time. A 50% inhibition in ATPase activity was observed within 2 min at 50 μM DCCD. The correlation between the binding of [^{14}C]DCCD to membrane proteins and the inhibition of ATPase activity reveals that up to 50% inhibition, the binding of DCCD is linear with respect to the inhibition observed. Extrapolation of the linear portion of the curve to 100% inactivation of ATPase activity indicated that 1.57 nmol [^{14}C]DCCD bound per mg *M. phlei* membrane protein. In mitochondria 0.4 nmol DCCD bound per mg protein was effective in maximal inhibition of ATPase activity [9]. Similarly, it has been shown that complete inhibition of ATPase activity in chloroplast membranes occurs when DCCD at a ratio of 20 nmol/mg membrane protein was incubated for short periods of time, corresponding to 0.17 nmol DCCD bound per nmol isolated proteolipid [28]. In *Neurospora crassa*, Sebald et al. [29] also observed a higher amount (2.5 nmol) of DCCD bound per mg mitochondrial protein. Since carbodiimides are well known nonspecific chemical modifiers [30–32] that covalently form stable adducts with carboxyl groups, cysteine, tyrosine and amino groups as well as activate carboxyl groups leading to the formation of unstable *O*-acylisourea, the data indicate that the number of DCCD-reactive sites are an underestimate in this and other reported studies. Recently, Kopecky et al. [33] have also shown, in studies of the binding of [^{14}C]DCCD to mitochondrial membranes, that the correlation of the inhibition of ATPase activity to DCCD binding did not reflect the accurate stoichiometry of the DCCD-ATPase interaction. These authors observed that [^{14}C]DCCD binding to bovine heart mitochondria was linearly proportional up to 50% inhibition in ATPase activity resulting in 0.6 mol DCCD bound covalently to the specific inhibitory site, while the kinetics of the inhibition of ATPase activity revealed that 1 mol DCCD per mol ATPase eliminated the ATPase activity.

A proteolipid soluble in $CHCl_3/CH_3OH$ and *n*-butanol, which specifically and covalently binds DCCD in *M. phlei* membranes, has been isolated and purified to homogeneity. The polypeptide has an apparent molecular weight of 8000 in SDS-urea gel electrophoresis, as has been observed for the DCCD-reactive proteolipid component present in mitochondria, chloroplasts and *E. coli* [8]. Analysis of lipids present in the purified proteolipid of *M. phlei* revealed the presence of phosphatidylinositol (67%), phosphatidylethanolamine (18%) and cardiolipin (8%). Qualitatively, the composition of phospholipids present in proteolipids is similar to that present in membranes. The possibility of contamination of phospholipids extracted directly from membranes contributing to proteolipid composition in unlikely, since membranes are extracted with $CHCl_3/CH_3OH$ or butanol followed by repeated precipitation with precooled (-20°C) diethyl ether to precipitate the proteolipid fraction. During this repeated ether precipitation procedure and subsequent chromatography on Sephadex LH-20, the adsorbed phospholipids, if any, on the proteolipid fraction should have been removed. It should be mentioned that proteolipid derived from *E. coli* is virtually free of lipids [23] while that from chloroplast membrane contains lipids [28].

The amino acid composition of the DCCD-binding proteolipid from *M. phlei* is comparable to that of the corresponding protein from *Halobacterium* (for a review, see Ref. 8), specifically in their higher (six residues per mol) content of proline. The content of hydrophobic amino acid residues is high. Most abundant are the amino acids glycine (15%), alanine (13%) and leucine (13%), comparable to proteolipids from yeast, chloroplast and mitochondria [9]. The polarity of proteolipids isolated from *M. phlei* is low (27%), lacking cysteine and tryptophan. The content of histidine is low, although this has been found to be lacking in most proteolipids which have been characterized with the exception of *Halobacterium* wherein histidine was found in trace amounts [8]. The low polarity of amino acids in the proteolipid from *M.*

phlei indicates that the segments of the polypeptide chain containing these amino acids are in proximity to the fatty acyl chain of phospholipids in the lipid bilayer, as has been observed for the integral proteins, glycophorin [34] and bacteriorhodopsin [35].

The isolated proteolipid from *M. phlei* when reincorporated into the liposomal system, mediated H^+ translocation in response to the potential difference via a K^+ diffusion gradient. Moreover, the H^+ translocation, indirectly measured by the quenching of fluorescence of 9-aminoacridine, was sensitive to DCCD. In addition, the rate of H^+ influx was sensitive to the external pH (data not shown), as has been observed in thermophilic F_0-reconstituted liposomes [36]. It is pertinent to mention that liposomes prepared without the proteolipid did not exhibit H^+ translocation. Thus, our studies indicate that proteolipid is the primary component of the H^+ channel of F_0. The H^+ translocation activity has previously been demonstrated in butanol-extracted proteolipid from chloroplasts [37] and bovine heart mitochondria [19] after incorporation into liposomes.

We utilized proteolipid-incorporated liposomes as a system to delineate whether vanadate inhibited the H^+ gradient in membrane vesicles by interacting with the proteolipid component of the F_0-F_1 complex. Our studies show that vanadate causes the reversal of quenching of fluorescence of 9-aminoacridine, induced by valinomycin, in proteolipid-incorporated liposomes. In addition, the reversal of quenching of fluorescence was enhanced by the addition of DCCD, supporting the earlier conclusion [12] that the effects of vanadate and DCCD on inhibition on ATPase activity and H^+ translocation are additive in nature.

A decisive conclusion regarding the details of the molecular mechanism of H^+ conduction by proteolipid would require structural analysis and functional reconstruction of fragmented proteolipid, as has been recently demonstrated for bacteriorhodopsin [38].

Acknowledgements

This work was supported by NIH grant AI-05637. We would like to thank Dr. Suresh C. Sikka for initiating these studies and Dr. Robert Farley, Department of Physiology, for the use of the Hewlett-Packard computer for analysis of the data. The excellent technical assistance of Morris Rehn is gratefully acknowledged. We thank Ms. Donna Kopitcke and Carol Papp for typing the manuscript. We are indebted to Mr. Vince Farnsworth of the California Institute of Technology, Pasadena, for carrying out the amino acid analysis of the proteolipid.

References

1 Kalra, V.K. and Brodie, A.F. (1971) Arch. Biochem. Biophys. 147, 653–659
2 Brodie, A.F. and Kalra, V.K. (1982) in Membranes and Transport (Martonosi, A.N., ed.), Vol. 1, pp. 473–477, Plenum Press, New York
3 Kumar, G., Daves, R. and Brodie, A.F. (1979) Eur. J. Biochem. 100, 365–375
4 Higashi, T., Kalra, V.K., Lee, S.H., Bogin, E. and Brodie, A.F. (1975) J. Biol. Chem. 250, 6541–6548
5 Beechey, R.B., Holloway, C.T., Knight, I.G. and Robertson, A.M. (1966) Biochem. Biophys. Res. Commun. 23, 75–80
6 Harold, F.M., Baarda, J.R., Baron, C. and Abrams, A. (1969) J. Biol. Chem. 244, 2261–2268
7 Fillingame, R.H. (1975) J. Bacteriol. 124, 870–883
8 Sebald, W. and Hoppe, J. (1981) Curr. Top. Bioenerg. 12, 1–64
9 Glaser, E., Norling, B., Kopecky, J. and Ernster, L. (1982) Eur. J. Biochem. 121, 525–531
10 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45–93
11 Kumar, G., Kalra, V.K. and Brodie, A.F. (1979) J. Biol. Chem. 254, 1964–1971
12 Yoshimura, F. and Brodie, A.F. (1981) J. Biol. Chem. 256, 12239–12242
13 Brodie, A.F. and Gray, C.T. (1956) J. Biol. Chem. 219, 853–862
14 Brodie, A.F. (1959) J. Biol. Chem. 234, 398–404
15 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1979) J. Biochem. 85, 503–509
16 Soto, E.F., Pasquini, J.M., Placido, R. and LaTorre, J.L. (1969) J. Chromatogr. 41, 400–409
17 Sigrist, H., Sigrist-Nelson, K. and Gitler, C. (1977) Biochem. Biophys. Res. Commun. 74, 178–184
18 Schindler, H. and Nelson, N. (1982) Biochemistry 4, 5787–5794
19 Celis, H. (1980) Biochem. Biophys. Res. Commun. 92, 26–31
20 Folch, J., Lees, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497–509
21 Chauhan, V.P.S., Sikka, S.C. and Kalra, V.K. (1982) Biochim. Biophys. Acta 688, 357–368
22 Marinetti, G.V. (1962) J. Lipid Res. 3, 1–20
23 Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630–6637
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

25 Bailey, J.L. (1967) Techniques in Protein Chemistry, 2nd edn., p. 340–342, Elsevier, Amsterdam
26 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659
27 Capaldi, R.A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 930–932
28 Sigrist-Nelson, K., Sigrist, H. and Azzi, A. (1978) Eur. J. Biochem. 92, 9–14
29 Sebald, W., Graf, T. and Lukins, H.B. (1979) Eur. J. Biochem. 93, 587–599
30 Kurzer, F. and Douraghi-Zadeh, K. (1967) Chem. Rev. 67, 107–152
31 Carraway, K.L. and Koshland, D.E. (1968) Biochim. Biophys. Acta 160, 272–274
32 Sebald, W., Machleidt, W. and Wachter, E. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 785–789
33 Kopecky, J., Dedina, J., Votruba, J., Svoboda, P., Houstek, J., Babitch, S. and Drahota, Z. (1982) Biochim. Biophys. Acta 680, 80–87
34 Tomita, M. and Marchesi, V.T. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2964–2968
35 Ovchinnikov, Y.A., Abdulaev, N.G., Fiegina, M.Y., Kisilev, A.V. and Lobanov, N.A. (1977) FEBS Lett. 84, 1–4
36 Okamoto, H., Sone, N., Hirata, H., Yoshida, M. and Kagawa, Y. (1977) J. Biol. Chem. 252, 6125–6131
37 Nelson, N., Eytan, E., Notsani, B.E., Sigrist, H., Sigrist-Nelson, K. and Gitler, C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2375–2378
38 Huang, K.S., Bayley, H., Liao, M.-J., London, E. and Khorana, H.G. (1981) J. Biol. Chem. 256, 3802–3809
39 Prasad, R., Kalra, V.K. and Brodie, A.F. (1975) J. Biol. Chem. 250, 3690–3698