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Chemiosmotic Energy Conversion of the Archaebacterial Thermoacidophile *Sulfolobus acidocaldarius*: Oxidative Phosphorylation and the Presence of an F_0-Related *N,N'*-Dicyclohexylcarbodiimide-Binding Proteolipid

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The energy-transducing mechanism of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* DSM 639 has been studied, addressing the question whether chemiosmotic proton gradients serve as an intermediate energy store driving an F_0F_1-analogous ATP synthase. At pH 3.5, respiring *S. acidocaldarius* cells developed an electrochemical potential of H^+ ions, consisting mainly of a proton gradient and a small inside-negative membrane potential. The steady-state proton motive force of 140 to 160 mV was collapsed by protonophores, while *N,N'*-dicyclohexylcarbodiimide (DCCD) caused a hyperpolarization of the membrane, as expected for a reagent commonly used to inhibit the flux through proton channels of F_0F_1-type ATP synthases. Cellular ATP content was strongly related to the proton motive force generated by respiration and declined rapidly, either by uncoupling or by action of DCCD, which in turn induced a marked respiratory control effect. This observation strongly supports the operation of chemiosmotic ATP synthesis with H^+ as the coupling ion. The inhibition of ATP synthesis by [^{14}C]DCCD was correlated with covalent reactions with membrane proteins. The extraction of labeled membranes with organic solvents specifically yielded a readily aggregating proteolipid of 6 to 7 kilodaltons apparent molecular mass. Its amino acid composition revealed significant similarity to the proteolipid found in eubacteria, such as *Escherichia coli*, as an extremely hydrophobic constituent of the F_0 proton channel. Moreover, the N-terminal amino acid sequence of the *Sulfolobus* proteolipid displays a high degree of homology to eubacterial sequences, as well as to one derived from nucleic acid sequencing of another *Sulfolobus* strain (K. Denda, J. Konishi, T. Oshima, T. Date, and M. Yoshida, J. Biol. Chem. 264:7119–7121, 1989). Despite certain structural similarities between eucaryotic vacuolar ATPases and the F_1-analogous ATPase from *Sulfolobus* sp. described earlier, the results reported here promote the view that the archaebacterial ATP-synthesizing complex functionally belongs to the F_0F_1 class of ATPases. These may be considered as phylogenetically conserved catalysts of energy transduction present in all kingdoms of organisms.

Sulfolobus acidocaldarius (9) is a typical representative of the sulfur-dependent archaebacteria, requiring high temperatures (70 to 80°C) and acidic medium (pH 2.0 to 3.5) for optimal growth. Although *S. acidocaldarius* metabolizes glucose by a modified Entner-Doudoroff pathway (19), it is not known whether under these conditions ATP is generated by direct carbohydrate degradation or by chemiosmotic utilization of a proton gradient, as reported for halophilic and methanogenic archaebacteria (6, 14, 27).

Proton-translocating activity coupled to respiration (48) has been found in *Sulfolobus* species. Also, the existence of a membrane-associated ATPase (34, 43) has been established which is immunologically related to the F_1 part of known energy-transducing H^+-ATPases (41, 42). However, little is known about the existence of an F_0-analogous proton channel in *Sulfolobus* species or other archaebacteria (40). The present study addresses the question of how *N,N'*-dicyclohexylcarbodiimide (DCCD), a classical covalently binding inhibitor of ion-translocating ATPases and ATP synthases (3), affects the components of the proton motive force in this archaebacterium. The data not only demonstrate electron transport-coupled phosphorylation in *S. acidocaldarius* but also reveal the presence of a low-molecular-mass DCCD-reactive proteolipid in the plasma membrane, which could be isolated and proved to display homology to the *Escherichia coli* F_0 subunit c (59, 63), as the essential constituent of the proton channel in F_0F_1 ATP synthase.

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MATERIALS AND METHODS

Cultivation and harvesting of the microorganism. *S. acidocaldarius* DSM 639, kindly provided by Wolfram Zillig, Martinsried, Federal Republic of Germany, was grown heterotrophically as reported by Brock et al. (9). Unless indicated otherwise, the bacteria were harvested in the midlogarithmic phase by spinning down 1-liter cultures at 4°C with the GS3 rotor of a RC-5B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 9,500 × *g* for 10 min. The cellular material was suspended in 1 to 2 ml of 20 mM potassium phosphate buffer, pH 7.0, and placed on ice. Upon storage for half a day, the biological activity of *Sulfolobus* cells, at least with respect to the functional parameters tested in this study, remained unaffected.

Polarographic determination of cellular oxygen uptake. O_2 consumption was measured by a Clarke-type electrode fitted with a 25-μm Teflon membrane in a water-jacketed closed vessel, thermostatted at 45 or 60°C, and filled with 2.84 ml (maximum volume) of BSG medium [10 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 10 mM glycyl-

glycine-sulfate pH 3.5] supplemented with 14 mM D-glucose. The total cell protein concentration was 2.5 mg/ml (measurements at 45°C) or 0.5 to 1.0 mg/ml (measurements at 60°C). Upon prolonged recordings after complete consumption of the physically dissolved O_2, pulses of oxygen were given by adding 0.5 to 1 μl of 3% (wt/vol) H_2O_2 and 10 μl of catalase (20 mg/ml; Boehringer GmbH, Mannheim, Federal Republic of Germany). If required, samples for ATP determination (see below) were withdrawn from the reaction vessel (measurements at 60°C).

Measurement of ΔpH and Δψ. In a total volume of 5 ml of BSG medium plus glucose (as described above), specific radioactive indicator substances were incubated with *Sulfolobus* cell suspensions (protein concentration, 2.5 mg/ml). Thermostatted at 45°C, the suspensions were stirred rapidly within a water-jacketed glass vessel with a large surface area in order to efficiently allow oxygen access. Samples (0.3 ml) were taken in 3-min intervals. The samples were immediately centrifuged (12,000 × *g*) for 2 min at room temperature through a 100-μl silicon oil layer in Eppendorf reaction tubes (density, 1.065 g/cm^3 at 20°C; mixture from AR20 and AR200 silicon oils; Wacker Chemie, Munich, Federal Republic of Germany). The total pellets, obtained by cutting away the bottom of the tubes with a razor blade, and samples of the clear supernatants were solubilized or dissolved, respectively, in a mixture of 0.5 ml of 5% sodium dodecyl sulfate (SDS)–10 μl of 10 N NaOH. After shaking overnight in a water bath at 40 to 50°C, 4.5 ml of the Miniria M20 scintillation cocktail (Zinsser, Frankfurt/Main, Federal Republic of Germany) was added to each sample. Radioactivity was measured with type 1215 or 1217 scintillation counters (Wallac-LKB).

The total bacterial pellet water space and cellular internal volume were obtained from the distribution of [^{3}H]H_2O (1 mCi/g; final concentration, 4 μCi/ml) and [^{14}C-carboxyl]carboxyldextran (1.2 mCi/g; final concentration, 0.75 μCi/ml). At 45°C, the internal volume (2.5 ± 0.1 μl/mg of cell protein) accounts for 69 ± 5% (average of n = 15 determinations each) of the total pellet water space.

For ΔpH ($pH_i - pH_a$; difference of internal and external pH) determination, the distribution of [1-^{14}C]benzoic acid (10 mCi/mmol) was analyzed by adding 0.2 μCi (18.7 μM total concentration) of radiolabel per ml. As Δψ ($\Delta\psi_i - \Delta\psi_a$; difference of internal and external electric potential) indicators, either [^{14}C]KSCN (58 mCi/mmol; total concentration, 0.2 μCi/ml [3.45 μM]) or [^{3}H]tetraphenylphosphonium bromide [^{3}H]TPP^+Br^-) (35 Ci/mmol; total concentration, 0.4 μCi/ml [11.3 nM]) was applied. When both ΔpH and Δψ had to be ascertained with ^{14}C-labeled probes, experiments were run in parallel for measuring each of the respective parameters. Also the cellular ATP level and the oxygen uptake (see above) were determined separately under identical conditions. Raw ΔpH and Δψ data were evaluated by the method of Rottenberg (56).

Correction for probe binding. The amount of energy-independent probe binding was determined after treatment of *Sulfolobus* cells with 5 or 10% (vol/vol) *n*-butanol in BSG buffer in the presence of the respective radioactive probe at room temperature for 2 to 12 h (4, 39). After separation by silicon oil centrifugation, the bound and free concentrations (C_b and C_f) of probe molecules were analyzed as described above. In a broad range of concentrations, the relation of C_b and C_f was linear (72), and the binding factors (k, where $k = C_b/C_f$) were determined to 3.8 for KSCN, 5.9 for TPP^+, and 4.0 for benzoic acid. Δψ and ΔpH were corrected with respect to nonspecific binding by the internal model of Zaritsky et al. (72), in which the extent of bound probe material is taken to be proportional to its intracellular concentration.

Measurement of cellular ATP level. To release the total cellular ATP content, the samples (20 to 50 μl) were pipetted on a mixture of 50 μl each of 20% (wt/vol) trichloroacetic acid and 0.3% (wt/vol) *N*-tetradecyl-*N*,*N*-dimethylammonio-3-propane sulfate (Sb-14), stored on ice for 5 to 20 min, and centrifuged for 5 min at 4°C (Eppendorf centrifuge). A 100-μl volume of the supernatants was neutralized by 30 μl of a solution containing 0.5 M Tris–2 M KCl; samples thereof were added into a 900-μl reaction mixture consisting of 50 mM Tris-acetate, 10 mM magnesium acetate, 1.5 mM EDTA, 1 mg of bovine serum albumin per ml, 10 μg of luciferin per ml, and 1.2 μg of luciferase per ml, pH 7.75. Light emission was recorded with a luminometer (Wallac-LKB) by calibrating the signals with 10 pmol of ATP as an internal standard. The ADP content was read after completion of the reaction induced by further addition of 1 μmol of phosphoenolpyruvate and 20 μg of pyruvate kinase.

Labeling of intact *Sulfolobus* cells by prolonged preincubation with [^{14}C]DCCD. After being collected, the *Sulfolobus* cells were suspended in a medium composed of 10 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 1 mM $MgSO_4$, and 0.5 mM $CaCl_2$, pH 6.5 (BS buffer) to a protein concentration of 10 mg/ml. The cells were incubated in a total volume of 200 μl with 0 to 10 nmol of [^{14}C]DCCD (specific radioactivity, 6 to 26 mCi/mmol) for 1.5 h at 37°C. Samples (50 μl) were diluted into 450 μl of BSG buffer (pH 3.5) and incubated at 60°C for 5 min; then samples were taken for determination of cellular ATP content. To remove adsorbed [^{14}C]DCCD, the residual 150 μl from the original incubation mixture was washed twice with 8 ml of 1:1 (vol/vol) ethanol-water. The pellets were dissolved in 300 μl of 10% (wt/vol) SDS and further treated for 30 min in a bath-type sonifier to decrease viscosity; after standing overnight at room temperature, samples were analyzed by gel electrophoresis in the presence of SDS (see below).

[^{14}C]DCCD labeling of membranes. Membranes of *S. acidocaldarius* and *E. coli* (fermentations and membrane preparations were as described previously [41, 43] were incubated at a protein concentration of 25 to 30 mg/ml in 2 ml of 100 mM Tris-chloride, pH 8.1, with 1.7 to 2.4 nmol of [^{14}C]DCCD (57 mCi/mmol) per mg of protein at 4°C for 24 h. Noncovalently bound DCCD was removed by repetitive dilution into 30 ml of 1:1 (vol/vol) ethanol–20 mM potassium phosphate buffer, pH 7.0, and sedimentation at 150,000 × *g* for 1 h. The final pellet was suspended in 2 ml of 20 mM potassium phosphate, pH 7.0.

Purification of the *Sulfolobus* proteolipid. For analytical reasons, [^{14}C]DCCD-labeled membranes (about 40 to 50 mg of protein) suspended in phosphate buffer (see above) were extracted by injection into 30 volumes of 2:1 (vol/vol) chloroform-methanol under rapid stirring for 24 h at 4°C. Further purification was done by elution with 10 mM ammonium acetate in 5:5:1 (vol/vol/vol) chloroform-methanol-water from a carboxymethyl cellulose column, by a procedure described previously (25), followed by Sephadex LH-60 chromatography (1, 20) of the sample equilibrated with 20 mM ammonium acetate in 2:1 (vol/vol) chloroform-methanol. Large-scale preparations were done the same way, starting with membrane material (1 to 2 g of protein) previously extracted with pyrophosphate buffer (42). Under these conditions, the chloroform-methanol extraction time was extended to 48 h to obtain higher yields.

Denaturating gel electrophoresis and protein determination.

For the identification of DCCD-binding proteins, the materials were solubilized with 5% (wt/vol) SDS, diluted with the same volume of sample loading buffer according to the respective gel system (see below), and kept at room temperature from 0.5 to 16 h to reduce aggregation effects. If initially dissolved in organic solvents, the proteins were recovered by centrifugation under reduced pressure. Total cell lysates were electrophoresed on 10 to 20% acrylamide gradient gels according to the Laemmli system (38). [^{14}C]DCCD-labeled proteolipids were analyzed by the SDS-polyacrylamide gel electrophoresis (PAGE) methods described by Schägger and von Jagow (57) or Fillingame (20). The gels were fixed in 20% (wt/vol) trichloroacetic acid for 1 to 2 h, stained with Coomassie blue, soaked in the aqueous scintillator Enlightning (New England Nuclear Corp., Boston, Mass.), and fluorographed at −70°C on AGFA Curix RP 2 film. Protein concentrations of cell and membrane suspensions were determined as described by Watters (71); solubilized protein was analyzed by a modified Lowry procedure (53).

Chemical analysis of the proteolipid. The amino acid composition was analyzed after performic acid oxidation and total hydrolysis (29). Cyanogen bromide fragmentation of the purified proteolipid was performed as described elsewhere (29), and the fragments generated were separated by SDS-PAGE (57). Partial amino acid sequences of the proteolipid or its cyanogen bromide fragments were determined by a type 470A device (Applied Biosystems), using on-line detection of phenylthiohydantoin-derivatized amino acids under standard conditions (29).

Other materials. All chemicals were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany; biochemicals were from Boehringer. 3,5-di(tert-butyl)-4-hydroxybenzylidenemalononitrile (SF 6847) was obtained from Wako Pure, and tetrachlorosalicylanilide (TCS) was from Eastman Kodak Co., Rochester, N.Y. 4,5,6,7-Tetrachloro-2′-trifluoromethylbenzi midazole (TTFB) was a kind gift from Evert Bakker, Osnabrück, Federal Republic of Germany. [^{14}C]KSCN and [^{14}C]DCCD were from Amersham Buchler; [1-^{14}C]benzoic acid, [^{3}H]H_2O, [^{14}C-carboxyl]carboxyldextrane, and [^{3}H]tetraphenylphosphonium bromide were from New England Nuclear.

RESULTS

Proton electrochemical gradient. Because of acidophilic nature, *Sulfolobus* species require active proton extrusion for the regulation of internal pH. The membrane potential and ΔpH of *Sulfolobus* cells were determined by distribution of radioisotopic probe molecules at 45°C. Though cells were grown at a higher temperature, this lower temperature was chosen in this set of experiments to allow for higher kinetic resolution at a simultaneously reduced respiratory rate and increased oxygen solubility. Upon incubation in mineral salt medium supplemented with glucose, a proton motive force (Δp) of −140 to −160 mV was generated (Table 1). The data were corrected for energy-independent probe binding by the method of Zaritsky et al. (72). Δp is composed largely of a ΔpH of greater than 2 U. Also, an inside-negative Δψ on the order of −20 to −40 mV contributed to the electrochemical proton potential. The values determined for membrane potential were somewhat dependent on the type of Δψ probe (cationic [TPP^+] or anionic [SCN^-]). Irrespective of the individual reporter molecule characteristics, at least under routine measuring conditions (external medium pH 3.5 and absence of inhibitors or ionophores, specifically), Δψ was measured as inside negative after subtraction of probe binding.

TABLE 1. Composition of the electrochemical proton gradient of *Sulfolobus* cells and response to cation-translocating ionophores[a]

Ionophore[b]	Amt (nmol/mg)[c]	pH_i[d]	−ZΔpH (mV)	Δψ (mV)	Δp (mV)
None		5.62	−134	−19	−153
TTFB (H^+)	6.7	5.20	−107	+54	−53
TCS (H^+)	6.7	4.67	−74	+69	−5
gr. D (H^+, K^+, Na^+)	3.4	4.77	−80	+57	−23
	30.0	4.56	−67	+63	−4

[a] ΔpH and Δψ of *Sulfolobus* cells were determined from the distribution of [^{14}C]benzoic acid and [^{14}C]SCN^- at an external pH of 3.5 in mineral salt medium after 10 min of incubation at 45°C. The data were corrected for the energy-independent binding of probe molecules (as described in reference 72, calculated with respect to the internal-binding model).

[b] The specificity of ion translocation of individual ionophores is given in parentheses. gr. D, Gramicidin D; TCS, tetrachlorosalicylanilide.

[c] Amounts refer to the total applied cellular protein.

[d] pH_i, Internal pH.

In the presence of protonophores, the proton motive force collapsed to different extents (Table 1), depending on the H^+-conducting potency of the respective agent. Due to the marked influx of positive charges induced by protonophores, the membrane potential was reversed to values up to approximately +70 mV, thus compensating for the residual pH difference across the membrane. Under these conditions, the proton motive force vanished almost completely.

Direct correlation of respiration and ATP synthesis. As shown in earlier experiments (2), sudden anaerobiosis caused a drop of cellular ATP concentration which recovered immediately after an oxygen pulse. Protonophores such as SF 6847 or TTFB caused a stimulation of cellular respiration by dissipation of Δp accompanied by a decay of ATP content. Figure 1 presents the time course of the components of the proton electrochemical gradient and of cellular ATP after TTFB addition. The respiratory rate under the respective conditions was approximately doubled (data not included). The parallel decrease of the indicated parameters and the increased O_2 uptake suggest direct coupling between Δp and ATP synthesis. The persistence of a residual Δp and a low ATP level even in the presence of uncoupler presumably results from limited efficiency of the agent due to an unfavorable combination of uncoupler pK value and the low medium pH (13). Evidently, by TTFB addition the internal pH was altered only insignificantly.

Effect of DCCD on ATP synthesis. Consistent with the above data, the proton was likely to be the main coupling ion. In fact, DCCD, a covalently binding and extremely efficient energy transfer inhibitor in F_0F_1 ATPases (3, 64), also affected *Sulfolobus* ATP synthase (40). However, fairly high concentrations of DCCD were required for substantial effects on the ATP level of *Sulfolobus* cells. This may be due to rapid thermal decomposition of the highly reactive DCCD (3). The addition of up to 400 nmol per mg of cell protein instantly inhibited cellular ATP regeneration and depressed respiratory activity at 60°C (data not shown). At a lower temperature (45°C), 180 nmol per mg of protein was sufficient for prompt inactivation of ATP synthesis. According to the expected blocking action on proton channels, DCCD caused a significant hyperpolarization of the plasma membrane when TPP^+ was chosen as a reporter ion for Δψ. An intensification of the (inside-negative) membrane potential (Δψ) by 20 to 30 mV was observed, indicating that the inward flux of positive charges (hydrogen ions) was impaired by the agent.

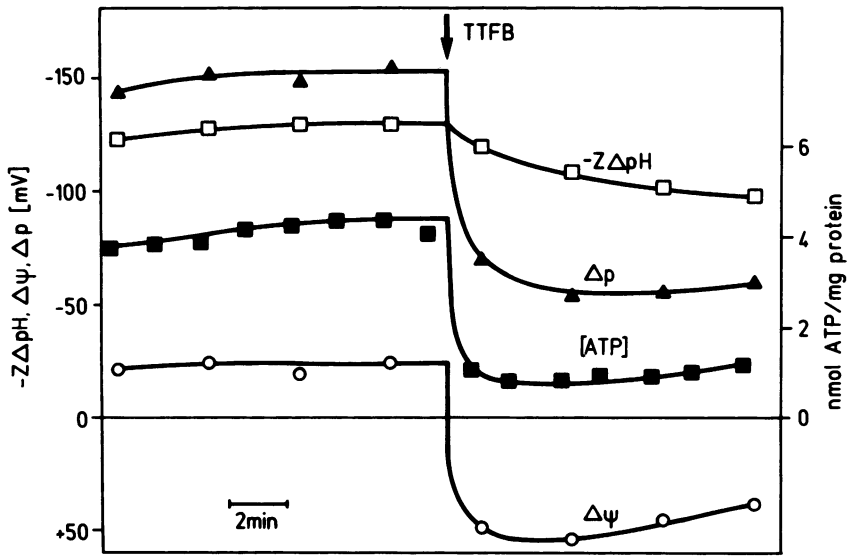

FIG. 1. Effect of the protonophore TTFB on cellular ATP content and the Δp components. At the time indicated (arrow), 6.7 nmol of TTFB per mg of *Sulfolobus* cell protein was added. The membrane potential (Δψ) indicator was [^{14}C]KSCN. ΔpH was recorded from the [^{14}C]benzoic acid distribution.

DCCD induced a kind of respiratory control phenomenon expressed by an unlowered proton motive force (Δp) at a simultaneously reduced respiratory rate (Fig. 2). This inhibition was counteracted by gramicidin D (or TTFB [data not shown]), with a concomitant collapse of the electrochemical proton potential. Thus, an interaction of DCCD with respiratory electron transport could be excluded, while the decay of ATP was likely to result from inhibition of an inward proton flux through an ATP synthase complex.

Unexpectedly, the intracellular ATP concentration rose slightly again when ionophores (Fig. 2) or protonophores (Fig. 3) were added to *Sulfolobus* cells pretreated with DCCD. To analyze this phenomenon in greater detail, the time course of the ADP level was also measured (Fig. 3A). ATP and ADP concentrations changed inversely, indicating that ATP generation and ATP consumption became imbalanced. The ATP/ADP ratio was lowered from about 10:1 in populations of functionally intact cells to 1:1 because of the effect of DCCD (Fig. 3B), while the adenylate energy charge dropped to values below 0.5, indicating drastic effects on the ATP-generating systems of the bacteria (15, 23). The slight recovery of ATP after the addition of DCCD plus uncoupler may be explained by scalar substrate level phosphorylation reactions possibly activated through H^+ influx or, alternatively, by partial inhibition of ATP-consumptive reactions.

Binding of [^{14}C]DCCD to *Sulfolobus* proteins. In order to identify the DCCD-reactive component(s) associated with inhibition of oxidative phosphorylation, the covalent incorporation of [^{14}C]DCCD into *Sulfolobus* proteins were studied. This could be achieved by prolonged preincubation of *Sulfolobus* cells with DCCD at much lower concentrations than described above (i.e., 1.5 h incubation time, 37°C, pH

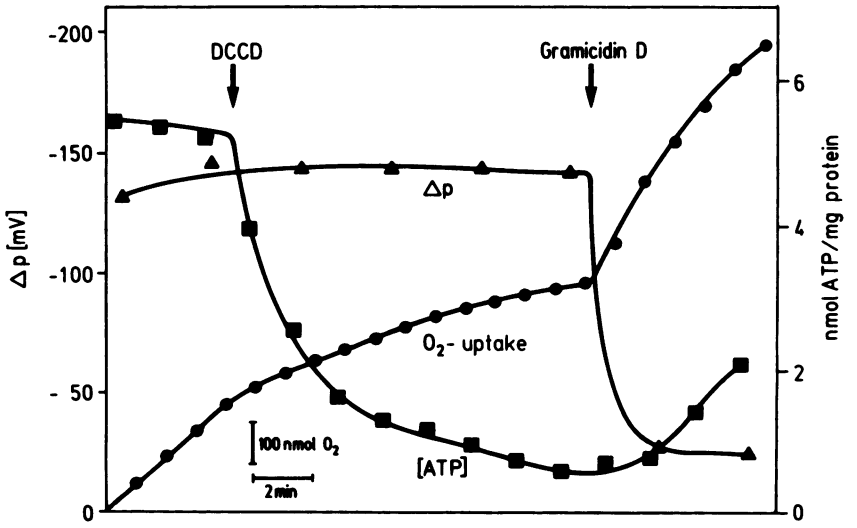

FIG. 2. Respiratory control effect: combined action of DCCD plus uncoupling ionophore gramicidin D on Δp, cellular ATP level, and respiration. Δψ and ΔpH were recorded as described for Fig. 1; oxygen uptake refers to the total volume of the reaction vessel (2.84 ml). At the times indicated (arrows), 180 nmol of DCCD and 3.4 nmol of gramicidin D were added.

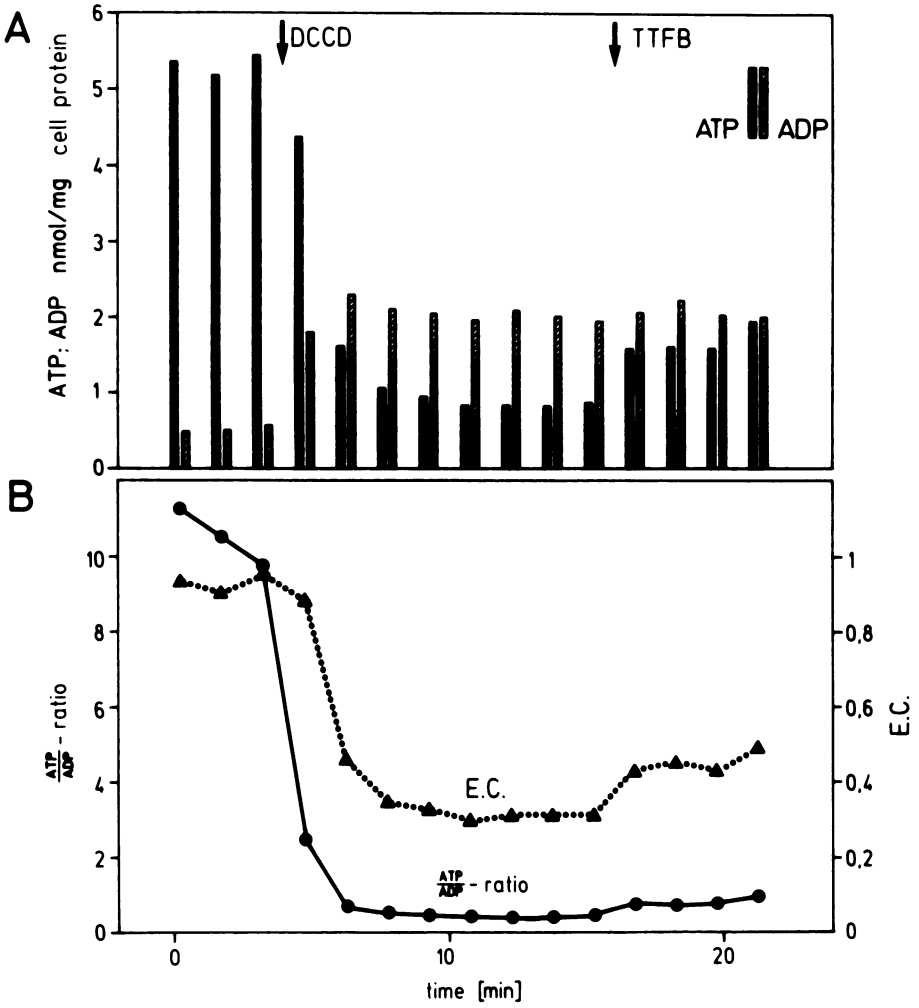

FIG. 3. Action of DCCD and TTFB on the adenine nucleotide pools of *Sulfolobus* species. The experiment was performed as described for Fig. 2, exhibiting qualitatively the same time courses of Δp and oxygen uptake, if TTFB is replaced for gramicidin D (as in Fig. 2). At the times indicated (arrows), 180 nmol of DCCD and 6.7 nmol of TTFB per mg of *Sulfolobus* cells were added. (A) Cellular ATP and ADP contents. (B) ATP/ADP ratio and adenylate energy charge (E.C.); assuming that the adenine nucleotide pool consists almost entirely of the sum of ATP and ADP concentrations (15) under normal conditions (i.e., before addition of DCCD), the AMP content could be calculated from the difference of total adenine nucleotide and ATP plus ADP concentrations.

6.5). Five minutes after transfer to acidic medium (pH 3.5) at 60°C, the ATP content of pretreated cells was determined (Fig. 4). An ATP level of only 10% with respect to the control was preserved in the presence of merely 8 nmol of DCCD per mg of cell protein. Concomitantly with the DCCD-induced inactivation of the ATP synthase, certain radioactive polypeptides were detected by SDS-PAGE of total cell lysates (Fig. 4, inset), exhibiting molecular masses of less than about 60 kilodaltons (kDa). The degree of labeling increased with the total amount of the inhibitor in the preincubation mixture. In the experiment depicted in Fig. 4 (inset), one band of 34 kDa apparent molecular mass (arrowhead) was already prominently marked after preincubation with <3 nmol of [^{14}C]DCCD per mg of cell protein (e.g., Fig. 4, point C).

To identify the molecular target of DCCD more precisely, [^{14}C]DCCD binding to isolated *Sulfolobus* membranes was analyzed under more-stringent reaction conditions (pH 8, 4°C). Analogous to procedures with known DCCD-reactive proteins of F_0F_1 ATPases, labeled membranes were extracted with organic solvents, and the resulting proteolipid extract was investigated by specifically developed SDS-PAGE systems (20, 57). As a reference, parallel experiments were done with [^{14}C]DCCD-labeled membranes of *E. coli* (containing subunit of c of F_0F_1 H^+-ATPase as the only DCCD-reactive protein species [20]). Fluorographs of gels run with chloroform-methanol extracts of *E. coli* and *S. acidocaldarius* membranes are shown in Fig. 5A and B. The DCCD-binding proteolipid of *E. coli* exhibits a molecular mass of 5.0 to 5.5 kDa either in the SDS (57) or in the SDS-urea (20)-PAGE systems, which indicates a slightly irregular electrophoretic mobility (molecular mass, 8,264 Da, as derived from amino acid sequence data [30]). The proteolipid fraction of *S. acidocaldarius* also exhibited radioactive bands of 7 kDa (SDS gel system; Fig. 5A) and 6 kDa (SDS-urea gel system; Fig. 5B). With the SDS system (Fig. 5A, lane 2), weaker additional signals detected at 14 and about 20 kDa might indicate the existence of dimeric and trimeric forms.

Properties of the DCCD-binding proteolipid. The DCCD-binding proteolipid from *Sulfolobus* membranes could be purified from crude chloroform-methanol extract by stan-

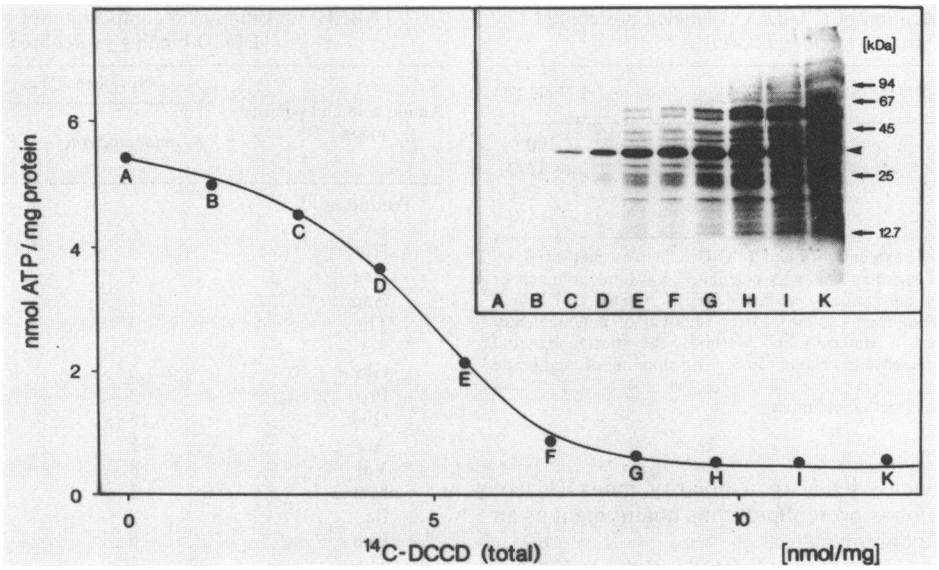

FIG. 4. Effect of prolonged preincubation of intact cells with [^{14}C]DCCD on cellular ATP level after transfer into BS6 medium at 60°C. The ATP content and amount of total [^{14}C]DCCD refer to *Sulfolobus* cell protein. After removing noncovalently bound DCCD from the protein (see Materials and Methods), the material was extracted with 10% SDS, and samples (190 μg per lane) corresponding to the incubation mixtures, (A to K) were analyzed by gradient SDS-PAGE. [^{14}C]DCCD bound covalently to polypeptides was localized after fluorography of the dried gel (inset).

dard methods (1, 20, 25). ^{14}C-labeled protein could be obtained in very small quantities only after carboxymethyl cellulose and LH-60 Sephadex chromatography (Table 2). This result was also found in the large-scale enrichment of nonradioactive material leading to low yields of about 150 μg per g of *Sulfolobus* membrane protein, compared with preparations from other organisms (reference 62 and references therein). ^{14}C-labeled and nonradioactive protein behaved identically with respect to electrophoretic mobility as well as chromatographic elution. Probably because of its extremely hydrophobic character, after SDS-PAGE the *Sulfolobus* proteolipid is faintly stained by Coomassie blue (results not shown). Therefore, it was more easily investigated in its radioactive form (Fig. 5C). Fluorographic analysis of the purified product exhibits several discrete bands likely to consist of different oligomeric forms, representing a ladder from $n = 1$ (7 kDa apparent molecular mass) to $n = 5$ or 6 (Fig. 5C). The *Sulfolobus* protein is made up of predominantly hydrophobic amino acids and reveals striking similarity to amino acid compositions found in the F_0-derived proteolipids from *E. coli* and the thermophilic bacterium PS3 (Table 3). As with eubacteria, the small contribution of

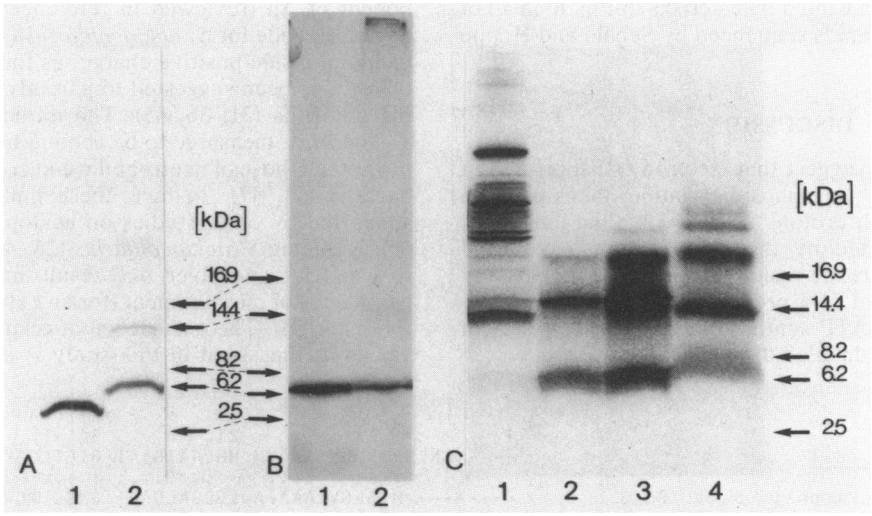

FIG. 5. [^{14}C]DCCD-binding proteolipids of *E. coli* and *S. acidocaldarius*. Fluorography of SDS-polyacrylamide gels from $CHCl_3$-methanol-extracted [^{14}C]DCCD-labeled membranes of *E. coli* and *S. acidocaldarius* (A) by a previously described method (57) and in the presence of 8 M urea (B) by a previously described method (20) is shown. Lanes 1, *E. coli* extract (5,600 dpm); lanes 2, *S. acidocaldarius* extract (6,800 dpm). (C) Fluorography of SDS-polyacrylamide gels (57) of fractions from the purification of the [^{14}C]DCCD-labeled *Sulfolobus* proteolipid, each loaded with approximately 7,500 dpm per lane. Lane 1, Membrane particles; lane 2, concentrated (as directed in reference 25) $CHCl_3$-methanol extract; lane 3, pooled carboxymethyl cellulose eluate; lane 4, pooled LH-60 Sephadex eluate.

TABLE 2. Purification of [^{14}C]DCCD-labeled proteolipid from *S. acidocaldarius*

Step	Radioactivity (10^{-3} dpm)[a]	Yield (%)
Membranes	2,584	100.0
$CHCl_3$ extract	360	13.9
CM-cellulose[b]	54	2.1
LH-Sephadex	26	1.0

[a] The radioactivity of covalently bound [^{14}C]DCCD was measured by scintillation counting of samples and was calculated for total volumes or pooled fractions. Initially membranes were labeled with [^{14}C]DCCD (1.7 nmol of DCCD per mg of membrane protein). After removal of noncovalently bound label as described in Materials and Methods, the starting material consisted of 45.5 mg of membrane protein (56.8×10^3 dpm/mg of membrane protein).

[b] CM-cellulose, Carboxymethyl cellulose.

hydrophilic components leads to a polarity index (11) of 18.2% for the *Sulfolobus* proteolipid, thus qualifying it as an integral membrane component.

N-terminal amino acid sequence analysis of the *Sulfolobus* proteolipid. Amino acid sequencing of the N terminus as well as of cyanogen bromide fragments revealed no indication of the presence of more than one polypeptide species in the purified protein. Though from the presence of two methionines (see amino acid analysis; Table 3) three fragments could theoretically be expected, only two CNBr cleavage peptides of 2 and 5 kDa molecular mass were found on SDS-PAGE, of which the 5-kDa species was inaccessible to amino acid sequencing because it was blocked.

Combined analysis of the N terminus and the 2-kDa CNBr fragment (starting from $n = 11$) led to a sequence information up to amino acid position 33 (Fig. 6). Alignment of this partial sequence with N-terminal sequences of F_0F_1 ATPase proteolipids from the eubacterial *E. coli* and the thermophilic bacterium PS3 discloses striking relationships. A number of invariant or conservatively substituted amino acids (Fig. 6, colons and periods, respectively) were determined in the *Sulfolobus* proteolipid. In addition, the archaebacterial protein also matches those positions exhibiting a conserved G or A or an invariant G (indicated by asterisks in Fig. 6) in all of the F_0-derived proteolipids sequenced by Sebald and Hoppe (62).

DISCUSSION

Our results greatly suggest that electron transport-driven phosphorylation (oxidative phosphorylation) takes place in *S. acidocaldarius* with protons used as coupling ions. This conclusion is supported by the identification and partial sequencing of a membrane-residing proteolipid presumably providing a constituent of a proton channel, as is typically present in F_0F_1-type ATP synthases. Nevertheless, certain aspects require more detailed discussion.

TABLE 3. Amino acid composition of various bacterial DCCD-binding proteolipids[a]

Amino acid and polarity index (%)[b]	No. of residues per molecule from:		
	S. acidocaldarius	*E. coli*[c]	Thermophilic bacterium PS3[c]
Amino acid			
Lys	1	1	
His			
Arg	1	2	4
Asp	2	5	1
Thr	4	1	3
Ser			3
Glu	6	4	5
Pro	3	3	3
Gly	15	10	11
Ala	15	13	9
Val	8	6	8
Met	2	8	2
Ile	9	8	9
Leu	6	12	10
Tyr	1	2	1
Phe	4	4	3
Cys			
Trp	ND[d]		
Polarity index	18.2	16.5	22.2

[a] The *S. acidocaldarius* proteolipid, recovered from 2:1 (vol/vol) $CHCl_3$-methanol, was dissolved in 98% formic acid, oxidized by H_2O_2-formic acid, and further analyzed, as described previously (29). Amino acid residues were determined as next integral numbers.

[b] The polarity index was calculated as in reference 11.

[c] Data taken from reference 62.

[d] ND, Not determined.

Electrochemical H^+ gradient of *S. acidocaldarius*. Consistent with their usual acidic milieu, *S. acidocaldarius* cells can generate a significant proton motive force by respiration. As with other acidophiles, Δp consists mainly of a ΔpH (24, 37, 47) rather than a membrane potential across the cytoplasmic membrane. This is in contrast to neutrophilic microorganisms, for which normally $\Delta\psi$ is the predominant component of Δp (reviewed in reference 33). $\Delta\psi$ is small but negative inside for *S. acidocaldarius* (Table 1). On the other hand, an inside-positive charge, as found in different acidophiles, has been suggested to partially compensate for large pH gradients (31, 36, 45). The intracellular pH of *S. acidocaldarius*, measured to be about 5.6 in this study, appears lower than those of neutrophilic and certain other acidophilic bacteria (33, 47). In fact, these findings in principle are supported by other studies on acidophilic bacteria (45, 60, 66), including *S. acidocaldarius* (26, 46). The divergency of reported data, however, may result simply from the different techniques of measurement (for an extensive discussion, see reference 33). The occurrence of relatively low internal pH values as measured in this study is also supported by the

```
                          1          11         21         31
E. coli                   ----MENLNM DLLYMAAAVM MGLAAIGAAI GIGILGGKFL
                                  :  :    :::.   ::.:.::.: : :..  . .
Thermophil. bact. PS 3    ---------M SLGVLAAAIA VGLGALGAGI GNGLIVSRTI
                                   .....: :::.:::::. . :
Sulfolobus acidocaldarius EAPQDTPQGF MGINIGAGLA VGLAAIGAGV AVG
                                     *      * * *    *
```

FIG. 6. Combined amino acid sequences from the N terminus and 2-kDa CNBr fragment (starting from position 11) of *S. acidocaldarius* determined by gas phase sequencing, as outlined in Materials and Methods. Partial sequences of proteolipids from *E. coli* and thermophilic bacterium PS3 were taken from reference 70. Invariant (:) and isomorphously substituted (·) residues are indicated. Amino acid positions that are invariant or conservatively replaced in any of the F_0F_1-affiliated proteolipids investigated (62) are marked (*).

slightly acidic pH optima of several characteristic enzyme activities (43, 67, 68).

Correction of energy-independent probe binding. As stressed by Michels and Bakker (47), serious technical problems may arise from the strict dependence of ΔpH and Δψ on the medium pH and the energy-independent probe binding to bacterial cell components. Because strong binding effects and a relatively low probe accumulation are involved for *S. acidocaldarius*, the internal-binding model of Zaritsky et al. (72) was chosen. However, the binding affinity of the ΔpH reporter itself must be assumed to be pH dependent; thus, protonated and deprotonated species would contribute to different extents to the overall effect. This seems to be a nontrivial problem. If correction factors obtained from nonspecific permeabilization in the acidic BSG medium (pH 3.5) are used to correct data recorded for energized bacteria under physiological conditions (i.e., under the omission of butanol), the calculated extent of binding of the reporter molecule might be overestimated. Thus, the computed cytoplasmic pH could be significantly lower than the real value.

On the other hand, the general validity of the correction is demonstrated when the distribution of Δψ indicators within energized *Sulfolobus* cells is considered. Without any adjustment, the recorded membrane potentials with either the cationic TPP^+ or the anionic SCN^- deviate about 100 mV from each other, and they even may indicate different degrees of polarity (data not shown). When corrected by the internal-binding model, the results with both Δψ reporter molecules differ by only 10 mV, leading to the measured membrane potential of 20 to 30 mV (inside negative).

Effect of ionophores on Δp and ATP synthesis. If suitable protonophores or general ionophores (gramicidin also preferentially translocates protons [54]), were added to respiring *Sulfolobus* cells, Δp disappeared by slightly diminishing the internal pH and markedly increasing the Δψ to positive values. This observation is strongly suggested to be induced by a massive H^+ influx. In neutrophilic bacteria, exhibiting an electrochemical proton gradient composed mainly of Δψ, the transfer of only a few charges is enough to generate complete uncoupling (Δp = 0). In contrast, for the acidophile *S. acidocaldarius*, a limited capacity of intracellular ions suitable to initiate counterfluxes against an inward proton flow may be concluded from the fact that even in the presence of gramicidin D neither $-Z\Delta pH$ nor Δψ approached zero, though Δp practically vanished. A significant residual ΔpH is still compensated for by the interior-positive membrane potential.

A striking feature is the instant decline of ATP following the partial TTFB-induced dissipation of the electrochemical H^+ gradient (5). This suggests that scalar phosphorylation reactions arising from glucose degradation pathways may contribute to only a small extent to the ATP pool of *Sulfolobus* species. Rather, the direct and immediate response to uncoupler action points toward ATP synthesis driven by vectorial proton flow as the prevailing mechanism of cellular energy conversion. Obviously, the thermodynamic equilibrium condition $n\Delta\tilde{\mu}_{H^+} = -\Delta G_p$ seems to be fulfilled when the values of Δp and ATP content of *Sulfolobus* cells are compared before and after partial uncoupling (Fig. 1).

Effects of DCCD on ATP synthesis. Upon binding to F_0F_1 H^+-ATPase, DCCD blocks proton permeability, and consequently membrane hyperpolarization as well as depressed oxygen uptake occur. Because DCCD is also competent to react with other proton pumps (3, 64), reversibility of respiratory depression in DCCD-prelabeled *Sulfolobus* cells necessarily had to be demonstrated. In fact, the addition of protonophores stimulated the O_2 uptake rate and concomitantly reduced the electrochemical proton gradient. Therefore, *S. acidocaldarius* exhibits a typical respiratory control effect, as previously described for mitochondria (12) and also the eubacterium *E. coli* (10).

An open question seems to be imposed by the phenomenon of apparent ATP synthesis after treatment of *Sulfolobus* cells with a combination of DCCD and protonophores. The analysis of time courses of ATP and ADP and of their sum indirectly demonstrates the buildup of significant amounts of AMP upon action of DCCD (Fig. 4A), suggesting a possible role for adenylate kinase (whose existence has been proven in cell extracts [M. Lübben, unpublished data]), partially compensating for the loss of ATP.

Localization of DCCD-binding protein. Although efficient blocking of ATP synthesis was achieved with *S. acidocaldarius* with relatively low concentrations of DCCD, the inhibition could not be assigned unambiguously to the covalent reaction with a unique specific membrane protein in intact *Sulfolobus* cells. If the complex reaction mixture was analyzed by gel electrophoresis, several polypeptide bands became successively radioactively labeled, displaying one prominent band at 34 kDa. Though distinct, this band cannot be safely ascertained to represent the respective ATP synthase protein. Because of its chemical reactivity (3), the DCCD inevitably intensifies nonspecific background labeling at high total concentrations. Therefore, additional discrete bands with apparent molecular masses greater and smaller than 34 kDa became tagged whether or not the respective protein(s) were engaged in the phosphorylation reaction in vivo.

On the other hand, it is known that the extremely hydrophobic DCCD-reactive protein from energy-transducing F_0F_1 H^+-ATPases, which is normally assembled from 6 to 12 copies attached to each other in the native complex (21, 22, 61), tends to assume various oligomeric states even when investigated by SDS gel electrophoresis (22, 61, 65). Similarly, the banding pattern in Fig. 4 may result from the predominance of multiple-association forms of a single low-molecular-mass polypeptide. In this context, the 34-kDa band may be provided with particular stability. Accordingly, a $CHCl_3$-methanol-extractable [^{14}C]DCCD-binding protein obtained by labeling isolated *S. acidocaldarius* membranes was regarded as the monomeric species of 7 kDa molecular mass. Again, on SDS gels the purified *Sulfolobus* proteolipid appeared as an ensemble of various oligomeric forms, displaying molecular masses attributable to integer monomers ranging up to about 6 (Fig. 5C). Presumably the tendency to generate stable aggregates results from altered conformations in various solvents and may be additionally overlaid by unusual electrophoretic mobility, which has been attributed at least to the mitochondrial proteolipid (35).

Relation of DCCD-binding protein to the ATP synthase. The archaebacterial proteolipid identified and characterized in this study is likely to be the constituent conferring DCCD sensitivity to the ATP synthase of *Sulfolobus* species. Further structural information, especially on the membrane-residing part of the complex, may provide clues for the decisive classification among F- and V-type ATPases (7, 52, 55, 59).

Beside catalytic subunits, the vacuolar ATPase from bovine chromaffin granules contains a 16-kDa proteolipid (44), which constitutes about twice the molecular mass resolved in this study for the corresponding 7-kDa *Sulfolobus* protein. Complete amino acid sequence analysis of the 16-kDa eu-

caryotic polypeptide reveals a composite primary structure of two portions, which are homologous to the F_0 proteolipids of *E. coli*, chloroplasts, or yeast mitochondria on the first half of the molecule and to those of bovine or *Neurospora* mitochondria on the second half (44). Thus, from an evolutionary point of view, the vacuolar-proteolipid gene seems to have been developed by duplication of a smaller ancestral proteolipid gene (44). On the other hand, the 7-kDa *S. acidocaldarius* DCCD-binding proteolipid is strictly related to equivalent eubacterial or yeast mitochondrial ATPase (62, 70) subunits with respect to primary structure. Hence, this archaebacterial proteolipid matches better to the low-molecular-mass F_0 constituent of eubacteria, mitochondria, and chloroplasts than to the 16-kDa integral membrane protein of vacuolar ATPase. This view is further confirmed by nucleotide sequence analysis of a gene from the strain *S. acidocaldarius* 9 encoding a proteolipid of a calculated molecular mass of 10,362 Da (18). When the predicted proteolipid amino acid sequence was aligned from its position 23 to the N terminus of our chemically determined partial sequence from *S. acidocaldarius* DSM 639, 82% of the amino acids were found to be identical, which strongly suggests that the polypeptides are homologous. Therefore, the protein may also exist in an immature form extended by 22 residues at the N terminus. After in vivo cleavage of the presequence, a polypeptide with a significantly reduced molecular mass should be left, which in turn fits fairly well with the value of 7 kDa determined by electrophoretic methods in this study.

At present there is no knowledge of additional polypeptides, which will be necessary to support the idea of a functionally competent proton channel as found in eubacterial F_0 (58).

An increasing number of soluble ATPases from archaebacterial sources have been purified and characterized (28, 32, 34, 43, 50). Considerable evidence from immunological cross-reactivity data obtained with antibodies directed against specific subunits (41) or against the whole complex of the soluble ATPase from *Sulfolobus* species (49) suggests that these enzymes are closely related to each other, irrespective of their origin from methanogens, halobacteria, or sulfur-dependent thermoacidophiles. A rather strong homology of the *Sulfolobus* ATPase to vacuolar than to F_1 ATPase has been suggested by nucleotide sequence data (16, 17, 51). It is not justified, however, to infer a confined, unequivocal role of vacuolar-type ATPase in cellular ATP synthesis of this archaebacterium, since V-type ATPases also exhibit significant structural homology to F-type ATPases (8, 73). On the other hand, only F_0F_1-ATPases have been established as universal catalysts of electron transport-driven phosphorylation as yet in energy-transducing membranes. In fact, the ATPase solubilized from *Sulfolobus* membranes is intimately related to eubacterial and organellar eucaryotic F_1 ATPase by immunological cross-reactivity of their structurally strongly conserved (69) β subunits (41), which are presumed to carry the catalytic sites of ATP synthase (reference 63 and references therein). In extension, observation of the F_1-characteristic pseudohexagonal quaternary structure also in the *Sulfolobus* ATPase via electron microscopy strikingly enhances this similarity (42).

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