HALOBACTERIAL A-ATP SYNTHASE IN RELATION TO V-ATPase

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Summary

The head piece separated from the A-ATP synthase of *Halobacterium halobium* hydrolyses ATP. This A_1-ATPase is inhibited by nitrate but not by other chaotropic anions. The nitrate inhibition is noncompetitive with respect to ATP, reversible, and partially protected by chloride. In contrast, ATP synthase *in situ* (A_1A_0-ATPase) is not inhibited by nitrate but apparently is inhibited by stronger chaotropic reagents, such as thiocyanate and trichloroacetate, which make the vesicle membrane permeable to protons. The mode of action of nitrate and chaotropic anions seems to differentiate A-ATPases from V-ATPases.

Other strains of *Halobacterium, Haloferax, Haloarcula, Halococcus* and *Natronobacterium,* contain at least two polypeptides immunochemically similar to the two major subunits, α ($86\times10^3 M_r$ on SDS–PAGE) and β ($64\times10^3 M_r$), of the A-ATPase of *Halobacterium halobium.* When solubilized, membrane vesicles of these halobacteria hydrolyse ATP. Their ATPases are commonly sensitive to nitrate. They require high concentrations of the supporting salt but depend differently on chloride or sulfate/sulfite. The A-ATPases of Halobacteriaceae appear to diverge with respect to salt preference.

Introduction

The ATP synthase in an extremely halophilic archaebacterium, *Halobacterium halobium (salinarium),* is an A-ATPase (Mukohata and Yoshida, 1987*b*; Mukohata and Ihara, 1990); it differs from the F-ATPase that had been considered to be ubiquitous throughout aerobic organisms and to be the central enzyme of ATP synthesis. The difference between the A-ATPase and F-ATPase was first shown enzymologically by the azide insensitivity of halobacterial ATP synthase (Mukohata and Yoshida, 1987*a,b*) and its A_1-ATPase (Nanba and Mukohata, 1987), then immunochemically, by the relatively small cross reaction of F-ATPase with an anti-halobacterial A_1-ATPase antibody (Mukohata *et al.* 1987*a*). (The halobacterial ATPase discussed here is the catalytic head-piece of the ATP synthase and thus corresponds to F_1-ATPase with respect to F_1F_0-ATP synthase. The ATPase is denoted as A_1-ATPase and the ATP synthase as A_1A_0-ATPase, when needed.) To our surprise, the antibody cross reacted with V-ATPase as much as it did with the A-ATPase of another kind of archaebacterium (Mukohata *et al.* 1987*a*). This

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close relationship between A-ATPases and V-ATPases and the remote relationship between F-ATPases and A/V-ATPases were confirmed by the identity (homology) values of the amino acid sequences deduced from the genes coding the ATPases (Denda *et al.* 1988*a*,*b*; Inatomi *et al.* 1989; Ihara and Mukohata, 1991).

The relationship was then discussed with reference to the evolution of the proton-translocating ATPase family (Gogarten *et al.* 1989; Iwabe *et al.* 1989; Mukohata *et al.* 1990). The diversion of the A-ATPase from the V-ATPase took place long after the F-ATPase had diverged from the A/V-ATPase.

V-ATPases have been modified on the various endomembranes: vacuole, chromaffin granule, synaptosome, Golgi apparatus, and so on (Forgac, 1989). The A-ATPase has also been modified, possibly in different ways, in the extreme environments of archaebacterial habitats.

Halobacterial ATP synthase (A_1A_o-ATPase) is not inhibited by nitrate, whereas its $\alpha_3\beta_3$ head piece (A_1-ATPase) is inhibited (Mukohata and Ihara, 1990). The V-ATPase *in situ*, which is thought to be equivalent to the ATP synthase (F_1F_o and A_1A_o), is inhibited by nitrate but its head piece does not hydrolyse ATP.

In this report, we describe some effects of nitrate and other chaotropic anions that differentiate A-ATPases and V-ATPases. We also describe some features which even differentiate the A-ATPase of one member of the Halobacteriaceae from that of other members.

Materials and methods

Halobacterium halobium (*salinarium*) R_1mR was separated as a spontaneous mutant from *Halobacterium halobium* R_1 (from D. J. Kushner, University of Ottawa). *Halobacterium halobium* DSM670, *Halobacterium saccharovorum* DSM1137, *Halobacterium sodomense* ATCC33755, *Haloferax volcanii* ATCC29605, *Haloarcula vallismortis* ATCC29715, *Halococcus morrhuae* DSM1307 and *Natronobacterium pharaonis* DSM2160 were purchased from the Institute for Fermentation (Osaka). *Halobacterium* and *Halococcus* were cultivated in media as reported previously (Matsuno-Yagi and Mukohata, 1977). *Haloferax* and *Haloarcula* were cultivated in 15.6% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.4% KCl, 1.3% $MgCl_2 \cdot 6H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, 0.02% $NaHCO_3$, 0.05% KBr, 0.1% glucose and 0.5% yeast extract, pH 7.0. *Natronobacterium* was cultivated in 25% NaCl, 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.1% sodium glutamate, 0.017% $CaSO_4 \cdot 2H_2O$, 0.024% $MgSO_4 \cdot 7H_2O$, 0.1% KCl, 0.5% casamino acid and 0.5% yeast extract at pH 9.0. All cultures were vigorously shaken for 1 week at 37–40°C. Cells were collected by centrifugation. Membrane vesicles were prepared by sonication as described by Mukohata *et al.* (1986). Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

Unless otherwise noted, ATP hydrolytic activity was determined at 40°C in a reaction mixture of 1.5 mol l^{-1} Na_2SO_4, 10 mmol l^{-1} $MnSO_4$, 4 mmol l^{-1} ATP and 40 mmol l^{-1} Mes at pH 5.8 (Nanba and Mukohata, 1987). The reaction was started by adding the membrane vesicles, which had been solubilized with 0.7% Triton X-165 at a final protein concentration between 0.2 and 0.6 mg ml^{-1}. Liberated inorganic phosphate (P_i) was

determined by the method of Taussky and Shorr (1953) or by the the Malachite Green method of Hess and Derr (1975). ATP synthetic activity was determined by the method of Mukohata *et al.* (1986), in which membrane vesicles of *Halobacterium halobium* were loaded with the reaction medium (1 mol l^{-1} NaCl or 1 mol l^{-1} sodium isethionate, 0.1 mol l^{-1} MgCl$_2$, 5 mmol l^{-1} ADP, 20 mmol l^{-1} P$_i$ and 50 mmol l^{-1} Pipes at pH 6.8) by sonication, and washed with substrate-free medium. When needed, 10 mmol l^{-1} chaotropic reagents were added to the medium. ATP was synthesized by base–acid transition (ΔpH=2.8) (Mukohata *et al.* 1986) and determined by the luciferin–luciferase method.

Western blotting was performed as described by Towbin *et al.* (1979). Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), transferred to a polivinylidene difluoride membrane (Millipore, Bedford) and hybridized with an antibody raised in rabbits against each of the subunits (α and β) of *Halobacterium halobium* A$_1$-ATPase. After the second hybridization with alkaline-phosphatase-conjugated goat anti-rabbit IgG, the hybridized proteins were visualized by mixing them with 5-bromo-4-chloro-3-indolylphosphate and tetrazolium.

All other reagents were purchased from Wako Chemical Co. (Osaka) and Nacalai Chemicals (Kyoto).

Results

Halobacterium halobium A$_1$-ATPase showed complex kinetics for ATP hydrolysis in a 1.5 mol l^{-1} Na$_2$SO$_4$ assay solution, as was found for the *Halobacterium saccharovorum* ATPase in 4 mol l^{-1} NaCl (Schobert and Lanyi, 1989) (Fig. 1). Apparently, the rate of P$_i$ release consists of two components: the rate V_1 found within the initial 1 min, which is not affected by nitrate concentration, and the rate V_2 after 3 min, which is affected by nitrate concentration. Here, we discuss only the nitrate-sensitive V_2. Lineweaver–Burk plots
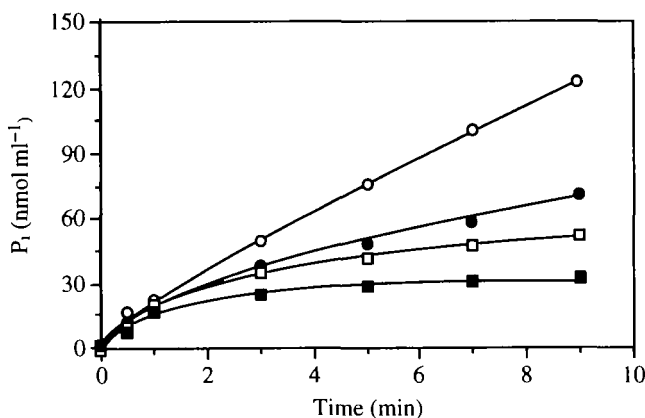

Fig. 1. An example of the effect of nitrate on ATP hydrolysis by *Halobacterium halobium* ATPase. The final protein concentration was 0.3 mg ml^{-1}. Nitrate was added at 0 mmol l^{-1} (O), 2 mmol l^{-1} (●), 10 mmol l^{-1} (□) and 50 mmol l^{-1} (■).

show that the nitrate inhibition is noncompetitive with a fixed K_m for ATP of 2.5 mmol l^{-1} and an apparent K_i for nitrate of about 3 mmol l^{-1} (data not shown).

Halobacterium halobium A$_1$-ATPase activity was examined for the effects of several anions, most of which appear in the Hofmeister series (Table 1). Nitrate inhibits the ATPase whereas other reagents, even more chaotropic ones, do not. Although chloride and acetate seem to activate ATPase activity to some extent, only chloride increased the I$_{50}$ value of nitrate by sixfold (Table 2). When the potassium salts of these anions were added, similar results were obtained.

The effects of these anions on ATP synthesis in the membrane vesicles of *Halobacterium halobium* were also examined (Table 3). Just as it had little effect in solutions containing 1 mol l^{-1} NaCl as supporting salt (the salt required for halophilic

Table 1. *The effects of anions on the ATP hydrolytic activity of the solubilized membrane of* Halobacterium halobium

Additional salt	Percentage activity		
	1 mmol l^{-1} salt	3 mmol l^{-1} salt	10 mmol l^{-1} salt
None	100	100	100
Sodium trichloroacetate	104±5	104±15	85±3
Sodium thiocyanate	101±11	102±6	93±8
NaI	102±6	100±4	98±7
NaClO$_4$	104±6	106±11	85±4
NaNO$_3$	59±5	47±6	26±4
NaBr	100±5	99±6	103±7
NaCl	102±2	105±11	107±4
Sodium acetate	107±4	103±1	118±10

The halobacterial membrane vesicles were suspended in 1.0 mol l^{-1} Na$_2$SO$_4$, 10 mmol l^{-1} Mes (pH 5.8) and dialyzed against 1.0 mol l^{-1} Na$_2$SO$_4$ to minimize the carrying-over chloride in the preparation. The membrane vesicles were then solubilized and assayed for ATP hydrolysis (100%=95±16 nmol P$_i$ min^{-1} mg^{-1} protein).

Values are expressed as the mean±standard deviation of four independent measurements.

Table 2. *The I$_{50}$ values of nitrate for* Halobacterium halobium *ATPase in the presence of various added salts*

Additions	I$_{50}$ (mmol l^{-1})
Control	4.1±0.5
Sodium isethionate	5.7±1.9
Sodium acetate	4.4±1.5
NaBr	7.5±2.2
NaCl	29.5±4.4

The salt (0.8 mol l^{-1}) was added to the reaction medium containing 1.0 mol l^{-1} Na$_2$SO$_4$, 10 mmol l^{-1} MnSO$_4$, 4 mmol l^{-1} ATP, 40 mmol l^{-1} Mes (pH 5.8). For the control, 0.5 mol l^{-1} Na$_2$SO$_4$ was added.

The I$_{50}$ value is almost constant between 1.0 and 2.0 mol l^{-1} Na$_2$SO$_4$ for the supporting salt solution. The means and standard deviations of three determinations are given.

enzymes to maintain their functions), nitrate did not inhibit ATP synthesis in solutions containing 1 mol l⁻¹ sodium isethionate, which does not protect the ATPase from nitrate inhibition (Table 2). Chaotropic reagents much stronger than nitrate, such as thiocyanate and trichloroacetate, did inhibit ATP synthesis.

The subunit sizes and salt-dependency of the ATPases of Halobacteriaceae were examined. Antibodies raised against each of the subunits (α and β) of *Halobacterium halobium* A_1-ATPase were tested for their cross reactions to the whole-cell proteins of various halobacterial strains. The sizes of the two major subunits of all the strains were similar to the individual subunits of *Halobacterium halobium* ATPase: $86 \times 10^3 M_r$ (α subunit) and $64 \times 10^3 M_r$ (β subunit) on SDS–PAGE (Fig. 2).

Membrane vesicles prepared from various halobacteria showed little ATPase activity without the addition of detergent. After the vesicles had been solubilized with Triton X-165, ATPase activity became detectable (Table 4). As has been demonstrated with

Table 3. *The effects of anions on ATP synthesis by the membrane vesicles of* Halobacterium halobium

Salt added (10 mmol l⁻¹)	Percentage activity	
	NaCl (1 mol l⁻¹)	Sodium isethionate (1 mol l⁻¹)
None	100	100
Sodium trichloroacetate	13	23
Sodium thiocyanate	12	25
NaI	91	113
NaClO₄	91	91
NaNO₃	98	107

The ATP synthesized in 30 s after a pH jump (external pH 6.8 to 4.3) was measured.

The activities of the control (none) in 1 mol l⁻¹ NaCl and 1 mol l⁻¹ sodium isethionate were 266 and 339 pmol ATP mg⁻¹ protein, respectively.

Results of two independent measurements were averaged.

Table 4. *The ATP hydrolytic activity of solubilized membrane of various halobacterial strains*

Strain	Activity (nmol P$_i$ min⁻¹ mg⁻¹ protein)	
	In 4 mol l⁻¹ NaCl (pH 7.0)	In 1.5 mol l⁻¹ Na₂SO₄ (pH 5.8)
Halobacterium halobium R₁mR	20±3	84±8
Halobacterium halobium DSM670	18±3	81±5
Halobacterium saccharovorum	69±7	62±8
Halobacterium sodomense	75±2	63±4
Haloarcula vallismortis	57±5	27±7
Haloferax volcanii	9±3	9±2

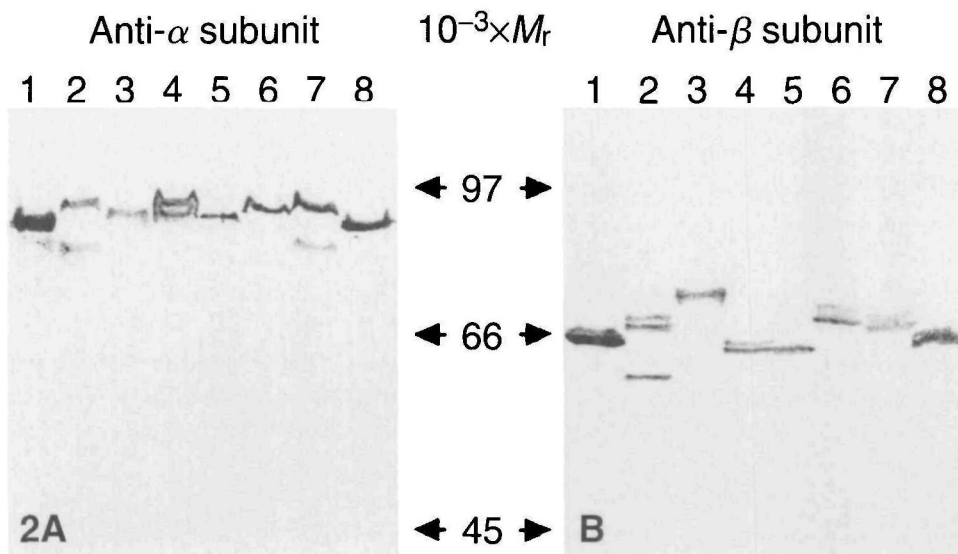The means and standard deviations of three determinations are given.

Fig. 2. Diversity of the subunit sizes in ATPases of Halobacteriaceae. Western blots of the proteins of various halobacterial strains were performed with the antibodies raised against the α and β subunits of *Halobacterium halobium* ATPase. Whole cells were solubilized and electrophoresed on a 10% SDS gel. Cross reactions were performed with (A) anti-α subunit and (B) anti-β subunit. The relative molecular masses are indicated. Lanes 1, 8, *Halobacterium halobium*; lane 2, *Natronobacterium pharaonis*; lane 3, *Halococcus morrhuae*; lane 4, *Haloferax volcanii*; lane 5, *Haloarcula vallismortis*; lane 6, *Halobacterium sodomense*; lane 7, *Halobacterium saccharovorum*.

Halobacterium halobium (Nanba and Mukohata, 1987), this result is found because the vesicles were in the right-side-out configuration with catalytic A_1 sectors inside. ATP was hydrolysed only after the membranes had been solubilized and the A_1-ATPase had been released. The ATPases of *Halobacterium halobium* R_1mR and DSM670 were much more active in $1.5\,mol\,l^{-1}$ Na_2SO_4 at pH 5.8 than in $4\,mol\,l^{-1}$ NaCl at pH 7.0. In contrast, the ATPases of *Halobacterium saccharovorum*, *Halobacterium sodomense* and *Haloarcula vallismortis* were more active in $4\,mol\,l^{-1}$ NaCl than in $1.5\,mol\,l^{-1}$ Na_2SO_4. The ATPase of *Haloferax volcanii* exhibited only low activity in both media.

Discussion

A-ATPases and V-ATPases

A-ATPases and V-ATPases, like F-ATPases, are H^+-translocating enzymes composed of a catalytic head piece, a stem and a membrane-embedded channel (Pedersen and Carafoli, 1987a; Penefsky and Cross, 1991; Mukohata *et al.* 1992). A-ATPases and F-ATPases usually synthesize ATP under the proton-motive force, whereas V-ATPases only hydrolyse ATP to pump protons across membrane into endosomal vesicles. The halobacterial ATP synthase of present interest is a type of A-ATPase (A_1A_o) that is similar to V-ATPases (V_1V_o). The halobacterial ATPase under discussion is the head

piece separated from the ATP synthase and thus designated the A_1-ATPase. The halobacterial A_1-ATPase appears to be composed of $\alpha_3\beta_3$ subunits (Mukohata *et al.* 1991); no other subunit is needed for ATP hydrolysis. The F_1-ATPase is composed of $\alpha_3\beta_3\gamma$ or $\alpha_3\beta_3\delta$ as the functional complex (Yoshida *et al.* 1977), although an $\alpha_3\beta_3$ complex of the thermophilic bacterium PS3 has been shown to hydrolyse ATP (Miwa and Yoshida, 1989).

The effects of nitrate and chloride on Halobacterium halobium *A₁-ATPase*

Halobacterium halobium A_1-ATPase is inhibited by nitrate with an I_{50} value of about 3 mmol l^{-1} in 1.5 mol l^{-1} Na_2SO_4 (Mukohata and Ihara, 1990). Since reagents that are even more chaotropic than nitrate, such as thiocyanate and chlorate, were less effective inhibitors (Table 1), one can conclude that nitrate inhibited the A_1-ATPase specifically. The addition of chloride or acetate apparently activated the ATPase; this effect became very obvious as the concentration of chloride or acetate increased. However, the I_{50} value increased as the chloride concentration was increased, but not as the acetate, isethionate or sulfate concentration was increased. Therefore, chloride seems to protect the ATPase from nitrate inhibition to some extent. This protective effect of chloride is not due merely to the increase in concentration of salts that support the integrity of the enzyme and/or activate it.

100 mmol l^{-1} nitrate completely inhibits the halobacterial A_1-ATPase. However, removal of nitrate by dialysis restores the initial activity of the sample to the level observed before the addition of nitrate (data not shown). Reversibility of nitrate inhibition was also reported for *Halobacterium saccharovorum* ATPase (Schobert and Lanyi, 1989).

The activity of *Halobacterium halobium* ATPase is very low in solutions of NaCl or KCl as the supporting salt, even at 4 mol l^{-1}, and is much higher in solutions of Na_2SO_3 (1.5 mol l^{-1}) and sodium citrate (0.8 mol l^{-1}) (Nanba and Mukohata, 1987). *Halobacterium saccharovorum* ATPase (Hochstein *et al.* 1987; Stan-Lotter and Hochstein, 1989; Schobert and Lanyi, 1989) and some halobacterial ATPases show higher activity at pH 7.0 in 4 mol l^{-1} NaCl than in 1.5 mol l^{-1} Na_2SO_4 (Table 4). Therefore, the requirement for a high (supporting) salt concentration of halobacterial ATPases in general is largely due to a salting-out type of action, which makes the subunit complex tight enough to hydrolyse ATP. The tightness needed would probably differ among ATPases even in the halobacterial family, depending on the structures of the subunits (see below). The preference for the supporting salt could be explained in a similar way. Activating effects of SO_4^{2-} and HSO_3^- are reported in the A_1-ATPase of *Sulfolobus acidocaldarius* (Konishi *et al.* 1987; Lübben *et al.* 1987) and *Methanosarcina barkeri* (Inatomi, 1986). However, the concentrations of the supporting salts in these reaction media were very low (20–30 mmol l^{-1}). The effect of sulfate may not be same on all A-ATPases.

Effects of chaotropic anions on ATP synthesis

Nitrate only inhibits the halobacterial ATP synthesis slightly in 1 mol l^{-1} NaCl, even at 100 mmol l^{-1}, a level that almost completely inhibits ATP hydrolysis in 1.5 mol l^{-1}

Na_2SO_4 (Mukohata and Ihara, 1990). As in the case of ATP hydrolysis (Table 2), 1 mol l^{-1} NaCl loaded into the vesicles may protect ATP synthesis from nitrate inhibition. However, ATP is synthesized in 1 mol l^{-1} sodium isethionate in amounts similar to or even greater than those in 1 mol l^{-1} NaCl. Nitrate does not inhibit the ATP synthesis either in sodium isethionate or in NaCl (Table 3). Since isethionate does not protect ATP hydrolysis from nitrate inhibition (Table 2), these results indicate that ATP synthesis is not affected by nitrate. The ATP synthase of *Halobacterium halobium* is the membrane-anchored holoenzyme (A_1A_0), whereas the isolated A_1-ATPase is the $\alpha_3\beta_3$ head piece of the synthase. There is probably a nitrate binding site(s), which is characterized by non-competitive inhibition. When the head piece is detached as the A_1-ATPase from the ATP synthase, the nitrate binding site should be exposed or its affinity for nitrate should be increased, which eventually inhibits ATP hydrolysis.

Slight inhibition of ATP synthesis by nitrate may be due to the chaotropic nature of nitrate. As shown in Table 3, the degree of inhibition of ATP synthesis roughly follows the Hofmeister series: SCN^-, $CCl_3COO^- > I^- > ClO_4^- > NO_3^-$. Additions of chaotropic anions to the external medium also depressed ATP synthesis. Moreover, these anions caused a faster decay of the light-induced pH shift of the membrane vesicle suspension (data not shown). Therefore, the reagents seem to make the membrane leaky to protons, which should diminish the proton-motive force and decrease ATP synthesis.

By contrast, chaotropic anions destroy V-ATPases by releasing the head piece, which carries no ATP hydrolysing activity (Rea *et al.* 1987; Bowman *et al.* 1989; Moriyama and Nelson, 1989; Arai *et al.* 1989). Halobacterial A_1-ATPase was not released from the membrane vesicles to any marked extent by various chaotropic anions (data not shown). Thus, the mode of action of chaotropic anions differs between A-ATPases and V-ATPases.

By analogy with F-ATPase, the N,N'-dicyclohexylcarbodiimide (DCCD)-binding protein is considered to be part of the membrane-embedded component of H^+-translocating A-ATPase/synthase. [^{14}C]DCCD labeled two polypeptides of 78 and $12 \times 10^3 M_r$ (by SDS–PAGE) in parallel with the inhibition of ATP synthesis in *Halobacterium halobium* (Mukohata *et al.* 1987*b*). The N-cyclohexyl N'-[4-(dimethylamide)-α-naphthyl] carbodiimide (NCD-4)-binding protein of $10 \times 10^3 M_r$ (by gel filtration in the presence of SDS) was isolated (K.-I. Sugimura, S. Watanabe, K. Ihara and Y. Mukohata, unpublished results). The DCCD-binding protein of 10 362 Da was also isolated from *Sulfolobus acidocaldarius* (Denda *et al.* 1989). The size of this DCCD-binding protein is almost in the range of the c subunit of F_1F_0-ATPase but not in the range of the corresponding subunit of V-ATPase, which has a duplicated mass of $16 \times 10^3 M_r$ (Mandel *et al.* 1988; Nelson and Nelson, 1989). This is another difference between A-ATPases and V-ATPases.

The A-ATPase family of Halobacteriaceae

The relative molecular masses of the two major subunits of *Halobacterium halobium* ATPase are similar to those of other A-ATPases and V-ATPases (Mukohata *et al.* 1991, 1992). The apparent relative molecular masses of the *Halobacterium halobium* ATPase subunits, estimated by SDS–PAGE, are 86×10^3 for the α subunit and 64×10^3 for the β

subunit (Nanba and Mukohata, 1987). These M_r values are much larger than those estimated from amino acid sequences deduced from the encoding genes: 64 104 Da for the α subunit and 51 956 Da for the β subunit (Ihara and Mukohata, 1991). This discrepancy is probably due to the excess content of acidic amino acids (approximately 20%) that is characteristic of halophilic enzymes. Western blot analysis using the antibody to *Halobacterium halobium* ATPase revealed that various halobacterial strains carry A-ATPase subunits of similar sizes (Fig. 2). The size diversity is a little wider among the β subunits than among the α subunits, which carry the ATP-binding region. Since the sizes as well as the amino acid sequences of the subunits are well conserved, even among the A-ATPases of the three different groups (Denda *et al.* 1988*a,b*; Inatomi *et al.* 1989; Ihara and Mukohata, 1991), the actual sizes of these ATPase subunits of Halobacteriaceae should be similar. The apparently larger subunit sizes on SDS–PAGE suggest that, like *Halobacterium halobium* ATPase, all of these subunits contain large amounts of acidic amino acids (Ihara and Mukohata, 1991). Such high contents (and the distribution) of acidic amino acids probably result not only in the apparent diversity of the subunit sizes on SDS–PAGE but also in the different salt preferences.

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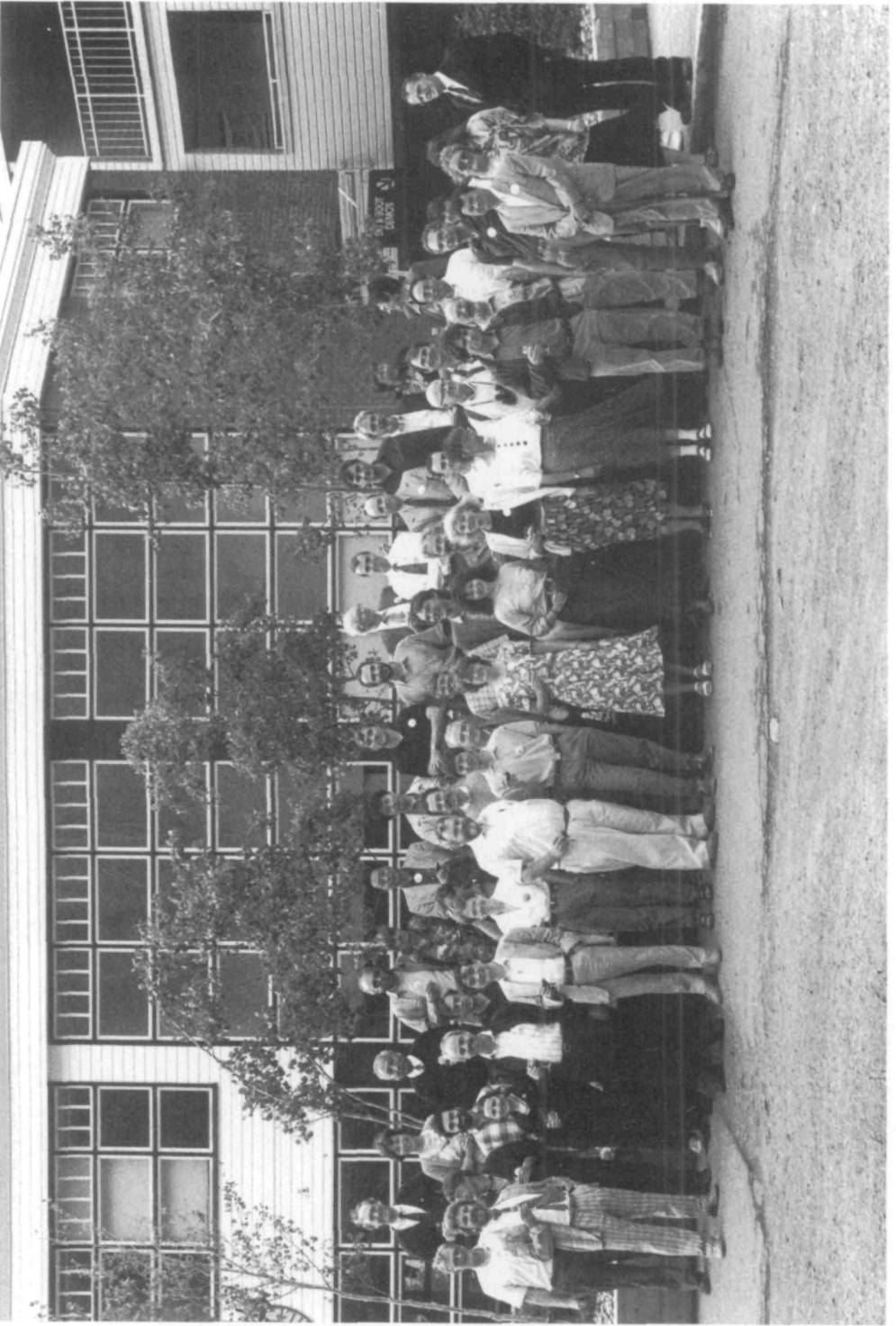
Photographs taken at Telluride

M. Clements

B. Gaddis

The jeep trip