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Amiloride Resistance in the Methanoarcheon *Methanothermobacter thermoautotrophicus*: Characterization of Membrane-Associated Proteins

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ABSTRACT. An amiloride-resistant mutant with diminished Na^+/H^+ antiporter activity was isolated from *Methanothermobacter thermoautotrophicus*. To define the protein basis of amiloride resistance, the composition of membrane-associated proteins was partially characterized and compared with that of the wild type strain. An abundant 670-kDa membrane-associated protein that was present only in the mutant strain was analyzed by MALDI-TOF MS and identified as a coenzyme F_{420}-reducing hydrogenase. The amiloride resistance was not accompanied by changes in protein size or changes in the level of subunits A or B of the A_1A_o-type ATP synthase; on the other hand, the SDS-PAGE patterns of the chloroform–methanol extract of membranes from both strains were different. Two bands with calculated molecular mass 16 and 11 kDa were identified as MtrD and AtpK, respectively. The observed over-expression of a 22.7-kDa protein in the mutant cells may represent the multimeric form of the MtrD subunit. These results show that the impairment of the Na^+/H^+ antiporter system in the amiloride-resistant mutant of *Methanothermobacter thermoautotrophicus* is accompanied by only small changes in a few membrane-associated proteins.

Evidence has been obtained that Na^+ ions can substitute for H^+ as the coupling ions in a number of different bacterial species (Skulachev 1994). The Na^+-energetic cycle composed of primary Na^+ electrochemical gradient generator(s) and consumer(s) was found in various bacteria in addition to the "classical" energetic of H^+-cycle based on circulation of protons (Skulachev 1989). Na^+/H^+ antiporters can therefore serve as a universal device for linking the H^+-cycle with Na^+-cycle (Padan and Schuldiner 1994; *cf*. Kinclová-Zimmermannová *et al.* 2004).

Similarly, Na^+ stimulated the growth, methane production and some membrane-bound processes in methanoarchaea (Perski *et al*. 1981, 1982). These findings indicate that Na^+ can be directly involved in energy transduction in this special branch of *Archaea*. During methanogenesis, H_2 reduction of CO_2 generates an electrochemical ion gradient of H^+ and Na^+ by two distinct primary ion pumps (Deppenmeier *et al.* 1996). Heterodisulfide reductase (EC 1.12.99.2) has been characterized as a proton-translocating system and N^5-methyltetrahydromethanopterin:coenzyme M methyltransferase (EC 2.1.1.86) has been shown to act as a primary Na^+ translocating enzyme (Becher *et al.* 1992). Both the ion gradients are directly coupled to ATP synthesis *via* two specific H^+- and Na^+-dependent processes (Deppenmeier *et al.* 1996). Furthermore, the circulation of H^+ and Na^+ across the cytoplasmic membrane seems to be coupled *via* Na^+/H^+ antiport in methanoarchaea. It was suggested that the Na^+/H^+ antiporter might participate in the transformation of a sodium electrochemical gradient into proton-motive force (Kaesler and Schönheit 1989). In such a case, the Na^+/H^+ antiporter might be a key regulatory element in the bioenergetic strategy of methanoarchaea and could be responsible for the adaptive energetic behavior observed under different physiological conditions (Majerník *et al*. 1997). In spite of many studies over the past decade, these processes have not yet been satisfactorily elucidated.

To understand the function and interrelationship of these bioenergetic subsystems, genetic elimination of the components of the bioenergetic machinery could be very fruitful. Therefore, we started with a systematic genetic approach to the problem of energy conservation in methanoarchaea (Šmigáň *et al.* 1997). This paper presents an analysis of membrane-associated proteins from a *Methanothermobacter thermoautotrophicus* mutant resistant to amiloride, an inhibitor of the Na^+/H^+ antiporter.

MATERIALS AND METHODS

Proteins from membrane vesicles were analyzed by SDS-PAGE using a standard technique (Laemmli 1970). Separated proteins were electroblotted onto nitrocellulose and probed with antibodies against the A and B subunits of A_1A_o ATPase from *Halobacterium salinarium.* Visualization was performed with pig anti-rabbit IgG secondary antibodies conjugated with alkaline phosphatase. After native PAGE, protein bands were excised from the gel, trypsin digested and analyzed using a MALDI TOF/TOF 4700 Proteomic Analyzer (*ABI*). The Mascot search engine was used to identify cognate proteins by matching MS detected peptides to *M. thermoautotrophicus* proteins. Selective extraction of subunit *c* of the A_1A_o ATPase from cytoplasmic membranes of *M. thermoautotrophicus* and the amiloride-resistant (AR) mutant was performed by chloroform–methanol extraction according to Ruppert *et al*. 2001. Protein was quantified according to Lowry method with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The AR mutant of *M. thermoautotrophicus* was isolated; the composition of the membrane-associated protein fraction of this mutant was compared with that of wild-type cells. An abundant, membrane-associated protein complex with a molar mass of 670 kDa was observed using native PAGE to be specifically expressed in AR mutant. A similar protein was also detected in an uncoupler-resistant and neomycin-resistant mutant (Čuboňová *et al*. 2004). The upregulated 670-kDa protein was identified as the coenzyme F_{420}-reducing hydrogenase by positive identification of peptides derived from all three of its subunits (coenzyme F_{420}-reducing hydrogenase reduces the central electron carrier – coenzyme F_{420} in methanoarchaea).

ATP synthesis under different experimental conditions driven by (*a*) methanogenic electron transport, (*b*) potassium diffusion potential in the presence of H^+ (electrochemical proton gradient dependent, (*c*) potassium diffusion potential in the presence of Na^+ (electrochemical sodium-ion-gradient dependent) was significantly elevated in the mutant strain (Šurín *et al*. 2006). For this reason, our study focused specifically on the comparison of the presence of different subunits of the *M. thermoautotrophicus* A_1A_o ATP synthase. An immunodetection was done of the two main subunits (A and B) of the A_1A_o ATPase/synthase in the wild-type and mutant cells. Two strong cross-reactive bands were observed after Western blotting of membrane vesicle proteins with a polyclonal antiserum reactive to either the A or B subunit of the halobacterial A_1A_o ATPase (Fig. 1) in both strains. The apparent molar mass of the cross-reacting bands was 80 and 60 kDa for the A and B subunit, respectively, which indicates that the impairment of ATPase and synthase activity in AR strain is not caused by or associated with remarkable changes in protein size or concentration of A and/or B subunits of the A_1A_o ATPase/synthase.

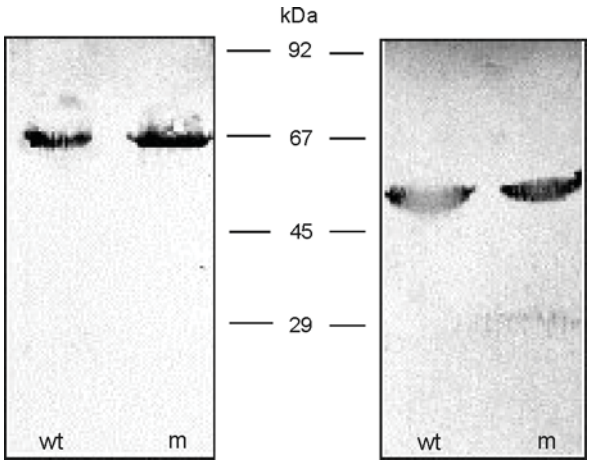

Fig. 1. Western blot of protein fractions derived from membrane vesicles of wild-type and amiloride-resistant mutant cells of *M. thermoautotrophicus* with antibodies against the A and B subunits of the A_1A_o ATPase from *H. salinarium*; *left* – subunit A, *right* – subunit B; wt – wild type, m – mutant.

We also compared chloroform–methanol extracts of the above strains. The extraction predominantly yielded two highly lipophilic proteins: subunit *c* (AtpK or proteolipid) of the A_1A_o ATPase/synthase and subunit D (MtrD) of the Na^+-translocating methyltetrahydromethanopterin:coenzyme M methyltransferase (Ruppert *et al*. 2001). Both these subunits were predicted to be directly involved in ion transport (Ruppert *et al*. 1998; Lienard and Gottschalk 1998). SDS-PAGE patterns of the chloroform–methanol extracts of membranes from wild-type and mutant strains are different (Fig. 2). Based on estimated molar mass, we were

able to positively identify only two smaller bands of calculated molar mass of 16 and 11 kDa represent MtrD and AtpK, respectively (Ruppert *et al*. 2001).

AR in our mutant increased because of mutation(s) that affect a specific locus coding for the Na^+/H^+ antiporter. This mutant probably posses either pleotropic or multiple mutations responsible for the observable changes of protein composition.

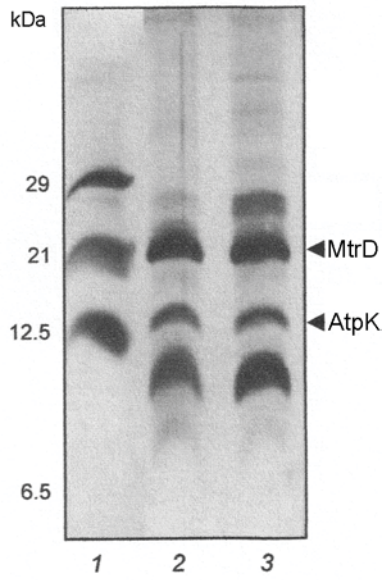

Fig. 2. SDS-PAGE of chloroform–methanol extracts of membranes from wild-type and amiloride-resistant mutant cells of *M. thermoautotrophicus*; protein band visualized by silver staining; *1* – molar-mass markers, *2* – wild-type strain, *3* – mutant; *arrowheads* – proteins MtrD and AtpK.

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