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Amino acid composition of bulk protein and salt relationships of selected enzymes of *Salinibacter ruber*, an extremely halophilic bacterium

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Abstract The extremely halophilic bacterium *Salinibacter ruber* was previously shown to have a high intracellular potassium content, comparable to that of halophilic Archaea of the family Halobacteriaceae. The amino acid composition of its bulk protein showed a high content of acidic amino acids, a low abundance of basic amino acids, a low content of hydrophobic amino acids, and a high abundance of serine. We tested the level of four cytoplasmic enzymatic activities at different KCl and NaCl concentrations. Nicotinamide adenine dinucleotide (NAD)-dependent isocitrate dehydrogenase functioned optimally at 0.5–2 M KCl, with rates of 60% of the optimum value at 3.3 M. NaCl provided less activation: 70% of the optimum rates in KCl were found at 0.2–1.2 M NaCl, and above 3 M NaCl, activity was low. We also detected nicotinamide adenine dinucleotide phosphate (NADP)-dependent isocitrate activity, which remained approximately constant between 0–3.2 M NaCl and increased with increasing KCl concentration. NAD-dependent malate dehydrogenase functioned best in the absence of salt, but rates as high as 25% of the optimal values were measured in 3–3.5 M KCl or NaCl. NAD-dependent glutamate dehydrogenase, assayed by the reductive amination of 2-oxoglutarate, showed low activity in the absence of salt. NaCl was stimulatory with optimum activity at 3–3.5 M. However, no activity was found above 2.5 M KCl. Although the four activities examined all function at high salt concentrations, the behavior of individual enzymes toward salt varied considerably. The results presented show that *Salinibacter* enzymes are adapted to function in the presence of high salt concentrations.

Key words *Salinibacter* · Amino acid composition · Malate dehydrogenase · Isocitrate dehydrogenase · Glutamate dehydrogenase · Halophilic

Introduction

Nature has devised two fundamentally different strategies for microorganisms to cope with the presence of high salt concentrations in their environment (Oren 1999). As was first shown by Christian and Waltho (1962), the halophilic Archaea of the family Halobacteriaceae accumulate high concentrations of KCl intracellularly, balancing the high concentrations of NaCl and other salts in their medium. Intracellular Na^+ concentrations are kept at a relatively low level. The second strategy, which is much more widespread, involves the synthesis or accumulation of high concentrations of organic osmotic solutes, while excluding salt as much as possible from the cytoplasm. This mode of osmotic adaptation is used by most aerobic heterotrophic halophilic Bacteria (Ventosa et al. 1998), photosynthetic prokaryotes and eukaryotes, and fungi, and also by halophilic methanogenic Archaea (Oren 1999).

The presence of high intracellular salt concentrations requires far-reaching adaptations of the enzymatic machinery to be active at molar concentrations of KCl. The proteins of halophilic Archaea of the family Halobacteriaceae are highly enriched in the acidic amino acids glutamic acid and aspartic acid, have a low content of basic amino acids, and contain relatively low levels of hydrophobic amino acids, while being enriched in serine (Dennis and Shimmin 1997; Lanyi 1974). Different mechanisms have been proposed to explain the special behavior of halophilic proteins. Acidic residues are more highly hydrated than other amino acids and can coordinate the organization of a hydrated salt ion network at the surface of the protein (Dennis and Shimmin 1997; Dym et al. 1995; Eisenberg 1995; Eisenberg and Wachtel 1987; Eisenberg et al. 1992). Moreover, certain acidic residues can be used to form salt bridges with strategically positioned basic residues. Salt bridges, positioned so

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that they are protected from shielding by solute ions, provide structural rigidity and are important determinants in the stabilization of the three-dimensional structure of halophilic proteins (Dennis and Shimmin 1997; Dym et al. 1995). Thus, an ordered water-molecule network and intersubunit salt bridges are formed, "locked" in by bound solvent chloride and sodium ions (Richard et al. 2000). The hydrophobic character of the surface may be further lowered by a reduction in surface-exposed lysine residues (Britton et al. 1998). Such proteins are not only functional at high salt concentrations, but they also generally require high salt for stability and activity.

The strategy of using high intracellular salt concentrations to balance the high osmotic pressure of hypersaline environments has been thus far found only in halophilic Archaea of the order Halobacteriales and in a specialized group of anaerobic halophilic Bacteria of the order Halanaerobiales (low G + C subgroup of the Firmicutes). Members of the Halanaerobiales contain high intracellular K^+ and Cl^- concentrations, their proteins show a large excess of acidic amino acids (Oren 1986), and their enzymes require high salt concentrations for optimal activity (Oren and Gurevich 1993; Rengpipat et al. 1988).

Recently a new type of red, rod-shaped, aerobic, extremely halophilic bacterium was isolated from saltern crystallizer ponds in Spain. The organism, *Salinibacter ruber* (Antón et al. 2002), is distantly related to the genus *Rhodothermus* (order Cytophagales). *Salinibacter* is an obligate halophile, growing optimally at salt concentrations between 200 and 300 g l^{-1}, and it is unable to grow at less than 150 g l^{-1} NaCl. By using fluorescent oligonucleotide probes, the organism has been shown to be abundant in certain hypersaline environments: between 5% and 25% of the total prokaryotic community in Spanish crystallizer ponds belongs to this species (Antón et al. 2000).

Preliminary attempts to assess the intracellular ionic concentrations within *Salinibacter* cells have shown K^+/protein ratios of the same order of magnitude as those found in the halophilic Archaea *Halobacterium salinarum* and *Haloarcula marismortui* (Antón et al. 2002), suggesting that *Salinibacter* may use a similar strategy of adaptation to high salt. More detailed measurements of its intracellular concentrations of inorganic ions and organic osmotic solutes have also been made (A. Oren, M. Heldal, S. Norland, and E.A. Galinski, manuscript submitted for publication).

In the present study, we compared the amino acid composition of the bulk protein of *S. ruber* and tested the behavior of several of its cytoplasmic enzymatic activities at different KCl and NaCl concentrations.

Materials and methods

Bacterial and archaeal strains and culture conditions

Salinibacter ruber strain M31 (DSM 13855) was grown on a rotatory shaker in 2-l Erlenmeyer flasks containing 1 l of medium of the following composition (all concentrations in g l^{-1}): NaCl, 195; $MgSO_4 \cdot 7H_2O$, 25; $MgCl_2 \cdot 6H_2O$, 16.3; $CaCl_2 \cdot 2H_2O$, 1.25; KCl, 5.0; $NaHCO_3$, 0.25; NaBr, 0.625; yeast extract, 1.0, pH 7.0.

The following reference strains were used in the amino acid composition assays: (1) *Halobacterium* (*Hbt.*) *salinarum* R1, grown in medium containing NaCl, 250; KCl, 5.0; $MgCl_2 \cdot 6H_2O$, 5.0; NH_4Cl, 5.0, and yeast extract, 10 g l^{-1}, pH 7.0; (2) *Haloarcula* (*Har.*) *marismortui* ATCC 43049, grown in medium composed of NaCl, 206; $MgSO_4 \cdot 7H_2O$, 36; KCl, 0.37; $CaCl_2 \cdot 2H_2O$, 0.5; $MnCl_2$, 0.013, and yeast extract, 5.0 g l^{-1}, pH 7.0; (3) *Halomonas* (*Hmn.*) *elongata* (ATCC 33173), grown in: NaCl, 100; KCl, 1.0; $MgSO_4 \cdot 7H_2O$, 10; and yeast extract, 20 g l^{-1}, Tris, 1.5%, pH 7.5; and (4) *Escherichia coli* K12, grown in LB broth (peptone, 10; yeast extract, 5.0, and NaCl, 10 g l^{-1}, pH 7.0). Cells were grown at 35°C on a rotatory shaker in 100-ml portions in 250-ml Erlenmeyer flasks. (5) *Halanaerobium* (*Haa.*) *praevalens* DSM 2228 was grown anaerobically as previously described (Oren 1986). Three-letter abbreviations for the species names of members of the Halobacteriaceae are used in this paper in accordance with published recommendations (Oren and Ventosa 2000).

Amino acid analyses

Cells in the late exponential phase of growth were collected by centrifugation (10 min, 12,000 *g*) and washed once with salt solution identical to that of the respective growth medium but without organic nutrients. Cells were then dried by lyophilization. Subsequently, the cells were subjected to acid hydrolysis for 20 h at 110°C in a Knauer protein hydrolyzer (Berlin, Germany).

The hydrolysates were analyzed by high performance liquid chromatography (HPLC), following precolumn derivatization with fluorenylmethyloxycarbonyl (FMOC) chloride as described by Betnér and Földi (1986, 1988), using a Merck-Hitachi (Darmstadt, Germany) HPLC setup including a RP8 Supersphere 60 Lichrocart 125-4 column, and a fluorescence detector. The procedure used does not detect Trp or Cys, and Gln and Asn are assayed as Glu and Asp following acid hydrolysis.

Enzymatic assays

Late-exponential-growth-phase cells were harvested by centrifugation (15 min, 6,000 *g*) at 4°C. Cell pellets were suspended in 3 M KCl + 50 mM Tris–HCl buffer, pH 8.0. Cells were then broken by sonication (3 × 30 s at 140 W) or by passing them through a French press (Aminco, Cambridge, UK; 152 MPa). Debris were removed by centrifugation (10 min, 12,000 *g*, 4°C), and the supernatant, containing between 6 and 15 mg protein ml^{-1}, was used as a crude enzyme preparation. Protein content was assayed by the procedure of Lowry et al. (1951), using bovine serum albumin as a standard.

Enzymatic assays were performed at 35°C in a Hewlett-Packard (Palo Alto, CA, USA) model 8452 diode array spectrophotometer provided with a temperature-controlled cuvette holder. Enzyme activity was assessed in 4-ml quartz cuvettes according to the reduction of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) or to the oxidation of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), measured by the absorbance change at 340 nm. The reaction mixture for the assay of isocitrate dehydrogenase (IDH) (EC 1.1.1.41 or 1.1.1.42) contained 20 µmol DL-isocitrate, 2.5 µmol NAD or NADP, 50 µmol Tris–HCl (pH 8.0), and NaCl or KCl as indicated, in a final volume of 2 ml. The assay of malate dehydrogenase (MDH) (EC 1.1.1.37) contained 0.76 µmol oxalacetate, 0.8 µmol NADH, 50 µmol Tris–HCl (pH 8.0), and NaCl or KCl as indicated, in a final volume of 2 ml. The composition of the assay mixture for L-glutamate dehydrogenase (GDH) (EC 1.4.1.2) was 40 µmol 2-oxoglutarate, 0.8 µmol NADH, 0.2 mmol ammonium acetate, 10 µmol Na_2-ethylenediaminetetraacetate (EDTA), 0.1 mmol glycine–NaOH buffer, pH 9.4, and NaCl and/or KCl as indicated, in a final volume of 2 ml. The reactions were started by the addition of the substrates (isocitrate, oxalacetate, and 2-oxoglutarate, respectively).

Results

Comparison of the amino acid composition of the bulk protein of *Salinibacter* with that of other halophilic and nonhalophilic prokaryotes

Comparison of the amino acid composition of the bulk protein of *S. ruber* with that of archaeal aerobic halophiles (*Hbt. salinarum*, *Har. marismortui*), the bacterial anaerobic halophile *Haa. praevalens*, the aerobic *Hmn. elongata*, and the nonhalophilic *E. coli* (Table 1) showed that *S. ruber* proteins have considerable resemblance to those of the archaeal halophiles of the order Halobacteriales. If it may be assumed that the values for Asx and Glx are representative of the relative contents of the acidic amino acids Asp and Glu, it can be concluded that the content of acidic amino acids in the *S. ruber* proteins (Asx + Glx = 29.2 mol%) is only little lower than that of *Hbt. salinarum* (31.8 mol%) and *Har. marismortui* (32.3 mol%), and is higher than that of *Hmn. elongata* and *E. coli* (26.0 mol% for both). The apparent excess of acidic amino acids [(Asx + Glx) – (Lys + Arg)] is high as well (21.2 mol%, as compared with 25.4 and 26.2 mol%, respectively, for the two archaeal species and 17.0 and 15.9 mol% for *Hmn. elongata* and *E.*

Table 1. The amino acid composition of the bulk protein of *Salinibacter ruber*, compared with the amino acid composition of halophilic Archaea and halophilic and nonhalophilic Bacteria

Amino acid	*Salinibacter ruber* M31	*Halobacterium salinarum*	*Haloarcula marismortui*	*Halanaerobium praevalens*	*Halomonas elongata*	*Escherichia coli*
Asx	12.49 ± 0.45	15.32 ± 0.50	14.47	12.51	10.98	11.99 ± 0.11
Glx	16.69 ± 0.22	16.48 ± 0.04	17.87	16.68	15.00	14.05 ± 0.32
Lys	2.77 ± 0.11	2.34 ± 0.04	2.20	7.68	3.70	6.03 ± 0.14
Arg	5.18 ± 0.20	4.10 ± 0.12	3.94	3.22	5.25	4.16 ± 0.02
His	2.01 ± 0.23	1.64 ± 0.12	1.58	ND	1.65	2.01 ± 0.01
Ser	5.50 ± 0.09	5.32 ± 0.25	5.60	4.84	5.20	4.59 ± 0.37
Thr	5.69 ± 0.25	6.19 ± 0.10	6.02	4.68	4.70	5.34 ± 0.06
Tyr	2.62 ± 0.04	2.35 ± 0.04	2.44	2.40	2.32	2.63 ± 0.01
Gly	9.13 ± 0.03	9.01 ± 0.07	9.18	8.68	9.40	8.91 ± 0.01
Pro	4.42 ± 0.06	3.86 ± 0.12	4.00	2.96	4.08	3.86 ± 0.01
Ala	9.09 ± 0.16	9.86 ± 0.06	8.98	8.56	11.24	10.13 ± 0.01
Val	7.29 ± 0.08	7.84 ± 0.21	8.04	6.95	8.04	7.19 ± 0.06
Leu	7.30 ± 0.17	6.36 ± 0.26	6.50	7.51	7.98	7.32 ± 0.31
Ile	4.38 ± 0.10	4.26 ± 0.05	4.33	7.64	4.50	5.43 ± 0.29
Phe	3.50 ± 0.07	3.13 ± 0.17	3.10	3.36	3.53	3.81 ± 0.03
Met	1.98 ± 0.16	1.81 ± 0.02	1.65	2.32	2.41	2.55 ± 0.08
Glx + Asx	29.18	31.8	32.34	29.19	25.98	26.04
Lys + Arg	7.95	6.44	6.14	10.90	8.95	10.19
(Glx + Asx) – (Lys + Arg)	21.23	25.36	26.20	18.29	17.03	15.85
Gly + Ser	14.63	14.33	14.78	13.52	14.60	11.54
Leu + Val + Ile + Phe	22.47	21.59	21.97	25.46	24.05	23.75

Data are presented as mol% of total amino acids, and are given as the mean ± standard deviation for *S. ruber*, *Hbt. salinarum,* and *E. coli* (analysis performed in triplicate); the other strains were analyzed once

Note that the procedure followed does not detect Trp or Cys and does not discriminate between Asp and Asn or between Glu and Gln, which are thus reported as Asx and Glx, respectively

ND, not detected

coli, respectively. The bacterial anaerobe *Haa. praevalens* shows intermediate values for the above indices.

S. ruber also shares with the halophilic Archaea a low content of hydrophobic amino acids (Leu + Val + Ile + Phe = 22.5 mol%, as compared with 21.6 and 22.0 mol% for *Hbt. salinarum* and *Har. marismortui* and 24.1 and 23.8 mol% for *Hmn. elongata* and *E. coli*, respectively. A high content of serine is also considered to be a characteristic feature of halophilic proteins (Dennis and Shimmin 1997; Lanyi 1974). The serine content of the *S. ruber* cellular proteins is 5.50 mol%, as high as the values measured in *Hbt. salinarum* and *Har. marismortui* (5.32 and 5.60 mol%, respectively), and higher than those in the nonhalophilic *E. coli* (4.59 mol%) or the moderately halophilic *Hmn. elongata* (5.20 mol%). The values for amino acid contents presented in Table 1 thus suggest a mode of adaptation of the cellular proteins of *S. ruber* similar to that of the halophilic Archaea of the order Halobacteriales.

Activities of *S. ruber* cytoplasmic enzymes as a function of salt concentration

The activities of the four cytoplasmic enzymes examined, comprising all of the enzymes participating in the central metabolic pathways, displayed markedly different relationships to salts, but all were shown to proceed in the presence of molar salt concentrations.

The NAD-dependent IDH proved to be a typical halophilic enzyme (Fig. 1A). In the absence of salt, activity was low. The enzyme functioned optimally at 0.5–2 M KCl, with rates of 60% of the optimum value at 3.3 M. NaCl provided less activation: the maximum reaction rate (about 70% of that measured in optimal concentrations of KCl) was found at 0.2–1.2 M NaCl, and above 3 M NaCl activity was low. *S. ruber* also contained NADP-dependent IDH activity. Its level remained approximately constant over a NaCl concentration range from 0 to 3.2 M, while molar concentrations of KCl proved stimulatory (Fig. 1B). Whether the organism possesses two different isocitrate dehydrogenases or a single enzyme with dual coenzyme specificity remains to be determined.

The NAD-dependent MDH was optimally active in the absence of salt, and activity decreased with increasing salt concentration (Fig. 2). KCl and NaCl had a very similar effect. In spite of the salt inhibition, the enzyme is able to function at KCl or NaCl concentrations exceeding 3 M, albeit at suboptimal rates (approximately 25% of those measured in the absence of salt).

The NAD-dependent GDH, as assayed by the reductive amination of 2-oxoglutarate, showed an unusual behavior (Fig. 3A): low activity was obtained in the absence of salt, and this activity further decreased with increasing KCl concentration to the extent that no activity was found above 2.5 M KCl. However, a specific stimulation by NaCl was observed, optimum activity being achieved at 3–3.5 M. The effect was specific for NaCl; when the total salt concentration was kept constant at close to 3 M while varying the ratio between NaCl and KCl in the assay mixture, activities dropped sharply when the concentration of KCl was

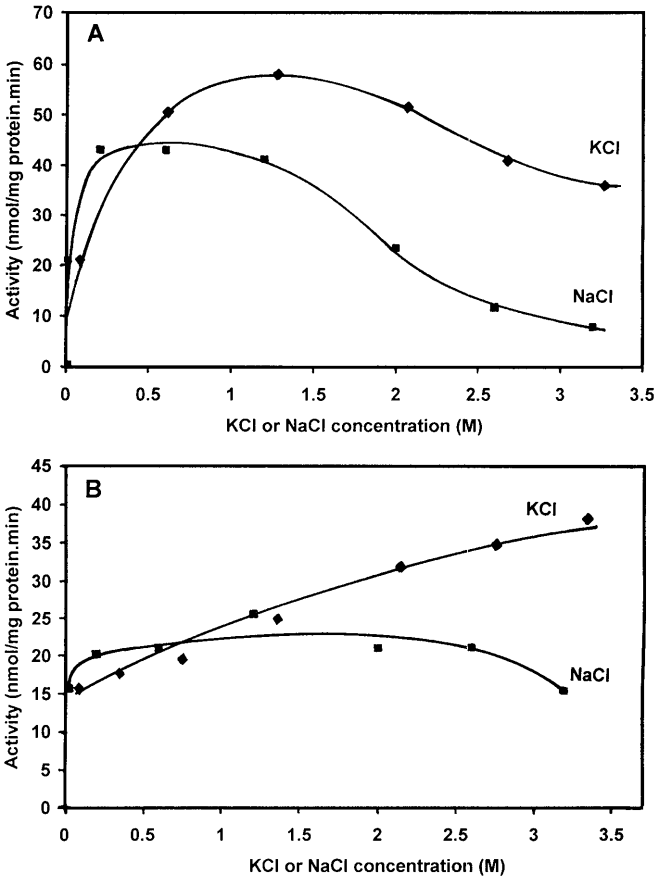

Fig. 1. Dependence of the *Salinibacter ruber* nicotinamide adenine dinucleotide (NAD)-dependent (**A**) and nicotinamide adenine dinucleotide phosphate (NADP)-dependent (**B**) isocitrate dehydrogenase (IDH) activity on the concentration of KCl (*diamonds*) and NaCl (*squares*). The NaCl-based systems all contained in addition 75 mM KCl, introduced with the enzyme preparation. The figure shows typical results obtained from three (**A**) or two (**B**) independent experiments

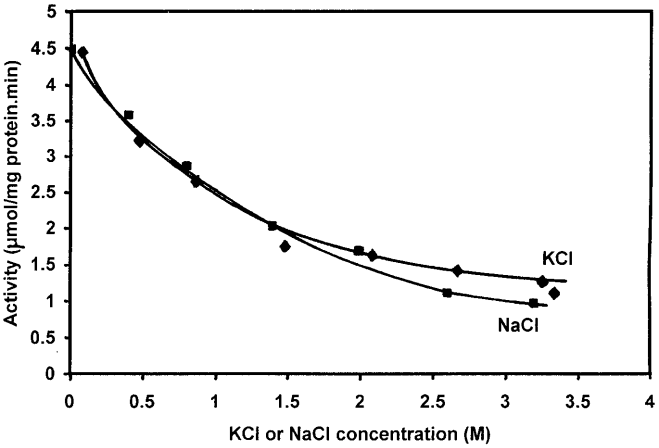

Fig. 2. Dependence of the *Salinibacter ruber* NAD-dependent malate dehydrogenase (MDH) activity on the concentration of KCl (*diamonds*) and NaCl (*squares*), as measured by the oxidation of nicotinamide adenine dinucleotide, reduced (NADH) in the presence of oxalacetate. The NaCl-based systems all contained in addition 75 mM KCl, introduced with the enzyme preparation. The figure shows typical results obtained from three independent experiments

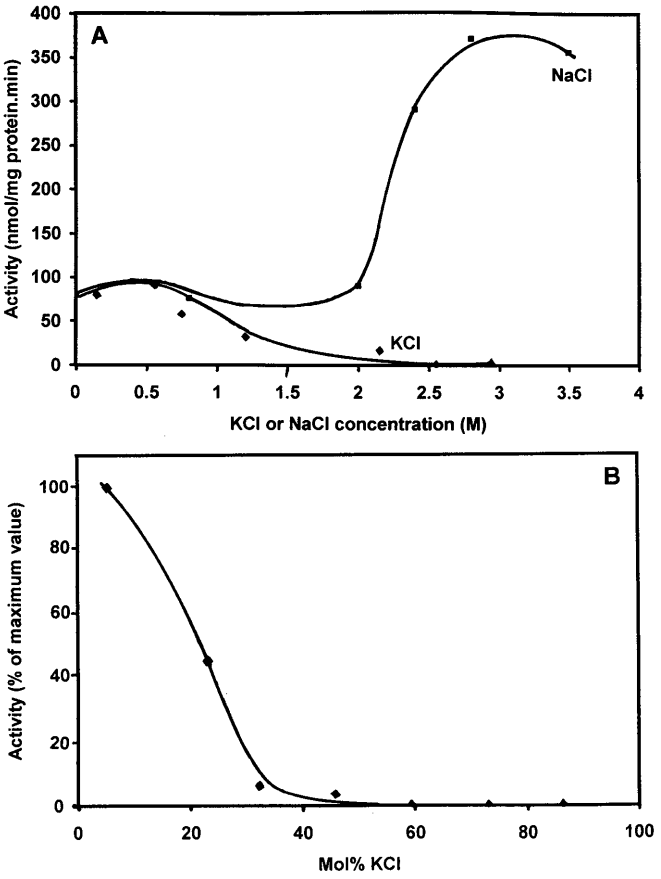

Fig. 3. Dependence of the *Salinibacter ruber* NAD-dependent glutamate dehydrogenase (GDH) activity on the concentration of KCl (*diamonds*) and NaCl (*squares*), as measured by the reductive amination of 2-oxoglutarate (**A**). The NaCl-based systems all contained in addition 75 mM KCl, introduced with the enzyme preparation. The figure shows typical results obtained from three independent experiments. The lower panel (**B**) shows the inhibition of the *Salinibacter ruber* NAD-dependent GDH activity by KCl. The reductive amination of 2-oxoglutarate was measured in mixtures of NaCl and KCl (total concentration 2.95 M) in which the ratio Na/K was varied. The figure shows typical results obtained from two independent experiments

increased (Fig. 3B). No oxidation of NADH was observed when ammonia was omitted from the reaction mixtures. We did not detect any activity of NADP-dependent GDH (EC 1.4.1.4) in *S. ruber* extracts.

The results presented show that *Salinibacter* enzymes are adapted to function in the presence of high salt concentrations, although there are far-reaching differences in behavior among the different enzymes.

Discussion

The results of the enzymatic assays presented above suggest that the intracellular enzymes of *S. ruber* may be expected to be functional at the high intracellular salt concentrations suggested to be present in this unusual bacterium. Intracellular KCl concentrations in the molar range have been found inside the cells, and no significant concentrations of organic compatible solutes have been detected (A. Oren, M. Heldal, S. Norland, and E.A. Galinski, manuscript submitted for publication).

The halophilic behavior of the enzymes could be predicted to a large extent from the amino acid composition of the bulk protein of the organism (Table 1). There is a high apparent content of acidic and a low content of basic amino acids. In addition, the content of hydrophobic amino acids is relatively low, and is balanced by a high frequency of serine. Serine appears to be an important determinant of hydrophobicity because of its compact size and borderline hydrophobic–hydrophilic character (Dennis and Shimmin 1997).

All aerobic halophilic Bacteria known before the discovery of the genus *Salinibacter* exclude salt to a large extent, and primarily use organic-compatible solutes for osmotic stabilization (Ventosa et al. 1998). Although most of their intracellular enzymes do not display a high tolerance toward salt, moderately halophilic Bacteria often show a slightly higher content of acidic amino acids than do the comparable proteins of nonhalophiles. Determinations of the abundance of different amino acids in the bulk protein of *Hmn. elongata* showed a moderate excess of acidic amino acids and a low frequency of basic amino acids, the values being intermediate between those for *E. coli* and the archaeon *Haloferax* (*Hfx.*) *mediterranei* (Gandbhir et al. 1995; see also Oren 1995). Convergent evolution of amino acid usage was suggested to have led to this somewhat halophilic character of the *Hmn. elongata* proteins. However, this convergent evolution did not lead to changes in the frequencies of the hydrophobic amino acids in this case. The ratio of the occurrence of the hydrophobic amino acids (Ala, Val, Leu, Ile, Phe, and Met) to that of the borderline hydrophobic amino acids (Ser, Thr) was 3.56 in *Hmn. elongata*, compared with 3.58 in *E. coli* and 2.46 in membranes of *Hbt. salinarum* (Oren 1995). On the basis of the data presented in Table 1, this ratio was 3.00 for *S. ruber*, 2.98 for whole cells of *Hbt. salinarum*, 2.81 for *Har. marismortui*, 3.81 for *Hmn. elongata*, and 3.67 for *E. coli*. This comparison once more shows the potential halophilic character of the *Salinibacter* proteins.

The *S. ruber* NAD-dependent IDH is a typically halophilic enzyme, being stimulated about threefold by 1.2 M KCl and retaining 60% of its maximal activity at 3.2 M KCl (Fig. 1). No comparable data on NAD-dependent IDH in other extreme halophiles was found in the literature. However, the properties of NADP-dependent IDH from *Hbt. salinarum* have been well documented (Aitken and Brown 1969; Aitken et al. 1970; Baxter and Gibbons 1956; Hubbard and Miller 1969). The enzyme is halophilic, having its optimum activity in 0.5 M KCl or 1 M NaCl (Aitken et al. 1970), at 2 M KCl or NaCl (Aitken and Brown 1969), or at 2–4 M NaCl (Baxter and Gibbons 1956). Aitken et al. (1970) reported activities at 4 M KCl and 4 M NaCl to be 60% and 40% of the optimal activity, respectively, while Aitken and Brown (1969) and Baxter and Gibbons (1956) found a much stronger inhibition at the highest KCl or NaCl concentrations tested. In comparison, the NADP-dependent IDH of *S. ruber* proved optimally active at the highest KCl concentrations, but also functioned in the absence of salt (Fig. 1B).

Extensive information is available on the NAD-dependent MDH of *Hbt. salinarum* (Aitken and Brown 1969; Baxter and Gibbons 1956; Holmes and Halvorson 1965) and *Har. marismortui* (Dym et al. 1995; Eisenberg 1995; Mevarech and Neumann 1977; Richard et al. 2000). The *Hbt. salinarum* enzyme is optimally active in 2–3 M KCl. KCl allows slightly higher activities than NaCl, and little activity is found below 1 M salt (Aitken and Brown 1969; Baxter and Gibbons 1956). The *Har. marismortui* MDH is inactivated below 2 M NaCl (Mevarech and Neumann 1977). The enzyme is a tetramer, and its structure is known in great detail, thanks to extensive crystallographic and physicochemical studies (Dym et al. 1995; Eisenberg 1995; Richard et al. 2000). Compared with these typical halophilic enzymes, the *S. ruber* MDH does not behave as a true halophilic enzyme because its optimal activity is found in the absence of salt. However, considerable activity is still present at KCl or NaCl concentrations above 3 M (Fig. 2).

The properties of GDH have been investigated in *Hbt. salinarum*. The organism possesses both a NAD-dependent and a NADP-dependent enzyme. The NAD-dependent enzyme acts in two directions, but is thought to be mainly catabolically active in the oxidative deamination of glutamate (Bonete et al. 1996). Optimum activity was found at 3.2 M NaCl or 0.8 M KCl. The deamination reaction is inhibited by high concentrations of KCl: no activity was observed above 2 M KCl, while 4 M NaCl allowed excellent activity. The amination reaction was also inhibited by KCl concentrations above 0.8 M, while activity increased with NaCl concentrations up to 4 M (Bonete et al. 1986). However, the enzyme studied by Britton et al. (1998) (coenzyme specificity not stated) is still active in 4 M KCl. The NADP-specific enzyme, assayed by the reductive amination of 2-oxoglutarate, required at least 0.5 M KCl or NaCl, was optimally active in 1.6 M KCl, and still showed excellent activity at 4 M NaCl (Bonete et al. 1987). NADP-dependent GDH was also detected in *Haloferax mediterranei*. The enzyme is a hexamer, functions optimally at 1–2 M KCl or NaCl, and is still almost fully active in 3.5 M KCl. In the absence of salt, activity was about one-third of that under optimum conditions (Ferrer et al. 1996). The NAD-dependent GDH of *S. ruber* appears highly unusual in its absolute specificity for NaCl, which at high concentrations cannot be replaced by KCl (Figs. 3A,B).

The results presented show that the intracellular enzymes of *S. ruber* are able to function in the presence of high salt concentrations, as expected for an organism that uses inorganic ions rather than organic solutes to provide osmotic balance. Thus far, the only group of halophilic organisms within the domain Bacteria known to use inorganic salts to balance the high osmotic pressure of their environment and to possess salt-adapted enzymes is the Halanaerobiales (low G + C branch of the Firmicutes) (Oren 1986; Oren and Gurevich 1993; Rengpipat et al. 1988). *Salinibacter* is an aerobe with an altogether different phylogenic affiliation: the *Flavobacterium*/*Cytophaga* group (Antón et al. 2002).

There is a surprising similarity between the physiology of the extremely halophilic Bacteria of the genus *Salinibacter* and the Archaea of the family Halobacteriaceae. Both groups are aerobic heterotrophs, both maintain high intracellular K^+ concentrations, and both possess enzymes that are functional at high salt concentrations. A comparative genomic analysis of *Salinibacter* and members of the Halobacteriaceae may therefore prove of great interest, and is expected to shed new light on the adaptations of halophilic microorganisms at NaCl concentrations approaching saturation.

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