

Characterization of alcohol dehydrogenase from the haloalkaliphilic archaeon *Natronomonas pharaonis*

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Abstract Alcohol dehydrogenase (ADH; EC: 1.1.1.1) is a key enzyme in production and utilization of ethanol. In this study, the gene encoding for ADH of the haloalkaliphilic archaeon *Natronomonas pharaonis* (NpADH), which has a 1,068-bp open reading frame that encodes a protein of 355 amino acids, was cloned into the pET28b vector and was expressed in *Escherichia coli*. Then, NpADH was purified by Ni-NTA affinity chromatography. The recombinant enzyme showed a molecular mass of 41.3 kDa by SDS-PAGE. The enzyme was haloalkaliphilic and thermophilic, being most active at 5 M NaCl or 4 M KCl and 70°C, respectively. The optimal pH was 9.0. Zn²⁺ significantly inhibited activity. The K_m value for acetaldehyde was higher than that for ethanol. It was concluded that the physiological role of this enzyme is likely the catalysis of the oxidation of ethanol to acetaldehyde.

Keywords *Natronomonas pharaonis* · Haloalkaliphilic · Alcohol dehydrogenase · Molecular cloning

Introduction

Halophiles are microorganisms that are able to live in hypersaline environments. According to the widely used definition (Kushner 1978), halophiles can be classified into three groups: slight halophiles (many marine organisms which live in seawater containing about 0.5 M NaCl), moderate halophiles (optimal growth at 0.5–2.5 M NaCl), and extreme halophiles (optimal growth around 4 M NaCl). There are also borderline extreme halophiles that tolerate above approximately 2.5 M NaCl (Margesin and Schinner 2001). In the domain Archaea, we find aerobic halophiles (family *Halobacteriaceae*) as well as anaerobic halophilic methanogens (Kamekura 1998).

Proteins from the *Halobacteriaceae* are generally functional at high salt concentrations, in the range of 2–4 M NaCl or KCl, and they need high ionic strength to sustain their structure and function (Dym et al. 1995; Danson and Hough 1997; Demirjian et al. 2001). Halophilic proteins usually possess a much higher content of acidic amino acid residues than nonhalophilic homologs. The acidic residues can interact with the water molecules and salt ions, and they contribute to the stability and activity of halophilic proteins in concentrated salt solutions (Mader et al. 2000, 2004; Fukuchi et al. 2003; Irimia et al. 2003). The crystal structure of the halophilic malate dehydrogenase from *Haloarcula marismortui* has been extensively studied, and it was demonstrated that sodium and chloride ions are found at the periphery of the protein, interacting with the acidic residues, and are involved in the complex of salt bridge clusters located at monomer–monomer interfaces; the sodium ions can be replaced with potassium ions (Richard et al. 2000; Irimia et al. 2003).

Many halophilic Archaea oxidize glucose via modified Emden–Meyerhof and Entner–Doudoroff pathways

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(Siebers and Schönheit 2005), by which the intermediate pyruvate is produced. Pyruvate serves as a precursor for the production of acetate and ethanol (Bräsen and Schönheit 2004). However, little is known about the metabolism of ethanol in the halophilic Archaea. The finding of a gene annotated as alcohol dehydrogenase (ADH) in the genome of *Natronomonas pharaonis* was the starting point for the current study. ADHs (EC: 1.1.1.1) are a family of oxidoreductases that catalyze the interconversion between alcohols and aldehydes, and are widely distributed in all the three domains of life (Reid and Fewson 1994), as well as in other members of *Halobacteriaceae* (*Halobacterium* sp. NRC-1, *Haloarcula marismortui*, and *Haloquadratum walsbyi*). Alcohol dehydrogenases can be distinguished based on their cofactor specificity, possible cofactors being (1) NAD⁺ or NADP⁺, (2) pyrrolo-quinoline quinone, haem or F₄₂₀, (3) FAD. The NAD(P)⁺-dependent ADHs are mainly sub-divided into zinc-dependent ADHs, short-chain ADHs, and iron-activated ADHs, based on their catalytic properties and sequence information (Reid and Fewson 1994; Forrest and Gonzalez 2000).

N. pharaonis, one of the haloalkaliphilic Archaea, was isolated from highly saline soda lakes in Egypt, where it is exposed simultaneously to two forms of stress: hypersaline conditions and high pH (Falb et al. 2005). The cells grow optimally in the presence of 3.5 M NaCl and at pH 8.5. Relatively little information is available on the properties of the enzymes from this organism. In this work, we report the properties of ADH from *N. pharaonis*, the first purified ADH from the halophilic Archaea.

Materials and methods

Microbial strains and growth conditions

Natronomonas pharaonis CGMCC 1.1965^T (=DSM 2160^T) was purchased from the China General Microbiological Culture Collection Center. *N. pharaonis* was grown at 40°C in a medium as described previously (Konstantinidis et al. 2007). As a host for DNA manipulations *Escherichia coli* DH5 α was used. Protein expression was carried out in *E. coli* Rosetta.

Construction of expression plasmids

The *NpADH* gene (GenBank accession no. YP326290) was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase. The template DNA was prepared from *N. pharaonis* cells. Based on the *adh* gene of *N. pharaonis*, the sequences of forward and reverse synthetic primers which contained *NdeI* and *XhoI* recognition sites

(underlined) were designed as 5'-TTTCATATGCGCGCTGTCGTCTTCGAGGAG-3' and 5'-AATCTCGAGTCAGAACTCGTCGACACCG-3', respectively. The expression vector was constructed using the plasmid pET28b (Novagen, Germany), which was digested with *NdeI/XhoI* (Takara, Japan). The PCR product was then ligated into the gap introducing an N-terminal histidine tag. The resulting recombinant plasmid was called pET-*NpADH*.

Protein expression and purification

pET-*NpADH* was transformed into *E. coli* Rosetta. The transformed Rosetta cells were grown in 4 L of Luria-Bertani medium (containing 20 $\mu\text{g mL}^{-1}$ kanamycin) at 37°C. After an OD₆₀₀ of 0.6–0.8 was reached, 1 mM isopropyl-D-thiogalactopyranoside (IPTG) was added. Following an induction period of 3 h at 30°C, the cells were harvested by centrifugation (7,000 $\times g$, 5 min, 4°C), resuspended in cold sodium phosphate buffer (50 mM NaH₂PO₄, pH 8.0; 4 M NaCl; 10 mM imidazole), and finally broken up by sonication in ice (100 W, 1 s sonifying vs. 2 s pause, 500 cycles). After centrifugation (18,000 $\times g$, 20 min, 4°C), the supernatant was loaded onto a chromatography column filled with Ni-NTA agarose (Qiagen, Germany) and washed extensively with sodium phosphate buffer with increased imidazole concentration (≤ 100 mM) to remove unspecifically bound proteins. Subsequently the histidine-tagged protein was eluted in sodium phosphate buffer containing 250 mM imidazole. The purified protein was ultrafiltrated against buffer containing 50 mM Tris-HCl (pH 8.0) and 4 M NaCl, and was stored at -80°C until used. The ADH containing fractions were detected by SDS-PAGE, using 12% polyacrylamide gels, which were stained with Coomassie brilliant blue R250 (Laemmli 1970). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Enzymatic assays

Standard enzymatic assays were performed by monitoring the substrate-dependent absorbance change of NAD(H) at 340 nm using a Beckman Coulter DU 800 nucleic acid/protein analyzer as previously described (Ziegenhorn et al. 1976). The reaction was performed in 1 mL reaction mixture containing 50 mM Tris-HCl (pH 9.0), 4 M NaCl, 2 mM DTT, 0.2 mM NADH, 50 mM acetaldehyde and enzyme for the reduction of aldehyde; the assay mixture (1 mL) used for the oxidation of alcohol contained 50 mM Tris-HCl (pH 9.0), 4 M NaCl, 2 mM DTT, 0.4 mM NAD⁺, 100 mM ethanol, and enzyme.

Sequence handling

DNA sequences and putative protein sequence homology comparisons were carried out using the BLAST tool at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>). The phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei 1987), using software in MEGA program (Kumar et al. 1994).

Results and discussion

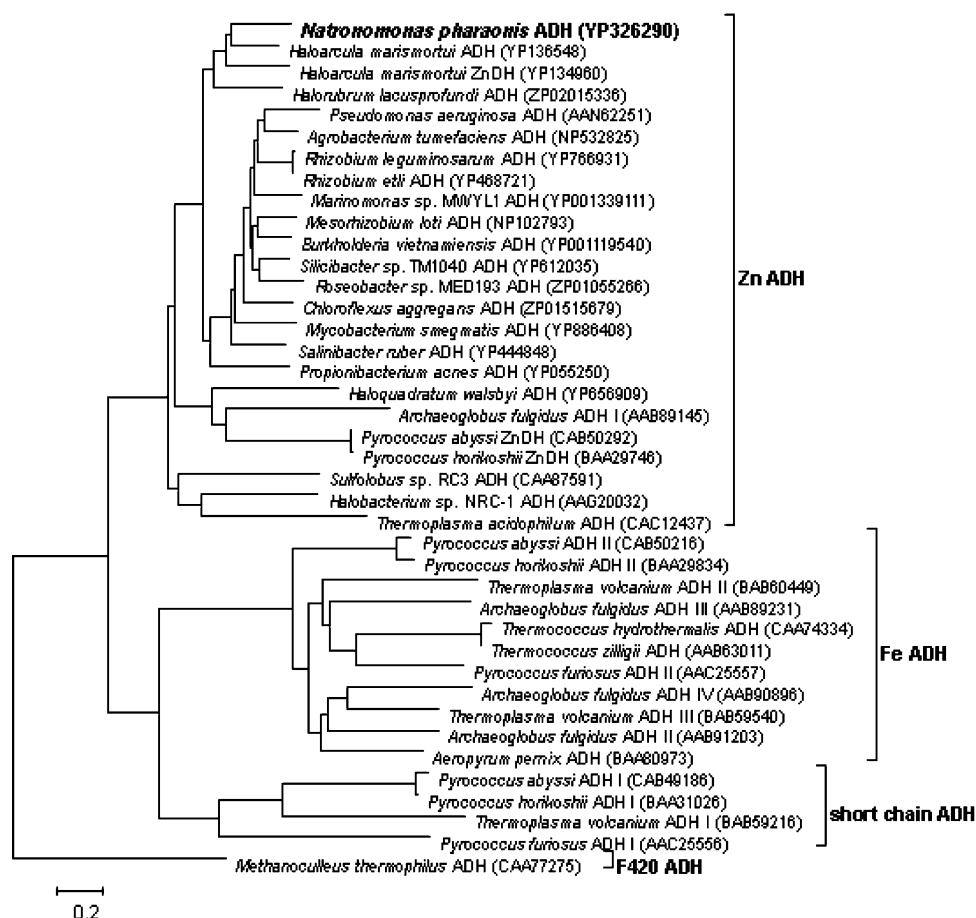
The phylogenetic tree presented in Fig. 1 shows a comparison of the deduced amino acid sequence of *N. pharaonis* ADH and 39 other sequences identified as ADHs from the NCBI protein database. The phylogenetic relationship of these amino acid sequences indicates that *NpADH* belongs to the family of zinc-dependent ADHs, which is one of the three NAD(P)-dependent subfamilies (Reid and Fewson 1994; Forrest and Gonzalez 2000).

Genome sequence analysis of *N. pharaonis* indicated that the *NpADH* gene including start codon (ATG) and

stop codon (TGA) is 1,068 bp in length. DNA sequencing showed that the *NpADH* gene cloned in the plasmid exhibited 100% identity to the coding region published in the database. In order to generate large quantities of *NpADH*, *E. coli* Rosetta carrying the plasmid pET-*NpADH* was grown in Luria–Bertani medium, and production of the recombinant enzyme was induced with IPTG. The *NpADH* was purified by Ni-NTA affinity chromatography methods. The expected 41.3 kDa fraction including one 6× histidine tag in the N-terminus was detected in 12% SDS-PAGE (Fig. 2). The yield of recombinant protein was 3.35 mg/L of culture.

The optimum pH for *NpADH* activity was 9.0 and the enzyme was inactive below pH 7.0, while the pH optimum for *NpADH* stability was rather broad, between 8.0 and 10.0 (Fig. 3a). The recombinant enzyme displayed optimal activity at 70°C, and its greatest stability was below 40°C (Fig. 3b). Consistent with other enzymes isolated from halophilic organisms, *NpADH* was highly salt dependent. The activity of *NpADH* increased with salt concentration to a maximum at 5 M NaCl or 4 M KCl. At salt concentrations below 2 M NaCl or 3 M KCl the stability was decreased (Fig. 3c).

Fig. 1 Phylogenetic tree derived from archaeal and bacterial ADH and related protein amino acid sequences extracted from NCBI database. The tree was constructed using the neighbour-joining method based on 1,000 resamplings. *N. pharaonis* ADH is shown in *bold*



The influences of several divalent cations (CaCl_2 , BaCl_2 , MnCl_2 , CoCl_2 , MgCl_2 , and ZnCl_2) on *NpADH* activity were investigated by adding the compounds at three concentrations of 0.5 mM, 2.0 mM, and 5.0 mM. The enzyme tolerated a range of divalent metal ions, except for zinc ions (89.1, 93.8, and 94.1% inhibition by 0.5, 2.0, and 5.0 mM).

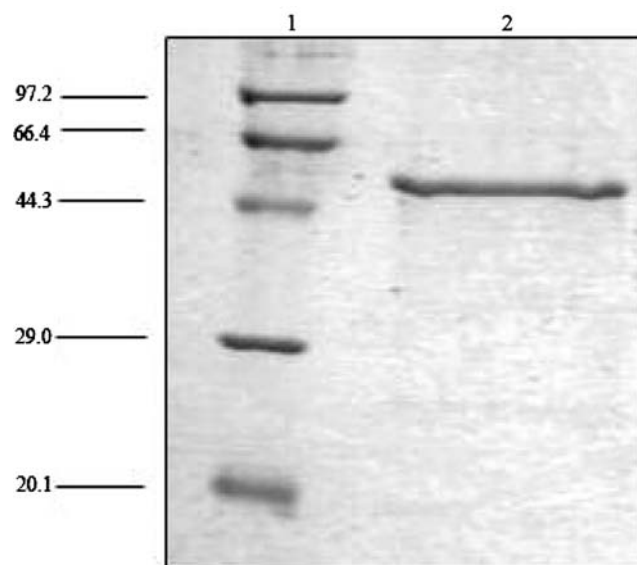


Fig. 2 SDS-PAGE of purified recombinant *NpADH*. Lane 1 protein molecular mass standards, Lane 2 purified recombinant *NpADH*. Molecular mass standards are indicated in kDa

Fig. 3 Effect of pH (a), temperature (b), and salt concentration (c) on *NpADH* activity and stability. **a** The pH dependence of *NpADH* activity (filled square) and stability (open square) were measured between pH 4 and 12, with three buffer systems, 50 mM Na_2HPO_4 -citric acid (pH 4.0–5.0), 50 mM Tris-HCl (pH 6.0–9.0), and 50 mM glycine-NaOH (pH 10.0–12.0). All buffers contained 4 M NaCl. Activity assays were performed at various pH values at 70°C for 30 min. For stability assays, 50 μL portions of enzyme solution in buffers with different pH were preincubated at 30°C for 1 h. Then 950 μL of 4 M NaCl, 50 mM Tris-HCl buffer (pH 9.0) with substrates were added, if necessary the pH was adjusted to pH 9.0, and the remaining activity was assayed as described above. **b** The effect of temperature on *NpADH* activity (filled square) and stability (open square) were determined at temperatures from 20 to 90°C. Activity was measured for 30 min at different temperatures. For stability assays, the enzyme was preincubated for 1 h at the indicated temperature, and residual activity was then assayed at 70°C as described above. **c** To test the effects of salts on *NpADH* activity and stability, the salt concentrations in the assay were varied from 0 to 5 M NaCl (filled square, open square) and from 0 to 4 M KCl (filled circle, open circle). Activity (filled square, filled circle) was determined at 70°C, pH 9.0 for 30 min. For stability measurements (open square, open circle), 50 μL portions of enzyme solution were preincubated at different concentrations at 30°C for 1 h. Then 950 μL substrates and salt solutions were added to adjust the final salt concentration to standard condition (4 M NaCl or KCl), and residual activity was assayed as described above

The purified enzyme catalyzed the oxidation of ethanol with NAD^+ and the reverse reaction of reducing acetaldehyde with NADH. The apparent kinetic constants, K_m , V_{max} , k_{cat} and catalytic efficiency k_{cat}/K_m , for the cloned *NpADH* with the substrates acetaldehyde and ethanol were determined at 40°C (the physiological temperature of organism) and at 70°C (the optimal temperature of enzyme activity) (Table 1). The four times higher K_m value for acetaldehyde compared to that for ethanol at the physiological temperature indicated that the enzyme may play a role in oxidation of alcohols, rather than in reduction of

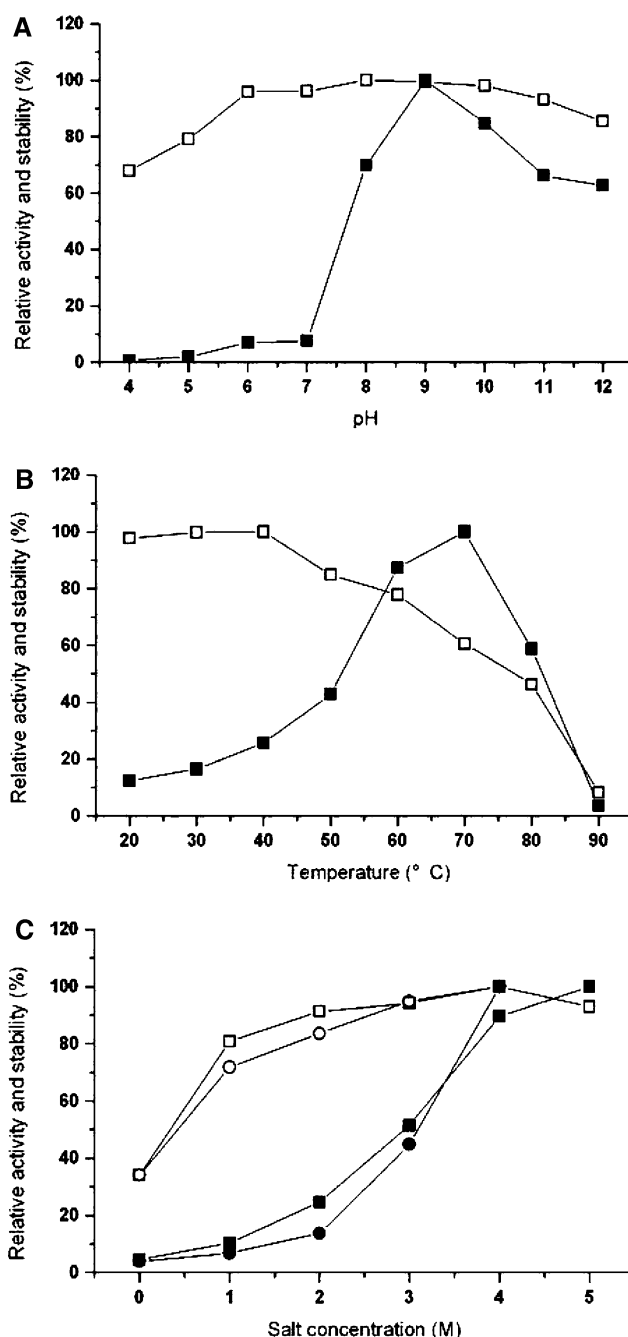


Table 1 Kinetic parameters of purified recombinant alcohol dehydrogenase from *N. pharaonis*

Substrate	Temperature (°C)	V_{\max} (mM s ⁻¹)	K_m (M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ M ⁻¹)
Acetaldehyde	40	2.1×10^{-5}	5.9×10^{-2}	0.3	5.1
Ethanol	40	2.7×10^{-5}	1.3×10^{-2}	0.4	30.8
Acetaldehyde	70	3.6×10^{-4}	3.4×10^{-1}	5.4	15.9
Ethanol	70	5.5×10^{-4}	2.2×10^{-2}	8.2	372.4

Enzyme activity was measured with 50 mM Tris-HCl (pH 9.0) and 4 M NaCl

aldehydes in vivo. The enzyme was highly specific for ethanol with NAD⁺ as the coenzyme. With methanol, 2-propanol, 1-butanol, and 1-pentanol the rates were 1.1, 15.7, 0.3, and 0% of that with ethanol. The results suggested that the *NpADH* was specific towards the transition between ethanol and acetaldehyde.

The present work shows that ADH from *N. pharaonis* is thermophilic and haloalkaliphilic. Studies to determine the optimum temperature reveal that the *NpADH* has its maximum activity at 70°C, so that it can be classified as a thermoactive enzyme. There are more cases of halophilic enzymes with temperature optima far above the maximum growth temperature of the organism that produces them: *Halorubrum sodomense* excretes an amyloglucosidase that is optimally active at 70°C (Oren 1973), and *Halobacterium salinarum* (“*halobium*”) has two glutamate dehydrogenases that are optimally active at 70°C (Bonete et al. 1986, 1987). *NpADH* is active and stable at alkaline pH, and this agrees well with the intracellular pH of 9.0 or slightly higher generally observed in alkaliphiles (Padan et al. 1981, 2005). *NpADH* depends on high salt concentrations for activity and stability, and shows a high percentage of excess acidic residues (18.3 %), similar to other halophilic enzymes that have a high content of aspartic acid and glutamic acid (Ng et al. 2000; Madern et al. 2000, 2004; Ebel et al. 2002; Fukuchi et al. 2003; Irimia et al. 2003). Three dimensional structures of halophilic proteins (Dym et al. 1995; Frolow et al. 1996; Pieper et al. 1998; Richard et al. 2000; Yamada et al. 2002; Bieger et al. 2003) imply that high densities of acids residues at the protein surface are involved in complex salt bridge networks with sodium or potassium and chloride ions compared with non-halophilic homologues. The amino acid sequence analysis indicates that *NpADH* belongs to the zinc-binding dehydrogenase family. The catalytic zinc binding residues (Cys38, His64, Cys154) and structural zinc binding residues (Cys97, Cys100, Cys108) are conserved in *NpADH*. We observed that Zn²⁺ significantly inhibited enzyme activity of *NpADH* at high salt concentration, while non-halophilic analogues depend on zinc ion for activity (Jörnvall et al. 1987; Ceccarelli et al. 2004). The nature of the interactions of *NpADH* with zinc ions deserves further study.

As a conclusion, the heterologously expressed ADH from *N. pharaonis* appears thermophilic, alkaliphilic, and

inactive at low salt concentration. The location of the *NpADH* gene within the *N. pharaonis* genome does not provide any clues on the possible function of the enzyme within a known metabolic pathway. Thus far there is no indication for the importance of alcohols or aldehydes in the alkaline hypersaline lake environment, and the function of the enzyme in *N. pharaonis* remains unclear.

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References

- Bieger B, Essen LO, Oesterhelt D (2003) Crystal structure of halophilic dodecin. A novel, dodecameric flavin binding protein from *Halobacterium salinarum*. Structure 11:375–385
- Bonete MJ, Camacho ML, Cadenas E (1986) Purification and properties of NAD⁺-dependent glutamate dehydrogenase from *Halobacterium halobium*. Int J Biochem 18:785–789
- Bonete MJ, Camacho ML, Cadenas E (1987) A new glutamate dehydrogenase from *Halobacterium halobium* with different coenzyme specificity. Int J Biochem 19:1149–1155
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
- Bräsen C, Schönheit P (2004) Regulation of acetate and acetyl-CoA converting enzymes during growth on acetate and/or glucose in the halophilic archaeon *Haloarcula marismortui*. FEMS Microbiol Lett 241:21–26
- Ceccarelli C, Liang ZX, Strickler M, Prehna G, Goldstein BM, Klinman JP, Bahnson BJ (2004) Crystal structure and amide H/D exchange of binary complexes of alcohol dehydrogenase from *Bacillus stearothermophilus*: Insight into thermostability and cofactor binding. Biochemistry 43:5266–5277
- Danson MJ, Hough DW (1997) The structural basis of protein halophilicity. Comp Biochem Physiol 117A:307–312
- Demirjian DC, Moris-Varas F, Cassidy CS (2001) Enzymes from extremophiles. Curr Opin Chem Biol 5:144–151
- Dym O, Mevarech M, Sussman JL (1995) Structural features that stabilize halophilic malate dehydrogenase from an archaeobacterium. Science 267:1344–1346
- Ebel C, Costenaro L, Pascu M, Faou P, Kernal B, Proust-De Martin F, Zaccari G (2002) Solvent interactions of halophilic malate dehydrogenase. Biochemistry 41:13234–13244
- Falb M, Pfeiffer F, Palm P, Rodewald K, Hickmann V, Tittor J, Oesterhelt D (2005) Living with two extremes: conclusions from the genome sequence of *Natronomonas pharaonis*. Genome Res 15:1336–1343
- Forrest GL, Gonzalez B (2000) Carbonyl reductases. Chem Biol Interact 129: 21–40

- Frolow F, Harel M, Sussman JL, Mevarech M, Shoham M (1996) Protein adaptation to a saturated salt environment: insights from the crystal structure of a halophilic 2Fe-2S ferredoxin. *Nature Struct Biol* 3:451–457
- Fukuchi S, Yoshimune K, Wakayama M, Moriguchi M, Nishikawa K (2003) Unique amino acid composition of proteins in halophilic bacteria. *J Mol Biol* 327:347–357
- Irimia A, Ebel C, Madern D, Richard SB, Cosenza LW, Zaccai G, Vellieux FMD (2003) The oligomeric states of *Haloarcula marismortui* malate dehydrogenase are modulated by solvent components as shown by crystallographic and biochemical studies. *J Mol Biol* 326:859–873
- Jörnvall H, Persson B, Jeffery J (1987) Characteristics of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases. *Eur J Biochem* 167:195–201
- Kamekura M (1998) Diversity of extremely halophilic bacteria. *Extremophiles* 2:289–295
- Konstantinidis K, Tebbe A, Klein C, Scheffer B, Aivaliotis M, Bisle B, Falb M, Pfeiffer F, Siedler F, Oesterhelt D (2007) Genome-wide proteomics of *Nastramonas pharaonis*. *J Proteome Res* 6:185–193
- Kumar S, Tamura K, Nei M (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. *Comput Appl Biosci* 10:189–191
- Kushner DJ (1978) Life in high salt and solute concentrations. In: Kushner DJ (ed) *Microbial life in extreme environments*. Academic Press, London, pp 317–368
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Madern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzymes. *Extremophiles* 4:91–98
- Madern D, Camacho M, Rodriguez-Arnedo A, Bonete MJ, Zaccai G (2004) Salt-dependent studies of NADP-dependent isocitrate dehydrogenase from the halophilic archaeon *Haloferax volcanii*. *Extremophiles* 8:377–384
- Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* 5:73–83
- Ng WV, Kennedy SP, Mahairas GG, Berquist B, Pan M, Shukla HD, Lasky SR, Baliga NS, Thorsson V, Sbrogna J, Swartzell S, Weir D, Hall J, Dahl TA, Welti R, Goo YA, Leithausen B, Keller K, Cruz R, Danson MJ, Hough DW, Maddocks DG, Jablonski PE, Krebs MP, Angevine CM, Dale H, Isenbarger TA, Peck RF, Pohlschröder M, Spudich JL, Jung KW, Alam M, Freitas T, Hou S, Daniels CJ, Dennis PP, Omer AD, Ebhardt H, Lowe TM, Liang P, Riley M, Hood L, DasSarma S (2000) Genome sequence of *Halobacterium* species NRC-1. *Proc Natl Acad Sci USA* 97:12176–12181
- Oren A (1973) A thermophilic amyloglucosidase from *Halobacterium sodomense*, a halophilic bacterium from the Dead Sea. *Curr Microbiol* 8: 225–230
- Padan E, Zilberstein D, Schuldiner S (1981) pH homeostasis in bacteria. *Biochim Biophys Acta* 650:151–166
- Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: New insights. *Biochim Biophys Acta* 1717:67–88
- Pieper U, Kapadia G, Mevarech M, Herzberg O (1998) Structural features of halophilicity derived from the crystal structure of dihydrofolate reductase from the Dead Sea halophilic archaeon, *Haloferax volcanii*. *Structure* 6:75–88
- Reid MF, Fewson CA (1994) Molecular characterization of microbial alcohol dehydrogenase. *Crit Rev Microbiol* 20:13–56
- Richard SB, Madern D, Garcin E, Zaccai G (2000) Halophilic adaptation: novel solvent protein interactions observed in the 2.9 and 2.6 Å resolution structures of the wild type and a mutant of malate dehydrogenase from *Haloarcula marismortui*. *Biochemistry* 39:992–1000
- Saitou N, Nei M (1987) The neighbour joining method: a new tool for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Siebers B, Schönheit P (2005) Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Curr Opin Microbiol* 8:695–705
- Yamada Y, Fujiwara T, Sato T, Igarashi N, Tanaka N (2002) The 2.0 Å crystal structure of catalase-peroxidase from *Haloarcula marismortui*. *Nat Struct Biol* 9:691–695
- Ziegenhorn J, Senn M, Bücher T (1976) Molar absorptivities of β -NADH and β -NADPH. *Clin Chem* 22:151–160