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ORIGINAL PAPER

Lars Redecke · Maria A. Brehm · Reinhard Bredehorst

Cloning and characterization of dihydrofolate reductase from a facultative alkaliphilic and halotolerant bacillus strain

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Abstract Elucidation of the molecular basis of the stability of enzymes from extremophilic organisms is of fundamental importance for various industrial applications. Due to the wealth of structural data from various species, dihydrofolate reductase (DHFR, EC 1.5.1.3) provides an excellent model for systematic investigations. In this report, DHFR from alkaliphilic *Bacillus halodurans* C-125 was cloned and expressed in *E. coli*. Functional analyses revealed that *Bh*DHFR exhibits the most alkali-stable phenotype of DHFRs characterized so far. Optimal enzyme activity was observed in a slightly basic pH region ranging from 7.25 to 8.75. Alkali-stability is associated with a remarkable resistance to elevated temperatures (half-life of 60 min at 52.5°C) and to high concentrations of urea (up to 3 M). Although the secondary structure shows distinct similarities to those of mesophilic DHFR molecules, *Bh*DHFR exhibits molecular features contributing to its alkaliphilic properties. Interestingly, the unique phenotype is diminished by C-terminal addition of a His-tag sequence. Therefore, His-tag-derivatized *Bh*DHFR offers the opportunity to obtain deeper insights into the specific mechanisms of alkaliphilic adaption by comparison of the three dimensional structure of both *Bh*DHFR molecules.

Keywords Diyhdrofolate reductase · *Bacillus halodurans* · Alkaliphily · Thermal stability · Biochemical characterization · His-tag

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L. Redecke (✉) · M. A. Brehm · R. Bredehorst
Institute of Biochemistry and Food Chemistry,
University of Hamburg, Martin-Luther-King-Platz 6,
20146 Hamburg, Germany
E-mail: Lars.Redecke@chemie.uni-hamburg.de
Tel.: +49-40-428382849
Fax: +49-40-428382848

Present address: M. A. Brehm
Center of Experimental Medicine,
Institute of Biochemistry and Molecular Biology I,
University Hospital Hamburg-Eppendorf, Hamburg, Germany

Abbreviations

DHFR	Dihydrofolate reductase
*Bh*DHFR	DHFR from *Bacillus halodurans*
MTX	Methotrexate
DLS	Dynamic light scattering

Introduction

During the last two decades proteins from microorganisms existing in extreme environments such as high temperatures, extremes of pH, high salt concentrations or high pressure have advanced in interest for industrial applications due to their outstanding stability. A large number of such organisms were isolated to achieve a more detailed understanding of the unique features of their proteins that distinguish them from their mesophilic counterparts (Demirjian et al. 2001; Niehaus et al. 1999). Significant progress has been made in identifying the structural and molecular basis for thermophilic (Vieille and Zeikus 2001 and references therein) and halophilic stability (Madern et al. 2000; Elcock and McCammon 1998). Moreover, structural studies of enzymes resistant to highly alkaline environments revealed some specific electrostatic features that appear to be important for alkali-stability, but systematic investigations have not been performed (Dubnovitsky et al. 2005; De Lemos Esteves et al. 2005; Shirai et al. 2001).

Comparative structural analyses of enzymes from different origins that exhibit the same function are useful in identifying the essential regions for activity and stability since active and/or substrate binding sites tend to be highly conserved. Based on this consideration, we selected dihydrofolate reductase (DHFR) [EC 1.5.1.3] as model enzyme to investigate the molecular basis of alkali-stability. DHFR catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a universal requirement of cell growth in both prokaryotes and eukaryotes. Due to this central position, DHFR is

an important target for the treatment of cancer (McGuire 2003) and for antifolate drugs against bacterial, fungal and protozoan infections (Then 2004; Schweitzer et al. 2000). During the past years, functional and structural characteristics of DHFRs from a wide range of organisms have been studied (Fierke et al. 1987; Thillet et al. 1990; Appleman et al. 1990) and high resolution structures determined by X-ray crystallography are available for several DHFR molecules including those from hyperthermophilic *Thermotoga maritima* (Dams et al. 2000) and moderate halophilic *Haloferax volcanii* (Pieper et al. 1998). Despite a low degree of sequence identity, the three-dimensional structures of DHFRs are closely related (Appleman et al. 1990). Therefore, DHFR appears to be a suitable model enzyme to identify structural properties conferring resistance to high alkaline pH values.

In the present report, we describe the cloning, expression, and characterization of DHFR from *Bacillus halodurans* C-125, a facultative alkaliphilic and halotolerant *Bacillus* strain. *B. halodurans* grows at pH values between 7.5 and 11 with an optimal temperature of 30°C, but it tolerates also temperatures up to 55°C (Ikura and Horikoshi 1979). This bacteria strain was extensively characterized regarding its physiology, biochemistry and genetics (Horikoshi 1999). Analysis of its entire genome revealed a close genetic relation to mesophilic *Bacillus subtilis* excluding genes supposed to be implicated in the alkali-stable phenotype (Takami et al. 2000). However, the DHFR molecules of both *Bacillus* species differ significantly which is reflected by a relatively low sequence identity of 51%. As shown in this study, DHFR from *B. halodurans* (*Bh*DHFR) exhibits resistance to alkaline conditions, high temperatures, and denaturing agents. These characteristics, which are not present in mesophilic DHFR molecules, are discussed in light of the ongoing search for the principles of alkaliphilic and thermophilic adaption of proteins.

Materials and methods

Bacterial strains and growth conditions

Bacillus halodurans C-125 (DSM No. 497) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The cells were grown aerobically at 30°C in medium consisting of 0.5% polypeptone and 0.3% beef extract that was adjusted to pH 9.7 with sodium sesquicarbonate solution. *Escherichia coli* strains DH5α and BL21 (DE3) acquired from Novagen (Madison, Wis., USA) were grown under conditions previously described (Sambrook et al. 1989).

Amplification and cloning of the drfA gene

The genomic DNA of *Bacillus halodurans* C-125 was isolated from 20 ml cultures according to Kalia et al. (1999). The drfA gene was amplified by polymerase chain reaction (PCR) using oligonucleotide primers designed on the basis of the drfA sequence of *B. halodurans* (GenBank accession no. NC002570). The primer sequences are as follows: PhalS (forward primer) 5′-GAA CTT GTC CGA TAT GAT CCG CAC CC-3′; and PhalAS (reverse primer) 5′-CTC TTA AAA CCC GCT CCT CTA CCC CA-3′. In order to clone the isolated drfA gene into the *E. coli* high expression vector pET26b(+) (Novagen, Madison, Wis., USA), a second PCR amplification was carried out to generate a *NdeI* restriction site at the 5′ end and a *NotI* restriction site at the 3′ end of the DHFR coding sequence using the previous PCR product as a template. Amplification products were digested with *NdeI* and *NotI* and ligated between the corresponding restriction sites of the previously digested expression vector in frame with its His-tag coding sequence. The resulting recombinant DHFR comprises a six-fold His-tag connected via an AAALG-linker to its C-terminal region. To express recombinant wild-type DHFR, the reverse primer of the second PCR was equipped with a TGA stop codon located in front of the *NotI* restriction site at its 3′ end. The generated plasmids were designated pET26b-*Bh*His and pET26b-*Bh*wt. All sequences were confirmed by automated DNA sequencing. Additional DNA manipulations and analyses followed standard methods (Sambrook et al. 1989).

Expression and purification of recombinant *B. halodurans* DHFR

The purified plasmids were transformed into *E. coli* BL21(DE3) cells. One single colony was inoculated into 200 ml of LB medium supplemented with 50 μg/ml kanamycin and grown at 37°C until the OD_{600} reached 1.0. Expression of target genes was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to give a final concentration of 1 mM. After further incubation for 4 h with continuous shaking at 37°C, the cells were harvested by centrifugation, washed in 50 mM Tris-HCl (pH 7.5), and disrupted by sonication. The soluble fraction was obtained by centrifugation at 20,800 × *g* for 20 min, followed by filtration of the supernatant through a 0.22 μm sterile filter.

For purification of the recombinant wild-type DHFR of *B. halodurans*, the crude extract was adjusted to pH 8.2 and loaded to a Poros HQ/M (anion exchange) column (4.6 × 100 mm, Boehringer, Mannheim, Germany) connected to a FPLC Äkta Protein Purifier (Amersham Biosciences, NJ, USA). After washing the column with 50 mM Tris-HCl (pH 8.2), an increasing linear salt gradient of 0–1 M NaCl was applied for protein elution. Fractions with DHFR activity eluted at a salt concentration of 0.1–0.2 M were combined, concentrated to a final volume of 2 ml by using Centricon YM-3 centrifugal filter devices (MWCO 3000, Millipore, Billerica, MA, USA), and loaded on a Superdex G75 gel filtration column (16 × 600 mm, Amersham Biosciences,

NJ, USA) that was pre-equilibrated with 50 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl. After passing of 60 ml buffer through the column, fractions with DHFR activity were collected and combined, containing approximately 5 mg recombinant wild-type DHFR.

The crude cell extract of *B. halodurans* DHFR with a C-terminal His-tag was dialysed against 50 mM sodium phosphate (pH 8.0) containing 0.3 M NaCl (buffer A) and applied to a nickel nitrilotriacetic acid (Ni-NTA) agarose affinity column (5 × 50 mm, Amersham Biosciences, NJ, USA). After washing of the resin with six column volumes of buffer A containing 20 mM imidazole, the DHFR was eluted using five column volumes of buffer A containing 300 mM imidazole. Fractions with DHFR activity were combined and dialysed against 50 mM Tris–HCl (pH 8.2). The second purification step was performed on a Poros HQ/M (anion exchange) column (4.6 × 100 mm, Boehringer, Mannheim, Germany) as described for the wild-type enzyme. Pooled fractions with DHFR activity contained approximately 7 mg enzyme.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue staining was applied to assess purity and homogeneity of the recombinant enzymes. Masses of the proteins were analyzed by linear flight MALDI-TOF mass spectrometry (Bruker Autoflex) with a sinapic acid matrix. Protein concentrations in crude samples were determined using the bicinchoninic acid (BCA) protein assay (Smith et al. 1985) and lysozyme as standard, whereas the molar extinction coefficient ε_{280} = 26,030 M^{-1} cm^{-1} was used to determine the protein concentration in fractions containing purified DHFR.

Steady-state kinetic analyses

The activity of DHFR molecules was determined spectrophotometrically by monitoring the decrease of absorbance at 340 nm, using an Ultraspec 3000 UV/VIS scanning spectrophotometer (Amersham Biosciences, NJ, USA). An extinction coefficient of 12,300 M^{-1} cm^{-1} was used to determine the conversion of dihydrofolate and NADPH to tetrahydrofolate and $NADP^+$ (Hillcoat et al. 1967). One unit DHFR activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol NADPH/h under standard assay conditions. Unless described otherwise, the reaction mixture contained 70 μM NADPH (Sigma-Aldrich, Munich, Germany), 75 μM dihydrofolate (Sigma-Aldrich, Munich, Germany), and varying units of DHFR in 30 mM Tris–HCl (pH 7.5). After pre-incubation of the enzyme with dihydrofolate for 5 min at ambient temperature, NADPH was added and the incubation of the reaction mixture was continued for 30 min at 37°C. For the determination of K_M values (substrate and cosubstrate) and IC_{50} values (methotrexate und trimethoprim) DHFR molecules purified to homogeneity were used.

Stability studies

To analyze the thermal stability samples containing 5 μM or 50 μM purified DHFR in 30 mM Tris-HCl (pH 7.5) were incubated for varying periods of time at indicated temperatures and subsequently assayed for remaining enzyme activity. Resistance to alkaline pH was analyzed by incubating purified DHFR molecules at different pH values in 0.1 M potassium phosphate or 0.1 M Tris–HCl for varying periods of time at 37°C followed by determination of the remaining enzyme activity. To investigate the effect of inorganic salts and denaturating agents on the activity of both DHFR constructs, the reagents to be tested were added to the standard reaction mixture at concentrations as indicated and the activity was assayed immediately thereafter. In a different set of experiments, the enzymes were incubated for 60 min in the presence of salts to be tested prior to the activity assays.

Dynamic light scattering (DLS) measurements

The hydrodynamic radius of *B. halodurans* apo-DHFR, of the DHFR-dihydrofolate complex, and the DHFR-methotrexate complex were determined by dynamic light scattering using the spectroscatterer 201 (RiNA GmbH, Berlin, Germany). The He–Ne laser provided a 690 nm light and an output power in the range of 10–50 mW. Samples containing 5 mg/ml purified enzyme in 10 mM sodium phosphate buffer (pH 7.5) were measured at ambient temperature using an autopilot function accumulating 50 measurements per sample. The theoretical hydrodynamic radii as well as the molecular masses of the protein complexes were calculated as previously described (Georgieva et al. 2004).

Circular dichroism (CD) measurements

CD spectra of *B. halodurans* apo-DHFR (final concentration: 0.1–0.2 mg/ml 10 mM sodium phosphate buffer, pH 7.5) and His-tagged *Bh*DHFR were recorded on a J810 Spectropolarimeter (Jasco, Easton, MD, USA) equipped with a temperature-regulated sample-chamber. Measurements were carried out in a cuvette with 1 mm path length in the far-UV region (185–260 nm) at ambient temperature. Each spectrum represents an average of 20 scans. Secondary-structure analysis of was performed using the neuronal network program k2d (Andrade et al. 1993).

Results

Analysis of the *B. halodurans* drfA gene

By alignment of published nucleotide sequences of various DHFR molecules, an open reading frame (ORF) of

489 bp coding for the DHFR (*dfrA*) gene of *B. halodurans* was identified between bp 3,563,029 and bp 3,563,517 of its genome. The ORF encodes a protein of 163 amino acids with a calculated molecular mass of 18.857 Da. The deduced amino acid composition is similar to those of mesophilic *Bacillus* strains including an increased amount of negatively charged amino acids (D and E) compared to positively charged residues (R and K). Moreover, the resulting theoretical isoelectric point (pI) of 5.25 is in the range of the corresponding mesophilic enzymes. A tendency to replace bulky, hydrophobic amino acids (M, F, L, and I) by small ones (A and V) was observed in *Bh*DHFR, but the total amount of hydrophobic residues remains comparable to that of mesophilic *Bacillus* strains. Unique characteristics of the *Bh*DHFR amino acid composition are restricted to a slight increase of the total number of charged residues and a significant replacement of Lys against Arg residues. Furthermore, the absence of Cys residues is an interesting specific property of the *Bh*DHFR molecules.

Alignment of the deduced amino acid sequence of *Bh*DHFR with those of various bacterial and eukaryotic DHFRs (Fig. 1) revealed maximum sequence identity with the genetically related DHFR of *B. subtilis* (52%), followed by those of *E. coli* (46%), human (31%), halophilic *H. volcanii* (31%), and hyperthermophilic *T. maritima* (27%). Despite the relatively low sequence homology, several amino acids that have been identified by crystallographic analyses of various DHFRs (Pieper et al. 1998; Volz et al. 1982) as essential residues for binding of dihydrofolate and NADPH, are also conserved or conservatively replaced in *Bh*DHFR (Fig. 1). These residues include Ala-7, Ser-49, and Leu-54, which are important for binding of dihydrofolate and methotrexate, Asp-27, which is involved in the protonation of the N-5 nitrogen of dihydrofolate, and Phe-31, which participates in forming the hydrophobic core of DHFR.

Cloning and expression of *Bh*DHFR

The coding sequence of the *drfA* gene was amplified by genomic PCR and cloned into the *E. coli* expression vector pET26b(+). In addition to the wild-type enzyme, a His-tagged construct was cloned by furnishing the 3′-end of the *drfA* gene with an AAALG-linker followed by a six fold His-tag. Expression of both constructs in *E. coli* BL21(DE3) yielded large amounts of active enzyme (approx. 10% of total cellular protein), the majority of which remained soluble.

Wild-type *Bh*DHFR was purified from extracts of *E. coli* by anion exchange chromatography and gel filtration chromatography, whereas immobilized metal ion affinity chromatography followed by anion exchange chromatography was performed for purification of the His-tagged *Bh*DHFR (Table 1). MTX agarose affinity chromatography was not applicable due to an extremely strong interaction of both enzymes with the resin, which would have required detrimental elution conditions. Homogeneity of the purified enzymes was confirmed by SDS-PAGE analysis (Fig. 2). Determination by mass spectrometry revealed molecular masses of 18.855 ± 3 Da and 20.157 ± 4 Da for wild-type and His-tagged *Bh*DHFR, respectively. These values are in excellent agreement with the molecular masses calculated from the deduced amino acid sequences. Apparently, the C-terminal His-tag does not affect the catalytic activity

Fig. 1 Alignment of DHFR sequences from different species**.** The deduced amino acid sequences of *B. halodurans* (*B. hal*), *B. subtilis* (*B. sub*) (GenBank accession no. M20012), *E. coli* (GenBank accession no. J01609), *T. matitima* (*T. mar*) (GenBank accession no. X81845), *H. volcanii* (*H. vol*) (GenBank accession no. J05088), and *H. sapiens* (*H. sap*) (GenBank accession no. J00140) were aligned. Identical amino acids are highlighted in *black*, homologous residues in *grey*. The essential amino acids involved in binding of NADPH or dihydrofolate are indicated with *asterisks*. Sequences are numbered from the start residue (1) for each enzyme

of *Bh*DHFR since both purified enzymes exhibited comparable specific activities (Table 1).

Structural characterization of *Bh*DHFR

Far-ultraviolet CD spectroscopy of wild type and His-tagged *Bh*DHFR yielded nearly identical spectra (data not shown), indicating that the secondary structure content of *Bh*DHFR is not affected by the His-Tag. Moreover, the spectra are highly similar to those of bacterial and eukaryotic DHFRs (Maglia et al. 2003; Kaufman and Kemerer 1977). A quantitative estimate of the secondary structure content indicated approximately 17% α-helices, 33% β-sheets and 50% coil structures. The predicted secondary structure in solution is in excellent agreement with the typical Rosmann fold, which is consistently observed in crystal structures of bacterial and eukaryotic DHFRs (Dams et al. 2000; Pieper et al. 1998; Wilquet et al. 1998).

Dynamic light scattering measurements revealed that wild-type *Bh*DHFR is characterized by a hydrodynamic radius of approximately 3 nm in solution. Similar radii were determined for the binary *Bh*DHFR complexes with dihydrofolate (3.1 nm) and methotrexate (2.9 nm). Apparently, binding of substrate or inhibitor does not alter the hydrodynamic radius of the apo-enzyme. Compared to the radii reported for monomeric DHFRs in crystal structures (approx. 2.5 nm, Kraut and Matthews 1987 and references therein), these data support the monomeric state of *Bh*DHFR assuming a hydrate shell of approximately 0.3 nm. All samples formed solutions that remained clear and monodisperse over a period of several days. Aggregated or precipitated material was not observed.

Kinetic properties of *Bh*DHFR

The catalytic turnover rate of purified wild-type and His-tagged *Bh*DHFR displayed the typical hyperbolic progression of a Michaelis-Menten kinetic. A hysteretic behaviour that has been reported for *E. coli* and *T. maritima* DHFR (Baccanari and Joyner 1981; Wilquet et al. 1998; Maglia et al. 2003) was not observed. The estimated K_M values for NADPH and dihydrofolate as well as the IC_{50} concentrations of methotrexate and trimethoprim are summarized in Table 2. Since similar values were obtained for wild-type and His-tagged *Bh*DHFR, the C-terminal His-tag does not appear to affect the catalytic behaviour of *Bh*DHFR. Furthermore, the K_M values determined for *Bh*DHFR are comparable to those of DHFRs from other bacterial species, suggesting a highly similar mechanism. Small discrepancies have only been detected concerning the IC_{50} concentrations of methotrexate and trimethoprim, which are approx. 100-fold higher than those reported for *E. coli* DHFR.

Alkaliphilic adaption of *Bh*DHFR

Recombinant wild-type *Bh*DHFR showed optimal activity in a broad, slightly basic pH region ranging from 7.25 to 8.75 (Fig. 3). Although a rapid decrease of activity was observed at pH values > 9, wild-type *Bh*DHFR exhibits the most alkaline pH activity range of all known DHFRs (Table 2). Surprisingly, the alkali-stable phenotype of wild-type *Bh*DHFR is lost by the attachment of a C-terminal His-tag. As shown in Fig. 3, optimal activity of the His-tagged *Bh*DHFR was observed at a pH region ranging between 6.75 and 7.75. Both enzymes exhibited only one activity to the maximum. A second pH optimum at acidic pH values which is characteristic for DHFRs of human, chicken and *E. coli* origin (Appleman et al. 1990; Kaufman and Kemerer 1977; Baccanari et al. 1977), could not be detected.

Table 1 Purification of recombinant wild-type *Bh*DHFR and His-tagged *Bh*DHFR expressed in *Escherichia coli* BL21 cells

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Wild-type *Bh*DHFR			
Crude extract	285.3	3446.3	12.1
Poros HQ/M	14.1	1212.3	86.0
Superdex G75	5.3	733.7	138.4
His-tagged *Bh*DHFR			
Crude extract	391.1	6762.0	17.3
Ni-NTA agarose	16.2	1121.0	69.2
Poros HQ/M	7.5	921.7	122.9

Enzyme activity was assayed under standard conditions
Protein concentration was determined as described in Materials and methods

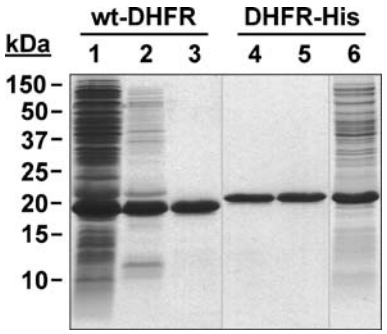

Fig. 2 SDS-PAGE (15%) analysis under reducing conditions of recombinant wild-type (wt-DHFR) and His-tagged (DHFR-His) *Bh*DHFR at different purification stages. The gel was stained by Coomassie Brilliant Blue. *Lane 1* crude cell extract of *E. coli* cells expressing wt-DHFR, *lane 2* wt-DHFR purified by Poros HQ/M anion exchange chromatography, *lane 3* wt-DHFR purified by Poros HQ/M anion exchange chromatography and subsequent gel filtration chromatography, *lane 4* DHFR-His purified by Ni-NTA agarose affinity chromatography and subsequent Poros HQ/M anion exchange chromatography, *lane 5* DHFR-His purified by Ni-NTA agarose affinity chromatography, *lane 6* crude cell extract of *E. coli* cells expressing DHFR-His

Table 2 Kinetic and biochemical parameters of DHFRs from different sources

Source and references	K_M		IC_{50}		Optimal pH range
	FH_2 (μM)	NADPH (μM)	MTX (μM)	TMP (μM)	
Bacillus halodurans wild-type	1.3 ± 0.7	8.2 ± 0.3	0.31 ± 0.05	8.9 ± 0.4	7.25–8.75
Bacillus halodurans His-tag	2.9 ± 1.9	5.9 ± 0.2	0.25 ± 0.02	10.1 ± 1.3	6.75–7.75
Bacillus subtilis (Iwakura and Tanaka 1992)	1.5 [a]	20.7 [a]	ND	ND	7.0
Escherichia coli (Baccanari et al. 1977)	8.9 ± 2.1	4.4 ± 1.0	0.003[a]	0.01[a]	4.0 and 7.0
Lactobacillus casei (Dann et al. 1976)	0.4 ± 0.1	16.6 ± 0.5	ND	ND	ND
Thermotoga maritima (Wilquet et al. 1998)	0.3[a]	4.0 [a]	0.07[a]	300[a]	6.5
Pneumocystis carnii (Delves et al. 1993)	2.3 ± 0.5	3.0 ± 0.5	0.0001[a]	24[a]	7.0
Gallus gallus (Kaufman and Kemerer 1977)	0.2[a]	1.8[a]	ND	ND	4.0 and 7.4
Homo sapiens (Appleman et al. 1990)	0.1 ± 0.02	0.2 ± 0.06	0.08[a]	2[a]	4.5 and 7.5

ND not detected
[a]Standard deviation not reported

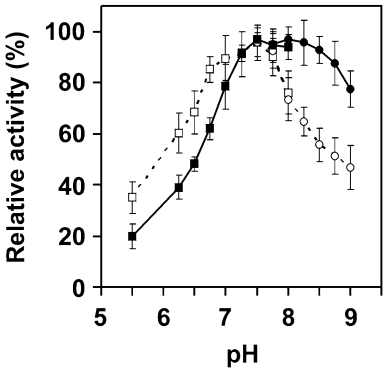

Fig. 3 Effect of pH on the activity of *Bh*DHFR. The activity of wild-type *Bh*DHFR (*straight line*) and His-tagged *Bh*DHFR (*dotted line*) was assayed at 37°C in 0.1 M potassium phosphate buffer (*squares*) or 0.1 M Tris-HCl buffer (*circles*) at indicated pH values

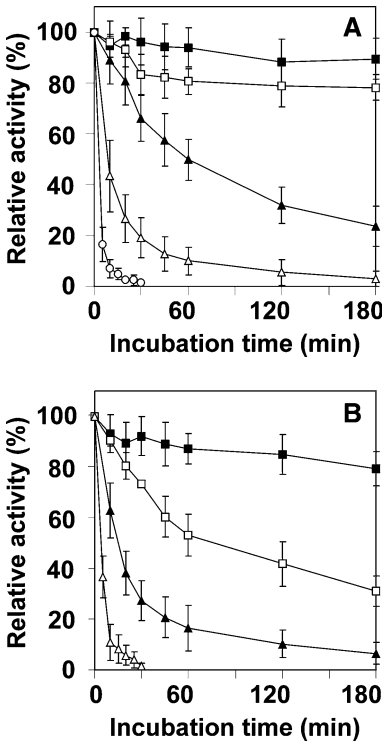

Fig. 4 Thermal stability of *Bh*DHFR. After incubation of wild-type *Bh*DHFR (5 μM) (**a**) and His-tagged *Bh*DHFR (5 μM) (**b**) at 45°C (*filled squares*), 50°C (*open squares*), 52°C (*filled triangles*), 55°C (*open triangles*) and 60°C (*open circles*) for indicated periods of time, the residual activity was determined

Thermo-stability of *Bh*DHFR

Although *B. halodurans* is not classified as thermophilic organism, wild-type *Bh*DHFR exhibited remarkable thermal stability. Even after incubation at 50°C for 3 h, the activity remained almost unchanged (Fig. 4a). However, at higher temperatures the DHFR activity decreased gradually resulting in complete inactivation at temperatures above 60°C. The half-life of wild-type *Bh*DHFR was determined to approximately 60 min at 52.5°C. The thermo-stable phenotype of wild-type *Bh*DHFR is also affected by attachment of a C-terminal His-tag (Fig. 4b). The His-tagged enzyme maintained activity only up to a temperature of 45°C, resulting in a significantly decreased half-life to approx. 15 min at 52.5°C. The different thermo-stability of both enzymes proved to be concentration-independent (data not shown).

Halotolerance of *Bh*DHFR and resistance to denaturing agents

The effect of inorganic salts and denaturating agents on the activity of *Bh*DHFR was determined immediately (Fig. 5) and 60 min (data not shown) after exposure of the enzyme to these reagents. Both experiments yielded comparable results. The activity of wild type *Bh*DHFR was not affected by NaCl or KCl up to a concentration of 0.3 M (Fig. 5a). At salt concentrations < 0.1 M the enzyme was even slightly activated. Inhibitory effects have only been observed at concentrations > 0.3 M NaCl or KCl, when the activity of wild-type *Bh*DHFR decreased asymptotically. This halo-tolerance is obviously reduced by attachment of a C-terminal His-tag. Even at low concentrations of NaCl or KCl the activity of the His-tagged enzyme is affected to a significant

extent (Fig. 5a). In contrast to monovalent salts, $CaCl_2$, and $MgCl_2$ proved to be strong inhibitors of both *Bh*DHFRs (Fig. 5b). In the presence of 0.3 M $MgCl_2$/$CaCl_2$, wild type and His-tagged *Bh*DHFR retained only 10 and 5% activity, respectively.

Wild type *Bh*DHFR showed remarkable resistance to urea (Fig. 5c). In fact, a slight increase in activity was observed at concentrations ranging from 1 to 3 M. Even in the presence of 4 M urea (limitation of the assay) the enzyme retained 50% activity. Attachment of a C-terminal His-tag to *Bh*DHFR reduced the resistance to urea by a factor of approximately 2. In contrast to urea, Gnd-HCl inactivates both wild-type and His-tagged *Bh*DHFR already at low concentrations, resulting in complete abolishment of DHFR activity in the presence of 1.0 M Gnd-HCl (Fig. 5c).

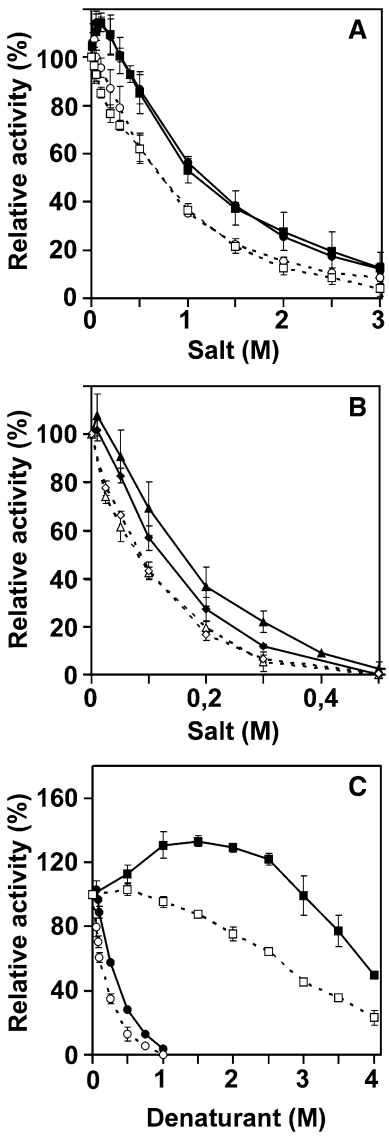

Fig. 5 Effect of inorganic salts and denaturing agents on the activity of *Bh*DHFR. The activity of wild-type *Bh*DHFR (*straight line*) and His-tagged *Bh*DHFR (*dotted line*) was determined in the presence of **a** NaCl (*squares*) and KCl (*circles*), **b** $CaCl_2$ (*triangles*) and $MgCl_2$ (*diamonds*), as well as **c** urea (*squares*) and guanidine hydrochloride (*circles*) at indicated concentrations

Discussion

In this study the DHFR from alkaliphilic *Bacillus halodurans* was cloned and expressed in *E. coli* to characterize its extremophilic properties. *Bh*DHFR exhibits the most alkaline activity range compared to all DHFRs analyzed so far, but its optimum pH for activity is still below the highly alkaline environment of pH 10.5 that is optimal for growth of *B. halodurans* (Nielsen et al. 1995). The presence of highly negative surface charges on the cell walls as well as Na^+/H^+ antiporters allows *B. halodurans* to maintain a cytoplasmic pH of 8.5 (Horikoshi 1999). Due to these cellular mechanisms of pH homeostasis, only moderate alkaliphilic adaption is required for cytosolic enzymes (Honda and Kitaoka 2004).

Based on crystallographic studies several structural features have been implicated in the alkaline adaption of extracellular enzymes, the majority of which are related to the modification of interactions mediated by hydrogen bonds, ion pairs, and hydrophobic effects (Dubnovitsky et al. 2005; Shirai et al. 2001). Similar molecular features could be postulated for cytolasmatic emzymes, even more, since the extent of adaption can be reduced. For *Bh*DHFR, these mechanisms possibly include a significant replacement of Lys by Arg residues. Since pK_a values of Arg are higher than those of Lys, these substitutions facilitate maintenance of the charge balance at higher pH values. However, the isolelectric point of *Bh*DHFR is not increased as it is essential for extracellular proteins from alkaliphilic organisms (De Lemos Esteves et al. 2005; Shirai et al. 2001). Compared to DHFR molecules from mesophilic *Bacillus* strains, *Bh*DHFR contains a higher percentage of small amino acids allowing optimized packing and, thereby, reduction of the solvent exposed hydrophobic surface area. While this assumption remains to be confirmed by three-dimensional structural analyses, the replacement of bulky hydrophobic residues by smaller ones has also been discussed as important mechanism of the alkaliphilic adaption of phosphoserine aminotransferase from *B. alcalophilus* (Dubnovitsky et al. 2005).

The lowered requirements for alkaliphilic adaption in the cytoplasmatic compartment also explains the distinct similarity of secondary structural features of *Bh*DHFR with those of DHFR molecules from mesophilic *Bacillus* strains, which is particularly revealed by the consistent far-ultraviolet CD spectra obtained for *Bh*DHFR as well as for other mesophilic bacterial DHFR molecules (Dams et al. 2000; Pieper et al. 1998; Smith et al. 1985).

The alkali-stable phenotype of *Bh*DHFR was found to be combined with a moderate thermo-stable phenotype. Apparently, resistance to alkaline conditions and thermo-stability are often associated since a combination of both properties has been reported for several proteins from alkaliphilic organisms (Wiegel 1989 and

references therein). *Bh*DHFR exhibits long-term stability up to 50°C which reflects adaption to the maximum temperature of cell growth (Nielsen et al. 1995). The thermo-stability of *Bh*DHFR is largely concentration independent, does not require the presence of substrates and, therefore, represents an intrinsic property of the DHFR molecule. Numerous mechanisms have been described that contribute to the intrinsic thermo-stability of proteins. Most of them are related to optimized interactions caused by specific structural modifications within the tertiary structure of proteins (Vogt et al. 1997; Ladenstein and Antranikian 1998). In *Bh*DHFR, the relatively high percentage of small amino acid residues allowing optimized packing represents a potential thermo-stabilizing mechanism, but a reliable evaluation requires again a detailed three-dimensional structural analysis. Based on the experimental data of this study, however, disulfide formation and dimerization of the DHFR molecule as observed in hyperthermophilic *T. maritima* (Dams et al. 2000; Wilquet et al. 1998) can be excluded as mechanisms of thermo-stabilization. *Bh*DHFR does not contain cysteine residues and DLS measurements have confirmed the monomeric structure of this enzyme.

Although *B. halodurans* tolerates inorganic salts up to a concentration of 3 M (Nielsen et al. 1995), *Bh*DHFR proved to be sensitive to high salt concentrations. In the presence of 3 M NaCl or KCl, only low residual enzyme activity was determined, and $CaCl_2$ as well as $MgCl_2$ inhibited *Bh*DHFR completely even at low salt concentrations. Apparently, *B. halodurans* has developed other cellular strategies to balance external high salinity. On the other hand, *Bh*DHFR exhibits a remarkable resistance to urea, which is reflected by the retained enzyme activity of 50% even in the presence of 4 M urea. The observed activation of *Bh*DHFR at lower concentrations has also been reported for DHFRs from chicken liver (Fan et al. 1996), *T. gondii* (Trujillo et al. 1996), and *T. maritima* (Wilquet et al. 1998). While the structural basis for urea-mediated activation remains to be determined in detail, loosening of the protein structure, particularly in vicinity of the active site, has been proposed as activating mechanism (Fan et al. 1996).

Interestingly, attachment of a C-terminal His-tag to wild-type *Bh*DHFR significantly decreased its thermo-stability and resistance to alkaline conditions, inorganic salts, urea, and Gnd-HCl, whereas kinetic properties and the secondary structure were not affected. Although speculative, the reduced thermo-stability could be a result of an increased flexibility of the C-terminal part of the structure due to the presence of the His-tag. On the other hand, modifications of electrostatic interactions on the protein surface caused by the inserted His residues may contribute to the reduced stability in alkaline and saline environments. While the precise molecular mechanisms remain to be established, the different structural properties of wild-type and His-tagged *Bh*DHFR emphasize the necessity to take great care in designing protein constructs for structural studies.

In conclusion, *B. halodurans* DHFR is the first DHFR from an alkaliphilic organism to be analyzed in detail. This DHFR is characterized by a unique accumulation of extremophilic properties and studies are in progress to determine its tertiary structure. In particular a comparative analysis of the three dimensional structures of both DHFRs, the wild type and the His-tagged enzyme, will provide deeper insights into the specific mechanisms of alkaliphilic and thermophilic adaption.

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