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ORIGINAL PAPER

Comparative study on dihydrofolate reductases from *Shewanella* species living in deep-sea and ambient atmospheric-pressure environments

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Abstract To examine whether dihydrofolate reductase (DHFR) from deep-sea bacteria has undergone molecular evolution to adapt to high-pressure environments, we cloned eight DHFRs from *Shewanella* species living in deep-sea and ambient atmospheric-pressure environments, and subsequently purified six proteins to compare their structures, stabilities, and functions. The DHFRs showed 74–90% identity in primary structure to DHFR from *S. violacea*, but only 55% identity to DHFR from *Escherichia coli* (ecDHFR). Far-ultraviolet circular dichroism and fluorescence spectra suggested that the secondary and tertiary structures of these DHFRs were similar. In addition, no significant differences were found in structural stability as monitored by urea-induced unfolding and the kinetic parameters, K_m and k_{cat}; although the DHFRs from *Shewanella* species were less stable and more active (2- to 4-fold increases in k_{cat}/K_m) than ecDHFR. Interestingly, the pressure effects on enzyme activity revealed that DHFRs from ambient-atmospheric species are not necessarily incompatible with high pressure, and DHFRs from deep-sea species are not necessarily tolerant of high pressure. These results suggest that the DHFR molecule itself has not evolved to adapt to high-pressure environments, but rather, those *Shewanella* species with enzymes capable of retaining functional activity under high pressure migrated into the deep-sea.

Keywords Deep-sea · DHFR · High pressure · Molecular adaptation · *Shewanella* species

Abbreviations

DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
ecDHFR	DHFR from *Escherichia coli*
sb21DHFR	DHFR from *Shewanella benthica* strain DB21MT-2
sb43992DHFR	DHFR from *S. benthica* strain ATCC43992
sb6705DHFR	DHFR from *S. benthica* strain DB6705
sfDHFR	DHFR from *S. frigidimarina* strain ACAM591
sgDHFR	DHFR from *S. gelidimarina* strain ACAM456
soDHFR	DHFR from *S. oneidensis* strain MR-1
spDHFR	DHFR from *S. putrefaciens* strain IAM12079
svDHFR	DHFR from *S. violacea* strain DSS12
THF	Tetrahydrofolate

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Introduction

Dihydrofolate reductase (DHFR) is an essential enzyme for the biosynthesis of purine nucleotides and certain amino acids and has, therefore, been studied as an indicator protein for bacterial adaptation to environments with extreme temperatures (Kim et al. 2005; Loveridge et al. 2009), pH (Redecke et al. 2007), salt concentrations (Wright et al. 2002), and pressures (Xu et al. 2003; Murakami et al. 2010). Although DHFRs isolated from these extremophiles show characteristic structures and properties, no definite correlation has been found between the observed molecular characteristics and the environmental conditions due to limited comparative studies.

It is known that DHFR from *Escherichia coli* (ecDHFR) living in ambient atmospheric conditions is highly flexible, and its structure and function are sensitively influenced by pressure (Kitahara et al. 2000; Ohmae et al. 2008). We previously found that the activity of ecDHFR decreases with increasing pressure, whereas DHFR from *Shewanella violacea* strain DSS12 (svDHFR), which was isolated from the Ryukyu Trench at a depth of 5,110 m, has an optimal activity at approximately 100 MPa (Ohmae et al. 2004). This finding suggests that DHFRs from deep-sea bacteria have characteristic pressure susceptibility and structural dynamics that are unique from those of organisms living at ambient atmospheric pressure.

Despite the apparent association between pressure and the function of DHFR from piezophilic *S. violacea*, other DHFRs from deep-sea isolates do not necessarily show such a relationship. For example, DHFRs from the genera *Moritella* and *Photobacterium* were not activated by pressure under the identical experimental conditions and no common amino acid sequences among DHFRs from high-pressure environments were identified (Hay et al. 2009; Murakami et al. 2010). However, as these results were obtained with bacteria of different genera, a direct comparison is unreasonable to clarify complicated pressure-adaptation mechanisms because their respective proteins may have undergone different molecular evolution. Therefore, comparative studies on the structure and function of DHFRs within an individual genus consisting of piezophilic and piezotolerant bacteria are necessary to give new insight into the pressure-adaptation mechanisms and molecular evolution of proteins. A target protein for such comparative studies has been so far limited to a single-stranded DNA-binding protein from marine *Shewanella* species: pressure tolerance of tetramer–monomer equilibrium of this protein correlated with the living pressure of these species (Chilukuri and Bartlett 1997; Chilukuri et al. 2002).

In this study, we therefore cloned and purified novel DHFRs from four piezophilic, one piezotolerant, and three piezosensitive *Shewanella* species inhabiting various extreme environments, as listed in Table 1, to survey the pressure-adaptation mechanisms of *Shewanella* species at the protein level. We followed up our previous work with svDHFR, which was pressure activated, to examine if DHFRs from *Shewanella* species too have the same pressure-tolerance behavior as has been seen in a single-stranded DNA-binding protein (Chilukuri and Bartlett 1997; Chilukuri et al. 2002). To our knowledge, this is the second example for the comparative study of proteins from *Shewanella* species inhabiting different pressure environments.

Materials and methods

Cloning and construction of *Shewanella* DHFR overexpression plasmids

Construction of the svDHFR overexpression plasmid was described previously (Ohmae et al. 2004). Genomic DNAs of seven bacteria (*S. oneidensis*, *S. frigidimarina*, *S. putrefaciens*, *S. gelidimarina*, *S. benthica* strain

Table 1 Description of *Shewanella* species and DHFR names used in this study

Species	Isolation source or depth	Piezophilicity of bacteria	References	DHFR names
S. benthica ATCC43992	4,575 m	Piezophilic	Deming et al. 1984; MacDonell and Colwell 1985	sb43992DHFR
S. benthica DB21MT-2	10,898 m	Piezophilic	Kato et al. 1998	sb21DHFR
S. benthica DB6705	6,356 m	Piezophilic	Kato et al. 1995; Nogi et al. 1998	sb6705DHFR
S. frigidimarina ACAM591	Antarctic sea ice	Piezosensitive	Bowman et al. 1997	sfDHFR
S. gelidimarina ACAM456	Antarctic sea ice	Piezotolerant	Bowman et al. 1997	sgDHFR
S. oneidensis MR-1	Oneida lake	Piezosensitive	Venkateswaran et al. 1999	soDHFR
S. putrefaciens IAM12079	Rancid butter	Piezosensitive	Owen et al. 1978	spDHFR
S. violacea DSS12	5,110 m	Piezophilic	Kato et al. 1995; Nogi et al. 1998	svDHFR

ATCC43992, *S. benthica* strain DB6705, and *S. benthica* strain DB21MT-2) were kindly provided by Dr. Nogi of JAMSTEC and were amplified prior to use with a GenomiPhi DNA Amplification kit (GE Healthcare UK Ltd., Buckinghamshire, UK). Plasmids overexpressing the DHFRs from *S. oneidensis* (soDHFR), *S. frigidimarina* (sfDHFR), and *S. putrefaciens* (spDHFR) were constructed by a PCR method using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) and primer sets derived from the genome sequences of the three species that were previously registered in the DDBJ/GenBank/EMBL database under accession numbers and locus tags SO_3647 of AE014299 (*S. oneidensis*), Sfri_3084 of CP000447 (*S. frigidimarina*), and Sputcn32_0984 of CP000681 (*S. putrefaciens*). The amplified DNA fragments were ligated to pUC118 vectors digested with *Sma*I, and used to transform competent *E. coli* HB101 cells (Takara Bio, Otsu, Japan). The transformants carrying the overexpression plasmids were selected on LB plates containing ampicillin (100 μg/ml) and trimethoprim (20 μg/ml) at 37°C. The DNA sequences of the purified plasmids were confirmed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare UK Ltd., Buckinghamshire, UK).

The overexpression plasmids for DHFRs from *S. benthica* strain ATCC43992 (sb43992DHFR), *S. benthica* strain DB6705 (sb6705DHFR), *S. benthica* strain DB21MT-2 (sb21DHFR), and *S. gelidimarina* strain ACAM456 (sgDHFR) were amplified by a PCR method with a mixed primer set based on sequences of the DHFRs from *S. oneidensis*, *S. frigidimarina*, *S. putrefaciens*, and six deep-sea bacteria (*S. violacea*, *Moritella profunda*, *M. yayanosii*, *M. japonica*, *Photobacterium profundum*, and *Colwellia psychrerythraea* with corresponding accession numbers and a locus tag of AB519243, AJ487535, AB505967, AB505968, AB505966, and CPS_4505 of CP000083, respectively). The amplified DNA fragments were purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and sequenced as described above. The up- and downstream regions were amplified using a DNA Walking Speed-up kit (Seegene, Seoul, Korea) with three sets of primers synthesized for each DHFR gene and were then sequenced. Finally, the entire DHFR gene of each species was amplified with a set of up- and downstream primers, KOD-Plus-DNA polymerase, and 0.1 ng of original genomic DNA. The DNA sequences of the amplified fragments were determined and registered in the DDBJ/GenBank/EMBL database under accession nos. AB538271 (sb43992DHFR), AB538272 (sb6705DHFR), AB538270 (sb21DHFR), and AB538273 (sgDHFR). The overexpression plasmids of these four DHFRs were then constructed using the primer set of svDHFR for sb43992DHFR, sb6705DHFR, and sb21DHFR, which have the identical amino acid sequences at the N- and C-terminal regions, and with the newly synthesized primers for sgDHFR. The DNA sequences of the resulting plasmids were confirmed as described above. The DNA sequences of the primers used in this study are listed in supplementary Table S1.

Protein purification

All DHFR proteins were over-expressed and purified from *E. coli* HB101 cells as described previously (Murakami et al. 2010). Briefly, cells were grown for 48 h at 25°C, harvested by centrifugation, washed with 20 mM Tris-hydrochloride (pH 8.0) containing 14 mM 2-mercaptoethanol and 0.1 mM EDTA (Buffer A), and broken by sonication. After centrifugation, 2% (wt/vol) streptomycin sulfate was added with stirring to the supernatant to precipitate nucleic acids. After centrifugation, a saturated ammonium sulfate solution was added with stirring to the supernatant to achieve a final concentration of 45%. The resulting precipitates were removed by centrifugation, and the supernatant was then loaded on a methotrexate-agarose affinity column (3 × 10 cm) (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with Buffer A. The column was washed with Buffer A containing 0.5 M NaCl and the DHFR protein was eluted with Buffer A containing 3 mM folate. DHFR-containing fractions were dialyzed against 20 mM Tris-hydrochloride (pH 9.0) containing 14 mM 2-mercaptoethanol and 0.1 mM EDTA (Buffer B) for 3 days and concentrated with a DE-52 anion-exchange column (1 × 5 cm) (Whatman, Kent, UK). The samples were then dialyzed against Buffer B containing 3 M guanidine hydrochloride to completely remove the folate and against Buffer B containing 6 M urea to completely remove the guanidine hydrochloride. Proteins were then refolded by dilution with Buffer B to a concentration of approximately 10 μM. Refolded DHFRs were dialyzed against 20 mM Tris-hydrochloride (pH 8.0) containing 0.1 mM dithiothreitol and 0.1 mM EDTA for 3 days and concentrated with an Amicon Ultra filter (Millipore, Billerica, MA, USA). The aggregates were then removed by centrifugation at 18,000×*g*. Whole cell lysates and purified proteins (15 μg) were separated on a 15% SDS-PAGE gel.

The concentrations of ecDHFR and svDHFR were determined from the absorbance at 280 nm using molar extinction coefficients of 31,100 $M^{-1}cm^{-1}$ (Fierke et al. 1987) and 22,900 $M^{-1}cm^{-1}$ (Murakami et al. 2010), respectively. The concentrations of sb6705DHFR, sb21DHFR, sfDHFR, soDHFR, and spDHFR were determined using molar extinction coefficients of 27,300, 34,600, 22,800, 23,000, and 24,400 $M^{-1}cm^{-1}$ at 280 nm, respectively, which were estimated from the fluorescence intensities of the fully unfolded DHFRs in 6 M guanidine hydrochloride solutions.

Circular dichroism spectrometry

Far-ultraviolet circular dichroism (CD) spectra of the DHFRs were measured using a Jasco J-720W spectropolarimeter. The temperature was maintained at 15°C using a Peltier-controlled thermobath (Jasco PTC-348W). The solvent used was 20 mM Tris-hydrochloride (pH 8.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol, and the protein concentration was 10 μM.

Fluorescence spectrometry

Fluorescence spectra of DHFRs were measured at 15°C using a Jasco FP-750 spectrofluorometer with an excitation wavelength of 290 nm and an emission wavelength ranging from 300 to 450 nm. The solvent used for the measurements was identical to that used for the CD measurements. The protein concentration was 0.3–0.8 μM and the intensities of the spectra were normalized to 1 μM protein.

Equilibrium unfolding

Equilibrium unfolding of the DHFRs in the presence of urea (ultrapure product from ICN Biomedicals, Irvine, CA, USA) was monitored by fluorescence measurements at 15°C with a Jasco FP-750 spectrofluorometer. The excitation and emission wavelengths, solvent, and protein concentration were identical to those used in the above spectrometric measurements. All samples were fully equilibrated at each denaturant concentration before the fluorescence spectra were measured. The center of spectral mass (CSM) of each spectrum was calculated from the following equation (Vidugiris and Royer 1998):

$$\mathrm{CSM} = \sum F_i \nu_i \Big/ \sum F_i \tag{1}$$

where F_i and ν_i are the fluorescence intensity and wave number at wavelength i, respectively. The obtained CSM values were plotted against the urea concentration and fitted directly to the two-state unfolding model, native (N) ⇔ unfolded (U), by non-linear least-squares regression analysis as follows:

$$\mathrm{CSM} = \{\mathrm{CSM_N} + \mathrm{CSM_U} \exp(-\Delta G_u/RT)\}/ \{1 + \exp(-\Delta G_u/RT)\} \tag{2}$$

where ΔG_u is the change in Gibbs free energy for unfolding, R is the gas constant, T is the absolute temperature, and $\mathrm{CSM_N}$ and $\mathrm{CSM_U}$ are the CSM values of the native and unfolded forms, respectively. $\mathrm{CSM_N}$ and $\mathrm{CSM_U}$ at a given urea concentration were estimated by assuming the same linear dependence of CSM values in the transition region as in the pure native (pre-transition region) and unfolded (post-transition region) states. The free energy change of unfolding, ΔG_u, was assumed to be linearly dependent on the urea concentration (Pace 1985):

$$\Delta G_u = \Delta G_u^\circ - m\,[\mathrm{urea}] \tag{3}$$

where ΔG_u° is the free energy change of unfolding in the absence of a denaturant, and the slope, m, is a parameter reflecting the cooperativity of the transition. The urea concentration at the midpoint of the transition ($\Delta G_u^\circ = 0$) was defined as C_m.

Steady-state kinetics at atmospheric pressure

Steady-state kinetics of the enzymatic reaction was measured at 25°C at atmospheric pressure with a Jasco V-560 spectrophotometer. The solvent used was 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol. The concentrations of DHF (Sigma-Aldrich, St. Louis, MO, USA) and NADPH (Oriental Yeast, Tokyo, Japan) were determined spectrophotometrically using molar extinction coefficients of 28,000 $\mathrm{M^{-1}cm^{-1}}$ at 282 nm for DHF and 6,200 $\mathrm{M^{-1}cm^{-1}}$ at 339 nm for NADPH (Dawson et al. 1969). The enzymatic reactions were measured under various concentrations of NADPH or DHF while maintaining either ligand at a saturating concentration of 50 μM. The concentration of the DHFRs was adjusted to 1–2 nM by a methotrexate titration method (Williams et al. 1979). The enzymatic reactions were initiated by mixing the DHF solution with the enzyme-NADPH solution. Prior to mixing, both solutions were preincubated for 10 min at 25°C to equilibrate the temperature and eliminate the hysteresis effect (Penner and Frieden 1985). The initial velocity (v) of the enzymatic reaction was calculated from the time course of absorbance at 340 nm using a differential molar extinction coefficient of 11,800 $\mathrm{M^{-1}cm^{-1}}$ (Stone and Morrison 1982). The Michaelis (K_m) and catalytic (k_{cat}) constants were determined with the program Origin (OriginLab, Northampton, MA, USA) by direct fitting of the initial velocity of the enzymatic reaction to the Michaelis–Menten equation using non-linear least-squares regression analysis.

Enzyme assay under high pressures

Pressure dependence of the enzyme activity was examined using a Jasco V-560 spectrophotometer equipped with a high-pressure absorbance cell unit (Teramecs PCI-400) and a hand pump (Teramecs PCI-500) as described previously (Ohmae et al. 2008; Murakami et al. 2010). Temperature was maintained at 25°C with a circulating thermobath (NESLAB RTE-5). The buffer used was 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 250 μM NADPH, and 250 μM DHF. The reaction mixture (1.5 ml) containing DHF and the enzyme-NADPH solution was loaded into the high-pressure cell,

and the absorbance at 370 nm was then measured for 1–5 min at pressures ranging from 0 to 250 MPa. The initial velocity of the enzymatic reaction was calculated from the time course of the absorbance using the differential molar extinction coefficient at each pressure (e.g., 3,180 and 3,710 $M^{-1}cm^{-1}$ at 0.1 and 250 MPa, respectively) (Ohmae et al. 2008). The activation free energy (ΔG^*) and the activation volume (ΔV^*) of the enzymatic reactions were calculated using the following equation, which is applicable at saturating substrate concentrations,

$$\Delta V^* = \partial \Delta G^* / \partial P = \partial(-RT \ln k_{cat})/\partial P = \partial(-RT \ln v)/\partial P \quad (4)$$

where R is the gas constant, T is the temperature, P is the pressure, and v is the initial velocity of the enzymatic reaction (Ohmae et al. 2008).

Results and discussion

Amino acid sequences of *Shewanella* DHFRs

To gain insight into the pressure-adaptation mechanisms and molecular evolution of DHFR proteins, we first compared the amino acid sequences of sb43992DHFR, sb6705DHFR, sb21DHFR, sfDHFR, sgDHFR, soDHFR, and spDHFR, which were determined from the DNA sequences, to those of ecDHFR and svDHFR (Fig. 1). From the alignments, the sequence identities of sb43992DHFR, sb6705DHFR, sb21DHFR, sfDHFR, sgDHFR, soDHFR, spDHFR, and ecDHFR to svDHFR were 89, 90, 89, 74, 76, 76, 74, and 55%, respectively. Overall, the N-terminal regions, particularly in the first 60 residues, showed high sequence conservation, whereas the C-terminal regions showed wider sequence variations, as previously found for DHFRs from other deep-sea species (Murakami et al. 2010). The Gly95-Gly96 residues, which are known to be essential for the proper folding of ecDHFR, were also completely conserved in all of the *Shewanella* DHFRs. Moreover, nearly all residues in the substrate and cofactor binding sites (with the exception of Val6 of sb43992DHFR, Asn17 of sb21DHFR, Gln19 of svDHFR and sb21DHFR, Lys76 of sfDHFR, Cys77 of sgDHFR, Met94 of spDHFR, and a residue 102) were conserved in all *Shewanella* DHFRs (Fig. 1). The Met20 loop comprising residues 10–24, which is a well-characterized and essential functional region, was also fully conserved in all of the *Shewanella* DHFRs, with the exception of only Gln19 of svDHFR, and Asn17 and Gln19 of sb21DHFR.

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                  10         20         30         40         50         60
ecDHFR         >M-ISLIAALAV DRVIGMENAM PWNLPADLAW FKRNTLNKPV IMGRHTWESI GRPLPGRKNI
svDHFR         >MKIAMIAAMAN NRVIGKDNQM PWHLPEDLKH FKAMTLGKPI VMGRKTYDSI GRALPGRLNI
sb6705DHFR     >MKIAMIAAMAN NRVIGKDNKM PWHLPEDLKH FKAMTLGKPI VMGRKTYESI GRALPGRLNI
sb43992DHFR     MKIAMIVAMAN NRVIGKDNKM PWHLPEDLKH FKAMTLGKPI VMGRKTYESI GRALPGRLNI
sb21DHFR       >MKIAMIAAMAN NRVIGKNNQM PWHLPEDLKH FKAMTLGKPI VMGRKTYESI GRALPGRLNI
sgDHFR          MKIAMIAAMAN NRVIGKDNKM PWHLPEDLRH FKAVTLGKPI VMGRKTYESI GRPLPGRHNI
sfDHFR         >MRIAMIAAMAN NRVIGKDNKM PWHLPEDLRH FKAMTLSKPV VMGRKTYESI GRPLPGRHNI
soDHFR         >MRIAMIAAMAN NRVIGKDNKM PWHLPEDLRH FKAMTLGKPV VMGRKTFESI GRPLPGRHNI
spDHFR         >MRIAMIAAMAN NRVIGKDNKM PWHLPEDLKH FKAMTLGKPV VMGRKTFESI GRPLPGRHNI
                ::****.**** ******:*:* ********:* ***:**.**: ******::** **.**** **

                  70         80         90        ↓↓100       110        120
ecDHFR          ILSSQPG-TDD RVTWVKSVDE AIAACGDVPE IMVIGGGRVY EQFLPKAQKL YLTHIDAEVE
svDHFR          VISRQEGLSIP GVTCVTSFEM AVEAAGECEE LVVIGGGQLY ASLLSKADKL YLTEINLDVA
sb6705DHFR      VISRQEGLSIA GVTCVTSFES AVAAAGECEE LVVIGGGQLY AALLSQADKL YLTEINLDVD
sb43992DHFR     VISRQEGLSIA GVTCVTSFES AVAAAGECEE LVVIGGGQLY AALLSQADKL YLTEINLDVD
sb21DHFR        VISRQQGLTIP GVTCVTSFES AVAAAGECEE LVVIGGGQLY AALLSQADKL YLTEINLDVD
sgDHFR          VISRQADLAID GVTTVTCFED AKLAAGDCEE LVVIGGGQLY TSLLCLSDVL YLTEISLDVE
sfDHFR          VISRNSQLSIE GVTCVKSFDE AITVAGDCEE IVVIGGGQLY QQLLPQADVL YLTLIDVDVD
soDHFR          VISRQADLQIE GVTCVTSFEA AKRVAGDCEE LVVIGGGQLY KQLLPQADRL YLTQINLDVD
spDHFR          VISRQADLVIE GVTSVTSFDA AKQAAGECEE LVIMGGGQLY RELLPQADIL YLTQINLDVE
                ****:  * *  *** *.:*:  *  .**:*** :*::****** **  :* * *** *.: **

                  130        140        150        160 Residues Identity  pI
ecDHFR          GDTHFPDYEP DDWESVFSEF HDADAQNSHS YCFEILERR-    159     54.7%   5.0
svDHFR          GDTFFPQWDD GSWERISQEM LVNGAG--LE YSFINLVKKC    160    100.0%   5.6
sb6705DHFR      GDTHFPRWDD GSWQRISQET AINDKG--LE YSFINLVKKC    160     90.0%   6.8
sb43992DHFR     GDTHFPRWDD GSWQRISQET AINDKG--LE YSFINLVKKC    160     89.4%   6.8
sb21DHFR        GDTHFPQWDD GSWQRVSQET SINDKG--VE YSFINLVKKC    160     88.8%   6.8
sgDHFR          GDTYFPHWDD GSWLEMSRDV AISGKQ--LE YSFIKMVKKC    160     76.3%   6.2
sfDHFR          GDTVFPAWDD GSWDLQNSVT ATNDKG--LQ YSFNTLVKKC    160     73.8%   5.9
soDHFR          GDTFFPAWED NEWQKTETQP SISADG--LE YNFINLVKKC    160     75.6%   6.8
spDHFR          GDTYFPEWDA RDWQETESLS CINADG--LE YRFINLTKKC    160     74.4%   5.6
                *** ** *:   .*   .       .      :: * * .:.***
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Fig. 1 Amino acid sequences of DHFRs from *E. coli* and eight *Shewanella* species. Multiple alignments were conducted with the CLUSTALW program (Thompson et al. 1994) on a DNA Data Bank of Japan server (http://www.ddbj.nig.ac.jp/). The numbering of residues is based on the ecDHFR sequence. The symbols ">" before sequences indicate the DHFRs used for protein purification. The symbols "*", ":" and "." below the alignment indicate fully, strongly, and weakly conserved residues, respectively. Amino acid residues different from those of svDHFR are indicated by *gray boxes*. Non-conserved residues in the substrate- and cofactor-binding sites are enclosed by *open boxes*. Fully conserved *cis*-glycine bond (Gly95-Gly96) is indicated by *arrows* above the sequences. Sequence lengths, levels of identity to svDHFR, and estimated isoelectric points (pI) are also indicated at the end of each sequence

It is noteworthy that the amino acid sequences of the *Shewanella* DHFRs from the deep-sea isolates (svDHFR, sb43992DHFR, sb6705DHFR, and sb21DHFR) are similar to those from the ambient-atmospheric isolates (sfDHFR, sgDHFR, soDHFR, and spDHFR), but largely different from that of ecDHFR. This result is consistent to those for the single-stranded DNA-binding protein (Chilukuri and Bartlett 1997) and the phylogenetic studies from 16S rDNA sequences (Venkateswaran et al. 1999; Kato and Nogi 2001), indicating that members within the genus *Shewanella* migrated to the deep sea after it separated from the genus *Escherichia*. From the DHFR alignments, it was also identified that only three residues were different between all four deep-sea isolates and the ambient atmospheric *Shewanella* species, including *E. coli*. The residues Ala53, Leu58, and Ser105 of the deep-sea DHFRs were substituted to proline, histidine or lysine, and cysteine or proline, respectively, in the ambient atmospheric bacteria.

Interestingly, for the translation of sgDHFR, *S. gelidimarina* appears to have adopted a unique initiation codon, TTG, since the nearest stop codon is located at the 36th upstream DNA base before the estimated N-terminus. However, we could not purify and determine the N-terminal residue of sgDHFR directly from this bacterium. Therefore, the structural and enzymatic properties of sgDHFR were not examined in the present study. In addition, sb43992DHFR was also omitted from the comparative studies of structure and function because this protein was predicted to have very similar properties to sb6705DHFR as they differed by only one amino acid residue (Ala6 or Val6). Thus, we compared the structure, stability, and activity of six *Shewanella* DHFRs, with the exception of sgDHFR and sb43992DHFR.

Following overexpression and purification of the six purified *Shewanella* DHFRs, the proteins were examined on a SDS-PAGE gel (Fig. 2). Although single bands corresponding to the DHFRs were predominantly observed, these proteins had additional 14 residues (MTMITNSSSVPGTS) at the N-terminal region that originated from the *lacZ′* gene on the vector as judged from the mass spectrometry. As the expression of the enzymes significantly decreased after elimination of these residues, we used the DHFRs with these additional residues in all experiments.

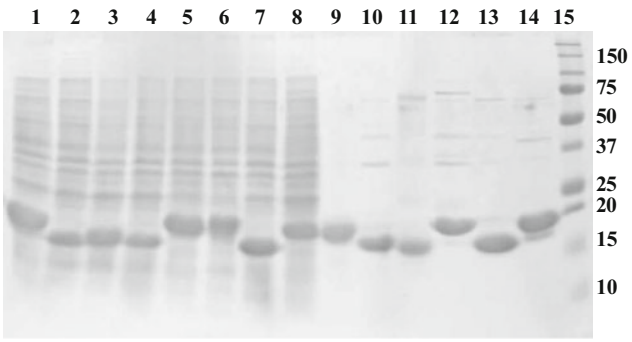

Fig. 2 SDS-PAGE of *E. coli* and *Shewanella* DHFRs. Lanes *1–8* whole protein extracts from *E. coli* transformants overexpressing svDHFR, sb43992DHFR, sb21DHFR, sb6705DHFR, sfDHFR, sgDHFR, soDHFR, and spDHFR. Lanes *9–14* affinity-column purified svDHFR, sb21DHFR, sb6705DHFR, sfDHFR, soDHFR, and spDHFR. Lane *15* molecular weight markers. The acrylamide concentration of the gel was 15%

Structure of *Shewanella* DHFRs

To examine the structure of the *Shewanella* DHFRs, they were subjected to far-ultraviolet CD spectrometry at 15°C and pH 8.0 at atmospheric pressure (Fig. 3a). Compared with ecDHFR, the CD spectra of the *Shewanella* DHFRs

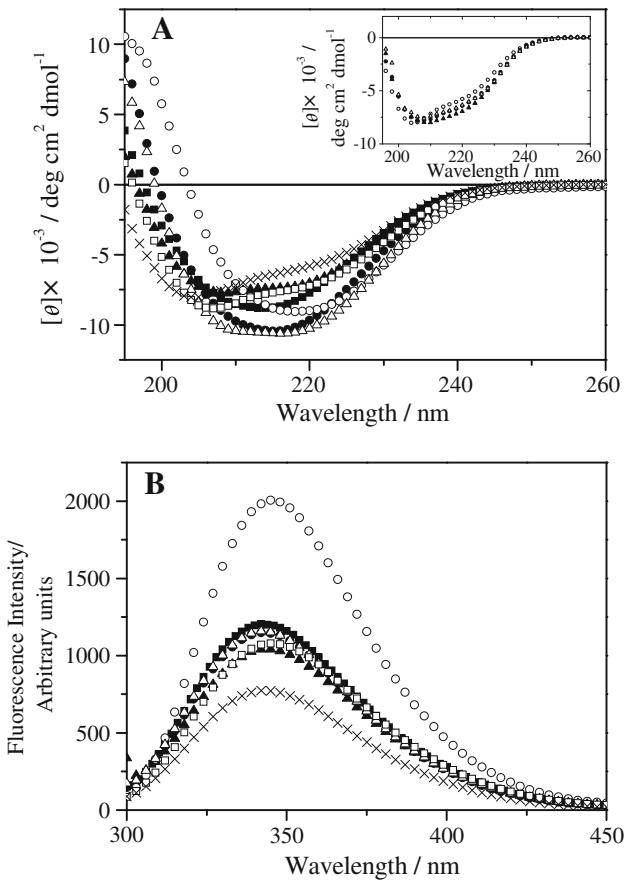

Fig. 3 Far-ultraviolet CD (**a**) and fluorescence (**b**) spectra of *E. coli* and *Shewanella* DHFRs at pH 8.0 and 15°C at atmospheric pressure. The solvent used was 20 mM Tris-hydrochloride containing 0.1 mM EDTA and 0.1 mM dithiothreitol. *Open circles* ecDHFR, *filled circles* svDHFR, *filled triangles* sb21DHFR, *open triangles* sb6705DHFR, *filled squares* sfDHFR, *open squares* soDHFR, and *cross symbol* spDHFR. *Inset* of panel **a** far-ultraviolet CD spectra of spDHFR with and without ligands. *Open circle* apo-spDHFR, *filled circle* spDHFR with 250 μM NADPH, *filled triangle* spDHFR with 250 μM dihydrofolate, and *open triangle* spDHFR with 250 μM NADPH and 250 μM folate

were largely blue-shifted. The peak intensities of sfDHFR and soDHFR were nearly identical to those of ecDHFR; however, svDHFR and sb6705DHFR clearly showed increased peak intensities whereas those of spDHFR and sb21DHFR were slightly decreased. These modified CD spectra do not necessarily indicate that the DHFR secondary structures differ from that of ecDHFR because even single amino acid substitutions in ecDHFR can induce large CD spectral changes (Ohmae et al. 1996, 2005). It has also been observed that DHFRs from other deep-sea bacteria, such as *P. profundum*, *M. yayanosii*, and *M. japonica*, show large changes in CD spectra (Murakami et al. 2010). Rather, these large CD spectral changes may be ascribable to the highly flexible structure of DHFR, which could perturb the CD excitation through movements of the secondary structure and/or altered contributions of aromatic side chains typically induced by exciton coupling of Trp47 and Trp74 in ecDHFR (Kuwajima et al. 1991, Ohmae et al. 2001). As shown in the inset of Fig. 3a, no significant difference in the CD spectra of spDHFR was induced by the addition of either cofactor (NADPH) or substrate (DHF), or by the addition of both cofactor and a substrate analog (folate). Similar results were also observed for the five other *Shewanella* DHFRs (data not shown). These results confirm that all *Shewanella* apo-DHFRs are in a folded (native) conformation even though they possess an additional 14 residues at the N-terminal region.

In addition to the CD spectra, the fluorescence spectra of the *Shewanella* DHFRs and ecDHFR were determined at 15°C and pH 8.0 at atmospheric pressure (Fig. 3b). The observed fluorescence spectra were mainly attributable to the content and environment of the tryptophan residues in the proteins. All *Shewanella* DHFRs, with only three tryptophan residues, showed reduced peak intensities compared with ecDHFR, which has five tryptophan residues. Small blue shifts of the peak wavelengths from 345 nm to 340–343 nm, with the exception of soDHFR, suggest that their tryptophan residues exist in more hydrophobic environments than those of ecDHFR. The spectrum of soDHFR showed the identical peak wavelength to that of ecDHFR, indicating that the tryptophan residues of soDHFR locate in more hydrophilic environments than those of the other *Shewanella* DHFRs. The observation that spDHFR showed a more significantly decreased peak intensity than the other *Shewanella* DHFRs even though the location of all three tryptophan residues is completely conserved (Fig. 1), suggests that the quantum yield of tryptophan residues and the surrounding polarity may be considerably different in spDHFR. Taken together, the spectral data of the *Shewanella* DHFRs presented here indicate that the enzymes have not undergone global structural changes, but rather, have retained localized structural alterations, as evidenced by changes in their CD and fluorescence spectra compared with those of ecDHFR. These observations are confirmed by the fact that these DHFRs retained enzymatic activities comparable to that of ecDHFR, as shown below.

Recently, we determined the crystal structure of the DHFR from the deep-sea bacterium *M. profunda* (PDB ID: 2zza), which has a 55% sequence identity with ecDHFR (Xu et al. 2003), in a ternary complex with $NADP^+$ and folate. The crystal structures of the apo form and DHFR–NADPH–MTX ternary complex of the identical protein have also been reported (Hay et al. 2009). These crystal structures are nearly identical to those of ecDHFR, which is consistent with our present spectroscopic observations that have revealed the *Shewanella* DHFRs have only marginal structural differences compared with ecDHFR.

Structural stability of *Shewanella* DHFRs

The structural stability of the *Shewanella* DHFRs was evaluated based on the CSM of the fluorescence spectra as a function of urea concentration at 15°C and pH 8.0 (Fig. 4). Although ecDHFR showed a cooperative transition as monitored by CD spectroscopy (Gekko et al. 1994; Ohmae et al. 1996), the transition curves of all *Shewanella* DHFRs were clearly shifted to lower urea concentrations with reduced cooperativity, suggesting that their structures were destabilized. The CSM values in the absence of urea varied considerably among the DHFRs from the different bacterial species (Fig. 4); however, they were nearly

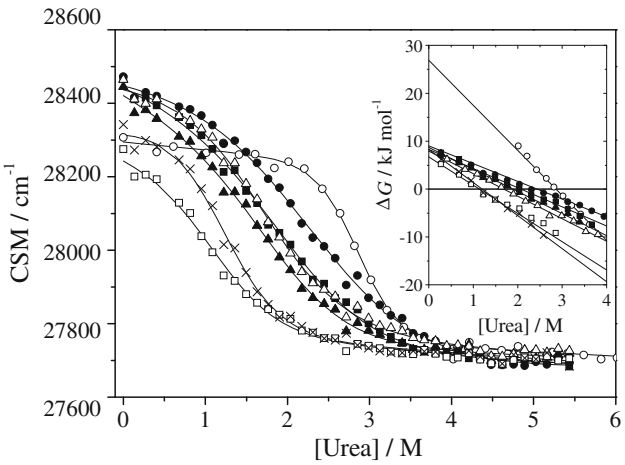

Fig. 4 Urea concentration dependence of the center of fluorescence spectral mass (CSM) of *E. coli* and *Shewanella* DHFRs at pH 8.0 and 15°C. The solvent used was 20 mM Tris-hydrochloride containing 0.1 mM EDTA, and 0.1 mM dithiothreitol. *Open circle* ecDHFR, *filled circle* svDHFR, *filled triangle* sb21DHFR, *open triangle* sb6705DHFR, *filled square* sfDHFR, *open square* soDHFR, and *cross symbol* spDHFR. The *solid lines* represent the theoretical fit to a two-state transition model with the parameter values shown in Table 2. The figure *inset* shows the dependence of the apparent free energy change of unfolding (ΔG_u) on the urea concentration

identical in the post-transition region (>4 M urea) for all DHFRs. These results indicate that the tryptophan residues of the DHFRs examined here, which are exposed to different environments in the native state, are fully exposed to the solvent in the unfolded state.

The transition curve of svDHFR obtained from the CSM values closely resembled the curve from the molar ellipticity data (data not shown), suggesting that the secondary and tertiary structures are disrupted by urea at the same time and the unfolding of *Shewanella* DHFRs follows the two-state unfolding model as found for ecDHFR (Touchette et al. 1986). As shown in Fig. 4, the transition curves of all *Shewanella* DHFRs showed a good theoretical fit to the two-state unfolding model as determined using Eqs. 2 and 3. The obtained thermodynamic parameters are listed in Table 2. The values of ΔG_u (26.9 ± 3.7 kJ/mol), m (9.4 ± 1.2 kJ mol^{-1} M^{-1}), and C_m (2.9 ± 0.5 M) for ecDHFR were consistent with our previous findings (25.4 ± 0.8 kJ/mol, 8.2 ± 0.3 kJ mol^{-1} M^{-1}, and 3.11 M, respectively) from the molar ellipticity changes under nearly identical conditions (Gekko et al. 1994). The ΔG_u values of *Shewanella* DHFRs ranged from 6.7 ± 1.0 kJ/mol (soDHFR) to 8.7 ± 1.2 kJ/mol (sb21DHFR), which were clearly smaller than that of ecDHFR. Reduced structural stability (3.2 kcal/mol) was also reported for another deep-sea DHFR from *M. profunda*, (Xu et al. 2003). However, the obtained ΔG_u values were not correlated with the environmental pressure from which the *Shewanella* species were isolated. Similar trends were also observed for the values of m and C_m. Taken together, these results suggest that the stability of the *Shewanella* DHFRs at atmospheric pressure does not largely vary between the strains and has not significantly changed during the evolutional adaptation of bacteria to the high-pressure environment of the deep sea.

Table 2 Thermodynamic parameters for urea denaturation of ecDHFR and six *Shewanella* DHFRs at 15°C and pH 8.0

DHFR	ΔG_u° (kJ mol^{-1})	m (kJ mol^{-1} M^{-1})	C_m (M)
ecDHFR	26.9 ± 3.7	9.4 ± 1.2	2.9 ± 0.5
svDHFR	8.0 ± 0.5	3.5 ± 0.2	2.3 ± 0.2
sb21DHFR	8.7 ± 1.2	4.6 ± 0.3	1.9 ± 0.3
sb6705DHFR	7.9 ± 0.9	4.7 ± 0.3	1.7 ± 0.2
sfDHFR	8.3 ± 0.9	4.0 ± 0.2	2.1 ± 0.2
soDHFR	6.7 ± 1.0	5.9 ± 0.3	1.1 ± 0.2
spDHFR	8.3 ± 1.4	6.9 ± 0.6	1.2 ± 0.2

The buffer used was 20 mM Tris-hydrochloride (pH 8.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol

Steady-state kinetics

To characterize the enzymatic function of the *Shewanella* DHFRs, their steady-state enzyme kinetics were examined at pH 7.0 and 25°C under atmospheric pressure. The kinetic parameters, K_m and k_{cat}, obtained for ecDHFR and the six *Shewanella* DHFRs are summarized in Table 3. The K_m values of soDHFR were 1.0 ± 0.1 and 1.4 ± 0.2 μM for DHF and NADPH, respectively, which were slightly smaller than the corresponding values (1.1 ± 0.1 and 1.9 ± 0.0 μM) of ecDHFR, suggesting that soDHFR has slightly increased affinities for the substrate and cofactor. However, the K_m values of the other *Shewanella* DHFRs ranged from 1.4 (sfDHFR) to 2.4 μM (sb6705DHFR) for DHF and from 2.6 (sb21DHFR) to 4.9 μM (spDHFR) for NADPH, indicating that they have a little weaker affinities for the substrate and cofactor than ecDHFR. In contrast, the k_{cat} values of the *Shewanella* DHFRs ranged from approximately 45 s^{-1} (soDHFR) to 100 s^{-1} (spDHFR), which were 2.3- to 5.2-fold larger than that of ecDHFR and resulted in 2.0- to 3.9-fold increases in k_{cat}/K_m. These results indicate that under these experimental conditions, the enzyme activities of the *Shewanella* DHFRs are higher than that of ecDHFR predominantly due to the enhanced turnover rates that serve to overcome the slightly decreased substrate and cofactor affinities. Three other DHFRs from

Table 3 Steady-state kinetic parameters of ecDHFR and six *Shewanella* DHFRs at 25°C and pH 7.0 under atmospheric pressure

DHFRs	DHF			NADPH		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
ecDHFR	1.1 ± 0.1	18.4 ± 0.2	16.7 ± 1.7	1.9 ± 0.0	19.2 ± 0.1	10.1 ± 0.1
svDHFR	1.9 ± 0.2	90.1 ± 2.9	47.4 ± 6.5	3.4 ± 0.3	86.8 ± 2.0	25.5 ± 2.8
sb21DHFR	1.7 ± 0.1	62.7 ± 1.2	36.9 ± 2.3	2.6 ± 0.2	68.3 ± 1.2	26.3 ± 2.1
sb6705DHFR	2.0 ± 0.2	79.1 ± 1.6	39.6 ± 4.0	2.9 ± 0.2	70.2 ± 1.1	24.2 ± 1.7
sfDHFR	1.4 ± 0.1	90.7 ± 1.6	64.8 ± 4.8	3.6 ± 0.2	93.3 ± 1.3	25.9 ± 1.5
soDHFR	1.0 ± 0.1	45.5 ± 0.8	45.5 ± 4.6	1.4 ± 0.2	45.1 ± 1.1	32.2 ± 4.7
spDHFR	2.4 ± 0.3	96.2 ± 2.9	40.1 ± 5.2	4.9 ± 0.3	98.7 ± 1.7	20.1 ± 1.3

The buffer used was 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA and 0.1 mM dithiothreitol

the deep-sea bacteria, *M. japonica*, *M. yayanosii*, and *P. profundum*, also exhibited larger K_m and k_{cat} values than ecDHFR (Murakami et al. 2010). Therefore, the increase of the K_m and k_{cat} values compared to ecDHFR seems common in many DHFRs. Further, the fact that no significant differences in the kinetic parameters were observed between *Shewanella* species suggests that in addition to structural stability, the enzyme function of these DHFRs at atmospheric pressure has not largely changed during the evolutional adaptation of bacteria to the high-pressure environment of the deep sea.

Pressure effects on enzyme activity

The effects of pressure on enzymatic activity were measured to investigate the potential high-pressure adaptation of DHFR function. Figure 5a shows the relative activity of ecDHFR and six *Shewanella* DHFRs as a function of the hydrostatic pressure over a range of 0–250 MPa at pH 7.0 and 25°C. It is clear that the enzyme activities of sfDHFR and spDHFR obtained from the ambient atmospheric species monotonously decreased with increasing pressure to 10–20% at 250 MPa. However, the DHFR from the ambient atmospheric species *S. oneidensis* showed pressure tolerance to approximately 50 MPa and retained 80% of its activity at 100 MPa, indicating that soDHFR also could function sufficiently in the deep sea. Although the biological meanings of this character are not clear, it is noteworthy that DHFR protein can obtain the pressure-tolerant activity independent of high-pressure environment. On the other hand, the DHFRs from two deep-sea *Shewanella* species, sb21DHFR and svDHFR, had clearly enhanced activities at moderate pressures of 25–150 MPa, suggesting that these DHFRs are adapted to the deep-sea environment. In contrast, the DHFR from the other deep-sea isolate, sb6705DHFR, showed only limited pressure tolerance up to 25 MPa and was monotonously inactivated at higher pressure at levels similar to sfDHFR and spDHFR, although these enzymes are still functional within the cells at their living pressure. Thus, DHFRs from ambient atmospheric species are not necessarily incompatible with deep-sea environments, and interestingly, DHFRs from deep-sea species are not necessarily tolerant to high pressure. These results also support the idea that DHFRs from deep-sea *Shewanella* species did not largely change functional characters during the evolutional adaptation to high-pressure environments after migrating into the deep sea.

The activation free energy (ΔG^*) calculated using Eq. 4 and the data from Fig. 5a were plotted against pressure (Fig. 5b). All *Shewanella* DHFRs showed a bimodal linear relationship between ΔG^* and pressure, whereas a linear relationship was observed for ecDHFR. The activation volumes, ΔV^*, of the enzyme reactions calculated from the slopes are listed in Table 4. Although the quantitative interpretation of these ΔV^* values is limited because the enzyme kinetics and structures of these DHFRs are unknown, these volumetric data clearly demonstrate a pressure-dependent function of the *Shewanella* DHFRs. All DHFRs show positive ΔV^* values in the pressure regions examined, except the low-pressure regions for svDHFR and sb21DHFR. The positive ΔV^* values indicate that the activated state has a larger partial volume than the reactant in the rate-limiting process of the enzyme reaction, leading to pressure-induced deactivation. Conversely, the negative ΔV^* values are indicative of pressure-induced activation. The DHFRs with a small positive ΔV^* value may also be pressure tolerant.

Interestingly, the calculated ΔV^* values for *Shewanella* DHFRs are considerably different in the low- and

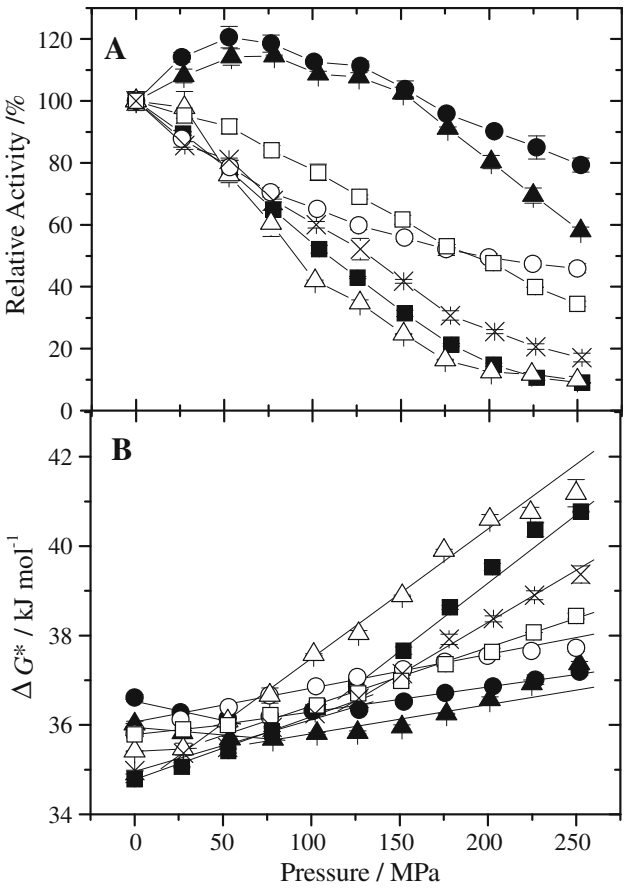

Fig. 5 Pressure dependence of relative enzyme activities (**a**) and activation free energies (**b**) of *E. coli* and *Shewanella* DHFRs at pH 7.0 and 25°C. The solvent used was 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 250 μM NADPH, and 250 μM dihydrofolate. *Open circle* ecDHFR data from Murakami et al. 2010, *filled circle* svDHFR, *filled triangle* sb21DHFR, *open triangle* sb6705DHFR, *filled square* sfDHFR, *open square* soDHFR, and *cross symbol* spDHFR. *Lines* in **b** were drawn using least-squares linear regression. The analyses were performed in duplicate and the values represent the mean ± error

Table 4 Activation volumes for enzymatic reactions of ecDHFR and six *Shewanella* DHFRs at 25°C and pH 7.0

DHFRs	ΔV^* (ml mol^{-1})[a]	
ecDHFR[b]	7.5 ± 0.2 (0.1–250 MPa)	
svDHFR	−8.6 ± 1.9 (0.1–50 MPa)	5.6 ± 0.1 (50–250 MPa)
sb21DHFR	−3.5 ± 0.6 (0.1–75 MPa)	6.5 ± 0.1 (75–250 MPa)
sb6705DHFR	2.0 ± 0.1 (0.1–25 MPa)	29.0 ± 0.3 (25–250 MPa)
sfDHFR	14.0 ± 0.1 (0.1–125 MPa)	30.5 ± 0.2 (125–250 MPa)
soDHFR	4.1 ± 1.4 (0.1–50 MPa)	13.1 ± 0.2 (50–250 MPa)
spDHFR	11.5 ± 0.2 (0.1–125 MPa)	23.3 ± 1.0 (125–250 MPa)

The buffer used was 20 mM Tris-hydrochloride (pH 7.0) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 250 μM NADPH, and 250 μM DHF

[a] The values in parentheses indicate the pressure range used for calculation

[b] Murakami et al. 2010

high-pressure regions. As the ΔV^* values are larger in the high-pressure regions, the rate-limiting step of the enzyme reaction likely shifts to a more pressure-sensitive step at pressures above a critical pressure between 25 and 125 MPa. Such modification of the enzyme reaction would be expected in the two deep-sea DHFRs, svDHFR and sb21DHFR, which have small positive ΔV^* values (5.6 and 6.5 ml/mol, respectively), because *S. benthica* strain DB21MT-2 has pressure tolerance to the deepest sea on the earth. It is likely that these DHFRs did not adapt to the deep-sea environment, but were able to retain function under high pressure by previously accumulated mutations. In contrast, the very large positive ΔV^* values of 30.5, 23.3, and 29.0 ml/mol observed for sfDHFR, spDHFR, and sb6705DHFR, respectively, suggest that more substantial conformational changes occur in these DHFRs under high pressure, which can also be inferred from their negligibly small enzyme activities.

Molecular adaptation of DHFR to high-pressure environments

As revealed in this study, there were no significant differences in the structure, stability, and function of DHFRs between *Shewanella* species living in deep-sea and ambient atmospheric-pressure environments. DHFRs from *Shewanella* species living at atmospheric pressures had more similar amino acid sequences to those from deep-sea *Shewanella* species than to ecDHFR (Fig. 1). The structural stability and steady-state kinetics of the *Shewanella* DHFRs were also similar to each other, but markedly differed from those of ecDHFR (Tables 2, 3). The finding that the DHFR from *S. oneidensis*, which typically inhabits ambient atmospheric-pressure environments, retained enzyme activity under moderate pressures (100 MPa) indicates that this DHFR also has the potential to adapt to deep-sea environments. In contrast, the DHFR from the deep-sea isolate *S. benthica* strain DB6705 was strongly inactivated by pressure as shown in Fig. 5a, and DHFRs from two other deep-sea bacteria, *M. japonica* (isolated from the Japan Trench at a depth of 6,356 m) and *P. profundum* (isolated from the Sulu Sea at a depth of 2,551 m), were monotonously inactivated by pressure similar to ecDHFR (Murakami et al. 2010). Although the pressure dependence of the enzyme activity may be influenced by the differences between in vitro and in vivo conditions such as temperature, pH, concentrations of salt, substrate, and cofactor, and the additional N-terminal residues presented in the DHFRs used, these results suggest that DHFRs from deep-sea *Shewanella* species need not undergo major structural and functional changes for adaptation within the chemical and physical constraints of their normal cellular milieu, and members whose enzymes could retain functional activity under high pressure migrated into the deep sea. This speculation is also supported by the fact that all five genera include piezophilic members, namely, *Shewanella*, *Moritella*, *Photobacterium*, *Psychromonas*, and *Colwellia*, also include nonpiezophilic species living at atmospheric pressure (DeLong et al. 1997; Nogi et al. 2002). Further comparative studies on other enzymes will provide useful information concerning the mechanism of molecular evolution and high-pressure adaptation of deep-sea bacteria.

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