Biosci. Biotechnol. Biochem., **69** (2), 364–373, 2005

Gene Cloning, Overproduction, and Characterization of Thermolabile Alkaline Phosphatase from a Psychrotrophic Bacterium

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Received September 24, 2004; Accepted November 1, 2004

The gene encoding alkaline phosphatase from the psychrotrophic bacterium *Shewanella* sp. SIB1 was cloned, sequenced, and overexpressed in *Escherichia coli*. The recombinant protein was purified and its enzymatic properties were compared with those of *E. coli* alkaline phosphatase (APase), which shows an amino acid sequence identity of 37%. The optimum temperature of SIB1 APase was 50 °C, lower than that of *E. coli* APase by 30 °C. The specific activity of SIB1 APase at 50 °C was 3.1 fold higher than that of *E. coli* APase at 80 °C. SIB1 APase lost activity with a half-life of 3.9 min at 70 °C, whereas *E. coli* APase lost activity with a half-life of >6 h even at 80 °C. Thus SIB1 APase is well adapted to low temperatures. Comparison of the amino acid sequences of SIB1 and *E. coli* APases suggests that decreases in electrostatic interactions and number of disulfide bonds are responsible for the cold-adaptation of SIB1 APase.

Key words: alkaline phosphatase; psychrotrophic bacterium; *Shewanella* sp.; cold-adaptation; thermal stability

Microorganisms that can grow at low temperatures, such as psychrophiles and psychrotrophs, usually produce cold-adapted enzymes. A cold-adapted enzyme has been specified by an increase in catalytic efficiency at low temperatures, a downward shift in the apparent optimum temperature for activity, and a reduction in stability at moderate temperatures, as compared to its mesophilic counterpart.[1–6] Because these properties are useful in various applications,[7,8] understanding of the structural determinants of the cold-adaptation is important not only to answer questions as to how these enzymes adapt to cold environments, but also to develop a technique to engineer cold-adapted enzymes in a rational manner. The molecular basis of cold-adaptation, however, remains largely unknown. One promising strategy to analyze the adaptation mechanisms of enzymes to cold environments is to compare the structure and function of a given enzyme from psychrophiles or psychrotrophs with those of a mesophilic counterpart and identify the amino acid substitutions responsible for these differences.

Alkaline phosphatase (APase) (EC 3.1.3.1) is a non-specific phosphomonoesterase that is widely distributed in various organisms from bacteria to humans.[9] Of these APases, *E. coli* APase has been the most extensively studied for structures and functions.[10–16] This enzyme is located in the periplasmic space as a homodimer and is involved in the acquisition of phosphate from esters when free inorganic phosphate is depleted. Each subunit is composed of 449 amino acid residues and contains two Zn^{2+} and one Mg^{2+} ions. The crystal structure of this enzyme has been determined, and the amino acid residues involved in the catalytic function, metal binding, and substrate binding have been identified. Hence, a system using *E. coli* APase as one of a pair of mesophilic and psychrophilic/psychrotrophic APases has potential for exploring the molecular mechanisms of cold-adaptation in proteins.

The genes encoding APases from the Antarctic psycrophile TAB5 (TAB5 APase),[17] the marine psycrophile *Vibrio* sp. G15-21 (*Vibrio* APase),[18] and the psycrophile *Shewanella* sp. (SCAPase)[19] were cloned and overexpressed in *E. coli*. These proteins show amino acid sequence identities of 32–34% to *E. coli* APase but these proteins may not be suitable as a partner of *E. coli* APase to analyze the cold-adaptation mechanism of enzymes for the following reasons. The

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Abbreviations: APase, alkaline phosphatase; *p*NPP, *p*-nitrophenyl phosphate; *p*NP, *p*-nitrophenol

recombinant protein of TAB5 APase shows typical features of a cold enzyme, but it is relatively small in size (353 amino acids) and apparently exists in a multimeric form[17] instead of a dimeric one. Likewise, the recombinant protein of *Vibrio* APase is very heat labile, but it apparently exists in a monomeric form.[18] The recombinant protein of SCAPase did not show typical features of a cold enzyme. Its optimal temperature for activity is 70 °C,[19] which is shifted upward by 30 °C compared to that of the native enzyme.[20]

Shewanella sp. strain SIB1 is a psychrotrophic bacterium that grows most rapidly at 20 °C.[21] This strain can grow even at 0 °C, but cannot grow at temperatures exceeding 30 °C. Ribonuclease HI[22] and FKBP22 with peptidyl prolyl *cis–trans* isomerase activity[23] from this strain have been shown to exhibit enzymatic properties characteristic of cold-adapted enzymes. In this study, we cloned the gene encoding APase from this strain (SIB1 APase), overexpressed it in *E. coli*, purified the recombinant protein, and compared its enzymatic activity and stability with those of *E. coli* APase. Based on these results, we discuss the cold-adaptation mechanism of SIB1 APase.

Materials and Methods

Cells and plasmids. The psychrotrophic bacterium *Shewanella* sp. SIB1 was isolated from Japanese oil reservoir water in our laboratory.[21] *E. coli* AC109 with the *phoA* mutation[24] was kindly donated by A. Nishimura. Plasmid pET-25b was from Novagen (Madison, WI, U.S.A.). *E. coli* AC109(DE3) was constructed by lysogenizing *E. coli* AC109 with λDE3, a recombinant phage carrying the cloned gene for T7 RNA polymerase under *lacUV5* control, using a λDE3 Lysogenization Kit (Novagen). λDE3 lysogens can be used to express the target genes cloned into pET vectors. The *E. coli* transformants were grown in NZCYM medium (Novagen) containing 50 mg/l ampicillin.

Materials. E. coli APase and *p*-nitrophenyl phosphate (*p*NPP) were obtained from Wako Pure Chemical (Osaka, Japan).

Cloning of the SIB1 APase gene. The genomic DNA of *Shewanella* sp. SIB1 was prepared as previously described[25] and used as a template to amplify part of the SIB1 APase gene by PCR. The sequences of the PCR primers were 5′-AGTGGTTATGTTACCGACTCGGCCGC-3′ for the 5′-primer and 5′-GTATGACCGCCCGTTGTCCAACC-3′ for the 3′-primer. PCR was performed with a GeneAmp PCR system 2400 (Perkin-Elmer Japan, Tokyo) using a KOD polymerase (Toyobo, Kyoto, Japan) according to the procedures recommended by the supplier. The amplified DNA fragment was used as a probe for Southern blotting and colony hybridization to clone the entire SIB1 APase gene. Southern blotting and colony hybridization were carried out using the AlkPhos Direct system (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the procedures recommended by the supplier. The DNA sequence was determined with a Prism 310 DNA sequencer (Perkin-Elmer Japan). Nucleotide and amino acid sequence analyses, including the localization of open reading frames, determination of molecular weight, and multiple alignment, were performed using DNASIS software (Hitachi Software, Tokyo). All DNA oligomers for PCR were synthesized by Hokkaido System Science (Sapporo, Japan).

Plasmid construction. Plasmid pET-SAP for overproduction of SIB1 APase was constructed by ligating the DNA fragment, which was amplified by PCR using the cloned DNA fragment containing the SIB1 APase gene as a template, into the *Xba*I–*Sal*I sites of pET-25b. The sequences of the PCR primers were 5′-CCTCTAGAAAGAAGGAGATATACATATGCTTATGGATGCCATCG-3′ for the 5′-primer and 5′-CAACCGTCGACGTTGATAGCGGAGG-3′ for the 3′-primer, where underlined bases show the positions of the *Xba*I (5′-primer) and *Sal*I (3′-primer) sites. In this plasmid, the initiation and termination codons of the SIB1 APase gene are located 70-bp downstream of the *Xba*I site and 40-bp upstream of the *Sal*I site respectively.

Overproduction and purification. E. coli AC109-(DE3) was transformed with pET-SAP, in which the transcription of the SIB1 APase gene is controlled by the T7 promoter, and grown at 37 °C. When the absorbance at 660 nm of the culture reached 0.5–1.0, 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture medium and cultivation was continued at 15 °C for 15–20 h. Cells were then harvested by centrifugation at 4000 *g* for 7 min. Recombinant proteins localized in the periplasmic space of the *E. coli* cells were released into the external medium by osmotic shock[26] and fractionated by ammonium sulfate precipitation. The protein precipitated between 20 and 40% ammonium sulfate was dissolved in 50 mM Tris–HCl (pH 8.0) containing 5 mM $MgCl_2$ and applied to a Hitrap Q column (Amersham Biosciences) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of 0–0.5 M NaCl. The fractions containing highly purified proteins were combined and used for further characterization. The purity of the protein was analyzed by SDS–PAGE,[27] followed by staining with Coomassie Brilliant Blue R250.

Protein concentration. The protein concentration was determined from UV absorption using an A_{280} value of 1.0 and 0.70 for 0.1% solutions of the mature forms of SIB1 APase (residues 28–455) and *E. coli* APase respectively. These values were calculated using $\varepsilon = 1576\ M^{-1}$ for Tyr and $5225\ M^{-1}$ for Trp at 280 nm.[28]

Biochemical characterizations. The molecular mass of the protein was estimated using a column (1.6 ×

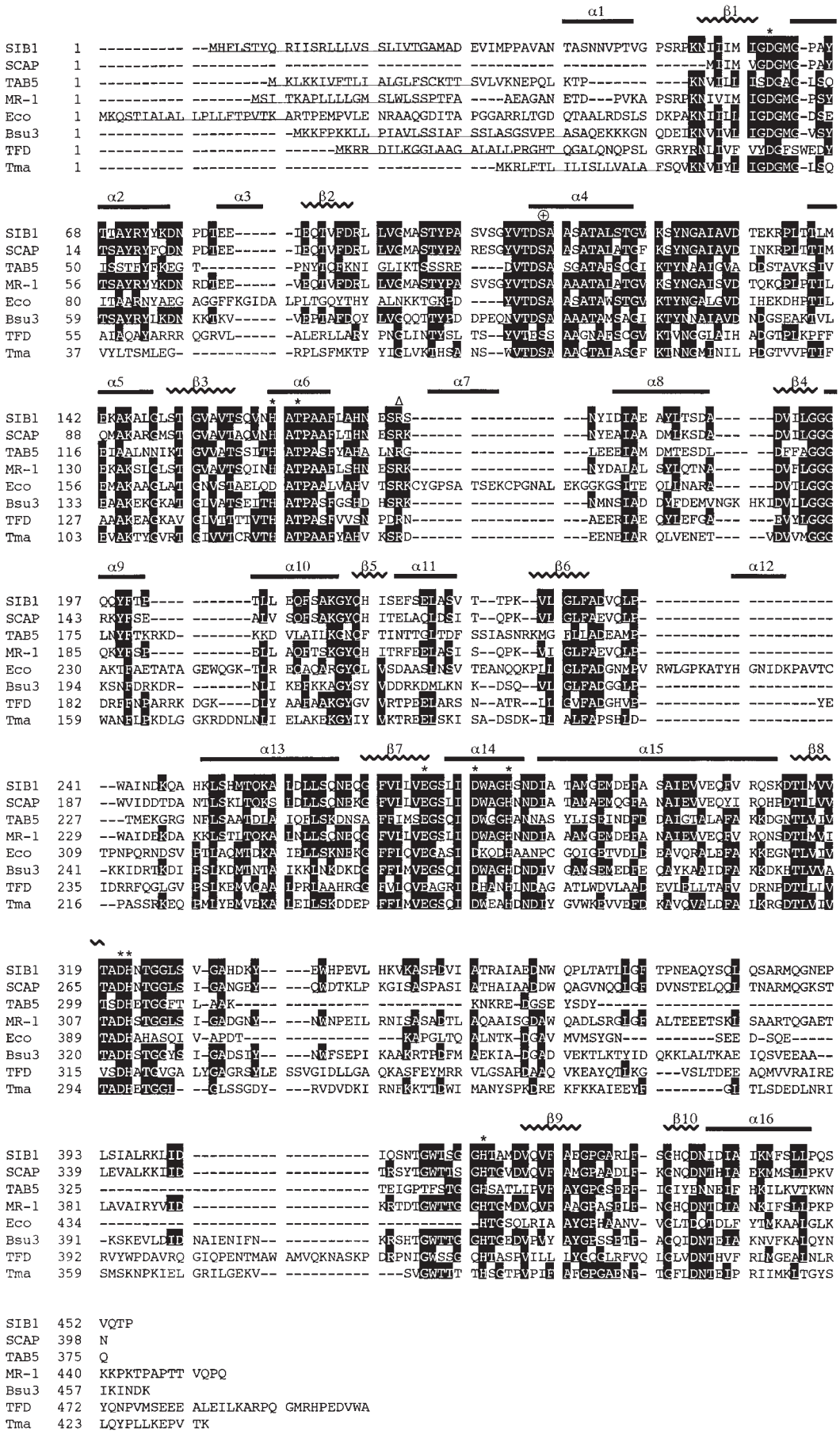
α1 β1
α2 α3 β2 α4
α5 β3 α6 α7 α8 β4
α9 α10 β5 α11 β6 α12
α13 β7 α14 α15 β8
β9 β10 α16
SIB1 1 ---------- --MHFLSTYQ RIISRLLLVS SLIVTGAMAD EVIMPPAVAN TASNNVPTVG PSRPKNIIIM IGDGMG-PAY
SCAP 1 ---------- ---------- ---------- ---------- ---------- ---------- ------MIIM VGDGMG-PAY
TAB5 1 ---------- ---------M KLKKIVFTLI ALGLFSCKTT SVLVKNEPQL KTP------- ----KNVIIL ISDQAG-LSQ
MR-1 1 ---------- -------MSI TKAPLLLLGM SLWLSSPTFA ----AEAGAN ETD---PVKA PSRPKNIVIM IGDGMG-PSY
Eco 1 MKQSTIALAL LPLLFTPVTK ARTPEMPVLE NRAAQGDITA PGGARRLTGD QTAALRDSLS DKPAKNIILL IGDGMG-DSE
Bsu3 1 ---------- ---------- -MKKFPKKLL PIAVLSSIAF SSLASGSVPE ASAQEKKKGN QDEIKNVIVL IGDGMG-VSY
TFD 1 ---------- ---------- ------MKRR DILKGGLAAG ALALLPRGHT QGALQNQPSL GRRYRNLIVF VYDGFSWEDV
Tma 1 ---------- ---------- ---------- ---------- ---MKRLFTL ILISLLVALA FSQVKNVIYL IGDGMG-LSQ
SIB1 68 TTAYRYMKDN PDTEE----- -IEQTVFDRL LVGMASTYPA SVSGYVTDSA ASATALSTGV KSYNGAIAVD TEKRPITTLM
SCAP 14 TSAYRYFQDN PDTEE----- -IEQTVFDRL LVGMASTYPA RESGYVTDSA ASATALATGF KSYNGAIAVD INKRPITTIM
TAB5 50 ISSTFYFKEG T--------- -PNYTQFKNI GLIKTSSSRE ----DVTDSA SGATAFSCGI KTYNAAIGVA IDSTAVKSIV
MR-1 56 TSAYRYMKDN RDTEE----- -VEQTVFDRL LVGMASTYPA SVSGYVTDSA AAATALATGV KSYNGAISVD TQKQPITTIL
Eco 80 ITARRNYAEG AGGFFKGIDA LPLTGQYTHY ALNKKTGKED ----YVTDSA ASATAWSTGV KTYNGALGVD IHEKDHETIL
Bsu3 59 TSAYRYLKDN KKTKV----- -VEPTAFDQY LVGQQTTYED DPEQNVTDSA AAATAMSAGI KTYNNAIAVD NDGSEAKTVL
TFD 55 AIAQAYARRR QGRVL----- -ALERLLARY PNGLINTYSL TS--YVTESS AAGNAFSCGV KTVNGGLAIH AEGTPIKPFF
Tma 37 VYLTSMLEG- ---------- -RPLSFMKTP YIGLVKTHSA NS--YVTDSA AAGTALASGF KTNNGMINIL PEGTVVPTIF
SIB1 142 EKAKALGLST GVAVTSQVNH ATPAAFLAHN ESRS------ ---------- ---NYIDIAE AYLTSDA--- ---DVILGGG
SCAP 88 QMAKARGMST GVAVTAQVNH ATPAAFLTHN ESRK------ ---------- ---NYEAIAA DMIKSDQ--- ---DVILGGG
TAB5 116 EIAALNNIKT GVVATSSITH ATPASFYAHA LNRG------ ---------- ---LEEEIAM DMTESDL----- --DFFAGGG
MR-1 130 EKAKSLGLST GVAVTSQINH ATPAAFLSHN ESRK------ ---------- ---NYDAIAL SYLQTNA--- ---DVFLGGG
Eco 156 EMAKAAGLAT GNVSTAELQD ATPAALVAHV TSRKCYGPSA TSEKCPGNAL EKGGKGSITE QLINARA--- ---DVILGGG
Bsu3 133 EAAKEKGKAT GIVATSEITH ATPASFGSHD HSRK------ ---------- ---NMNSIAD DYFDEMVNGK HKIDVILGGG
TFD 127 AAAKEAGKAV GIVTTTTVTH ATPASFVVSN PDRN------ ---------- ---AEERIAE QYLEFGA--- ---EVVLGGG
Tma 103 EVAKTYGVRT GIVVTCRVTH ATPAAFYAHV KSRD------ ---------- ---EENEIAR QLVENET--- --VDVVMGGG
SIB1 197 QQYFTE---- --------TLL EQFSAKGYQH ISEFSELASV T--TPK--VL GLFADVQLP- ---------- ----------
SCAP 143 RKYFSE---- --------AIV SQFSAKGYQH ITELAQLDSI T--QPK--VL GLFAEVQLP- ---------- ----------
TAB5 175 LNYFTKRKD- --------KKD VLAILKGNQF TINTTGLTDF SSIASNRKMG PLLADEAMP- ---------- ----------
MR-1 185 QKYFSE---- --------ELL AQFTSKGYQH ITRFEELASI S--QPK--VI GLFAEVQLP- ---------- ----------
Eco 230 AKTFAETATA GEWQGK-TLR EQAQARGYQL VSDAASLNSV TEANQQKPLL GLFADGNMPV RWLGPKATYH GNIDKPAVTC
Bsu3 194 KSNFDRKDR- --------NLI KEFKKAGYSY VDDRKDMLKN K--DSQ--VL GLFADGGLP- ---------- ----------
TFD 182 DRFFNFARRK DGK----DLY AAFAAKGYGV VRTPEELARS N--ATR--LL GVFADGHVE- ---------- --------YE
Tma 159 WANGLFKDLG GKRDDNLNLI ELAKEKGYIY VKTREELSKI SA-DSDK-IL ALFAPSHID- ---------- ----------
SIB1 241 --WAINDKQA HKLSEMTQKA LDLLSQNEQG FVLLVEGSII DWAGHSNDIA TANGEMDEFA SAIEVVEQSV RQSKDTIMVV
SCAP 187 --WVIDDTDA NTLSKITQKS LDLLSQNEKG FVLLVEGSII DWAGHNNDIA TANAEMQQFA NAIEVVEQYI RQHPDTLLVV
TAB5 227 ---TMEKGRG NFLSAATDHA IQELSKDNSA FFIMSEGSQI DWGGHANNAS YLISEINDFD DAIGTALAFA KKDGNTLVIV
MR-1 229 --WAIDEKDA KKLSTLTQKA LNLLSQNEQG FVLLVEGSII DWAGHNNDIA AANGEMDEFA NAIEVVEQSV RQNSDTIMVI
Eco 309 TPNPQRNDSV ETLAQMTDKA IELLSKNEKG FFLQVEGASI DKQDHAANPC GQIGETVDLD EAVQRALEFA KKEGNTLVIV
Bsu3 241 -KKIDRIKDI ESLKDMTNTA IKKLNKDKDG FFLMVEGSQI DWAGHDNDIV GAMSEMEDFE QAYKAAIDFA KKDKHTLVVA
TFD 235 IDRRFQGLGV ESLKEMVQAA LPRIAAHRGG FVLQVEAGRI DHANHLNDAG ATLWDVLAAD EVLDLLLTAE VDRNPDTLLV
Tma 216 --PASSRKEQ EMLYPNVEKA LEILSKDDEP FFLMVEGSQI DWEAHDNDIY GVWKEVVEFD KAVQVALDFA LKRGDTLVIV
SIB1 319 TADFNTGGLS V-GAHDKY-- ---EWHPEVL HKVKASPEVI ATRAIAEDNW QPLTATLLGF TPNEAQYSQL QSARMQGNEP
SCAP 265 TADFNTGGLS I-GANGEY-- ---QWDTKLP KGISASPASI ATHAIAADDW QAGVNQQIGF DVNSTELQQL TNARMQGKST
TAB5 299 TSDFETGGFT L-AAK----- ---------- ---------- KNKRE-DGSE YSDY------ ---------- ----------
MR-1 307 TADFSTGGLS I-GADGNY-- ---NWNPEIL RNISASAETL AQAAISGDAW QADLSRGIGF ALTEEETSKL SAARTQGAET
Eco 389 TADFAHASQI V-APDT--- ---------- ---KAPGLTQ ALNTK-DGAV MVMSYGN--- -------SEE D-SQE-----
Bsu3 320 TADFSTGGYS I-GADSIY-- ---NWFSEPI KAAKRTPDFM AEKIA-DGAD VEKTLKTYID QKKLALTKAE IQSVEEAA--
TFD 315 VSDFATGVGA LYGAGRSYLE SSVGIDLLGA QKASFEYMRR VLGSAFDAAQ VKEAYQTLKG ---VSLTDEE AQMVVRAIRE
Tma 294 TADFETGGL- --GLSSGDY- ---RVDVDKI RNFKKTTDWI MANYSPKDRE KFKKAIEEYE --------GL TLSDEDLNRI
SIB1 393 LSIALRKLID ---------- ---------- IQSNTGWISG GHTAMDVQVF AEGPGARLF- SGHQDNIDIA IKMFSLIPQS
SCAP 339 LEVALKKLID ---------- ---------- TRSYTGWTIS GHTGVDVQVF AMGPAADLF- KGNQDNTEIA EKMMSLIPKV
TAB5 325 ---------- ---------- ---------- TEIGPTFSTG GHSATLIPVF AYGPGSEEF- IGIYENNEIF EKILKVTKWN
MR-1 381 LAVAIRYVID ---------- ---------- KRTDTGWTIG GHTGMDVQVF AAGPASELF- NGHQDNTDIA NKIFSLIPKP
Eco 434 ---------- ---------- ---------- ---------- -HTGSQLRIA AYGPHAANV- VGLTDQTDLF YTMKAALGLK
Bsu3 391 -KSKEVLDID NAIENIFN-- ---------- KRSHTGWTTG GHTGPDVPVY AYGPSSELF- ACQIDNTEIA KNVFKAIQYN
TFD 392 RVYWPDAVRQ GIQPENTMAW AMVQKNASKP DRPNIGWSSG QHTASFVILL LYGCGLRFVQ LGLVDNTHVF RIMGEALNLR
Tma 359 SMSKNPKIEL GRILGEKV-- ---------- ---SVGWTIT THSGTPVPIE AFGPGAENF- TGFIDNTEIP RIIMKITGYS
SIB1 452 VQTP
SCAP 398 N
TAB5 375 Q
MR-1 440 KKPKTPAPTT VQPQ
Bsu3 457 IKINDK
TFD 472 YQNPVMSEEE ALEILKARPQ GMRHPEDVWA
Tma 423 LQYPLLKEPV TK

Fig. 1. Alignment of the Bacterial APase Sequences.

The most conserved amino acid residue at each position is highlighted in black. Gaps are denoted by dashes. The serine and arginine residues forming the catalytic site (phosphorylation site) and phosphate binding site of *E. coli* APase are denoted by ⊕ and △ respectively. The amino acid residues forming the three metal binding sites of *E. coli* APase are denoted by asterisks (*). Numbers along the sequences indicate the positions of the amino acid residues which start from the initiator methionine for each protein. The ranges of the α-helices and β-strands of *E. coli* APase are indicated above the sequences. The accession numbers of these sequences are AB073982 for *Shewanella* sp. APase (SCAP), Y18016 for Antarctic strain TAB5 APase (TAB5), AE015527 for *Shewanella* sp. MR-1 APase (MR-1), M29670 for *E. coli* APase (Eco), D88802 for *B. subtilis* APase III (Bsu3), AF079878 for *Thermus* sp. FD3041 APase (TFD), and AE001701 for *T. maritima* APase (Tma). Underlined sequences represent the N-terminal signal sequences, which were either experimentally determined or are estimated using the SignalP V2.0 World Web Server. The signal sequences have been determined for SIB1 APase (this study), TAB5 APase,[17] *E. coli* APase,[31] and *B. subtilis* APase III.[32]

60 cm) of Superdex 200 (Amersham) equilibrated with 20 mM Tris–HCl (pH 8.0) containing 150 mM NaCl. The standard proteins contained in a marker kit (Amersham) were aldolase (158 kDa), bovine serum albumin (67 KDa), ovalbumin (43 KDa), chymotrypsinogen A (25 KDa), and RNase A (14 KDa).

The far-UV (200–260 nm) and near-UV (250–320 nm) CD spectra were measured on a J-725 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) at 25 °C in 100 mM Tris–HCl (pH 8.0). The protein concentration was 0.18–0.27 mg/ml and 1.2–1.6 mg/ml, and the optical path length of the cell was 2 mm and 10 mm, for the measurement of far-UV and near-UV CD spectra respectively. The mean residue ellipticity (θ, deg cm^2 dmol^{-1}) was calculated using an average amino acid molecular weight of 110.

The NH_2-terminal amino acid sequence was determined using a Procise 491 automated protein sequencer (Perkin-Elmer Japan).

Enzymatic activity. The APase activity was determined using *p*-nitrophenyl phosphate (*p*NPP) as a substrate. The enzymatic reaction was carried out for 10 min in 50 mM Gly–NaOH (pH 10.5) containing 20 mM $MgCl_2$, 5 mM $ZnCl_2$, 100 mM KCl, and 5 mM *p*NPP at 20 °C for SIB1 APase, or in 0.1 M CAPS–NaOH (pH 10.0) containing 0.4 M NaCl, 10 mM $MgCl_2$, and 5 mM *p*NPP at 60 °C for *E. coli* APase, unless otherwise described. The buffer for *E. coli* APase was identical to that reported previously.[29] The reaction was initiated by the addition of the enzyme and terminated by the addition of 0.2 M NaOH. The amount of *p*-nitrophenol (*p*NP) produced by the reaction was determined from the molar absorption coefficient value of 16,200 M^{-1}cm^{-1} at 410 nm. One unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol *p*NP per min. The specific activity was defined as the enzymatic activity per milligram of protein. Dependencies of the SIB1 APase activity on pH, divalent cation, and salt were analyzed in a condition in which other conditions are optimal for this activity.

Stability against heat inactivation. Stability against heat inactivation was analyzed by incubating the enzyme (0.05–0.1 mg/ml) in 50 mM Tris–HCl (pH 8.0) containing 5 mM $MgCl_2$ at various temperatures. At appropriate intervals, aliquots were withdrawn and the enzymatic activity was determined at 20 °C for SIB1 APase and 60 °C for *E. coli* APase.

Results

Cloning

When the amino acid sequences of various bacterial APases are compared with one another, the sequences around the active-site residues are well conserved.[17–19] Previously, we reported that RNase HI[22] and FKBP22[23] from *Shewanella* sp. SIB1 show the highest amino acid sequence identities, of 78% and 85% respectively, to those from *Shewanella oneidensis* MR-1. These results suggest that SIB1 APase shows a high amino acid sequence identity to MR-1 APase as well. Therefore, we constructed the PCR primers based on the sequences encoding Ser^{92}–Ala^{100} and Gly^{396}–Thr^{403} of MR-1 APase (accession no. AE015527), and used them to amplify part of the SIB1 APase gene. These regions contain the phosphorylation site and one of the metal binding sites (Ser^{124} and His^{434} for *E. coli* APase respectively). A PCR using the genomic DNA of *Shewanella* sp. SIB1 as a template produced only a 936-bp DNA fragment, which encodes part of the SIB1 APase sequence. Southern blotting and colony hybridization using this DNA fragment as a probe indicated that a 2.5-kbp *Kpn*I fragment of the SIB1 genome contained the entire SIB1 APase gene (data not shown). Determination of the nucleotide sequence of the SIB1 APase gene indicated that SIB1 APase is composed of 455 amino acid residues with a calculated molecular weight of 49,104 and an isoelectric point (p*I*) of 4.9. A potential Shine Dalgarno (SD) sequence (AGGA) is located seven nucleotides upstream of the initiation codon for translation, and a possible promoter sequence is located upstream of this SD sequence. The nucleotide sequence of the SIB1 APase gene is deposited in DDBJ under accession no. AB190867.

Amino acid sequence

The amino acid sequence of SIB1 APase is compared with those of various APases from psychrophiles, mesophiles, and thermophiles in Fig. 1. SIB1 APase

shows the highest amino acid sequence identity, of 66%, to MR-1 APase.[30] It shows amino acid sequence identities of 62% to SCAPase,[19] 33% to TAB5 APase,[17] 37% to *E. coli* APase,[31] 42% to *B. subtilis* APase III,[32] 35% to *Thermus* sp. FD3041 APase (unpublished, accession no. AF079878), and 38% to *Thermotoga maritima* APase.[33] The residues forming the active site of *E. coli* APase[10] are fully conserved in the SIB1 APase sequence, except for Asp^{175}. They are Asp^{61}, Ser^{110}, Thr^{163}, Arg^{174}, Glu^{274}, Asp^{279}, His^{283}, Asp^{321}, His^{322}, and His^{414}, which correspond to Asp^{73}, Ser^{124}, Thr^{177}, Arg^{188}, Glu^{344}, Asp^{349}, His^{353}, Asp^{391}, His^{392}, and His^{434} of *E. coli* APase respectively. Of these, the serine and arginine residues form the catalytic site (phosphorylation site) and phosphate binding site respectively. Other residues form the three metal binding sites. These results suggest that SIB1 APase structurally and functionally resembles *E. coli* APase.

Asp^{175} of *E. coli* APase, which is one of the constituents of the metal binding sites, is replaced by His (His^{161}) in the SIB1 APase sequence. This residue is conserved as His, instead of Asp, in mammalian APases,[34] as well as the other APases listed in Fig. 1. It has been reported that Asp^{175} of *E. coli* APase is responsible for its low specific activity as compared to those of mammalian APases,[34] and the substitution of this residue by His results in a great increase in enzymatic activity due to the increased binding affinity of Mg^{2+}.[35] Another significant difference between the SIB1 and *E. coli* APase sequences is a deletion or an insertion of the sequence. The α7 helix and its flanking region (Cys^{190}–Gly^{208}) and a long loop containing α12 helix (Val^{288}–Cys^{308}) of *E. coli* APase are deleted in SIB1 APase. On the other hand, a long loop consisting of 37 residues is inserted in SIB1 APase at a position corresponding to that between the β8 and β9 strands of *E. coli* APase, but the other APase sequences have similar deletions and insertions when compared to the *E. coli* APase sequence. Hence, these differences are attributable to the unique *E. coli* APase sequence.

Biochemical properties of the recombinant protein

We used *E. coli* AC109(DE3) as a host for overproduction of SIB1 APase to avoid contamination of host-derived APase. The recombinant protein was purified from the periplasmic space of the *E. coli* cells to give a single band on SDS–PAGE (Fig. 2). The amount of purified protein obtained from 1-liter culture was roughly 1.0 mg. The N-terminal amino acid sequence of the purified protein was found to be Asp–Glu–Val–Ile–Met–Pro–Pro–Ala, indicating that the N-terminal signal peptide (Met^1–Ala^{27}) was removed when the recombinant protein was secreted across the cytoplasmic membrane into the periplasmic space of the *E. coli* cells.

The molecular mass of the purified protein was estimated to be 44 kDa by SDS–PAGE and 100 kDa by gel filtration column chromatography (data not shown), suggesting that SIB1 APase exists as a dimer, like

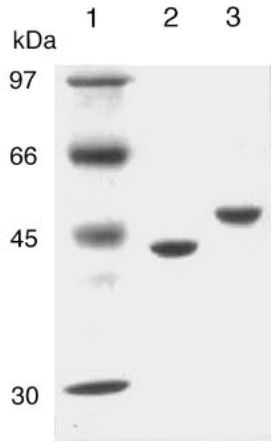

Fig. 2. SDS–PAGE of Purified SIB1 APase.

The samples were subjected to 12% SDS–PAGE and stained with Coomassie Brilliant Blue. Lane 1, a low molecular weight marker kit (Amersham Biosciences); lane 2, SIB1 APase; lane 3, *E. coli* APase. Numbers along the gel represent the molecular masses of individual standard proteins.

E. coli APase. The molecular mass of the protein estimated by SDS–PAGE was slightly smaller than but comparable to that (46,100) calculated from the amino acid sequence (Asp^{28}–Pro^{455}). The far- and near-UV CD spectra of SIB1 APase were similar to those of *E. coli* APase, except for the height of a broad peak around 280 nm (Fig. 3). These results suggest that local conformations around the aromatic residues of SIB1 APase are slightly different from those of *E. coli* APase, but that its overall main chain fold is similar to that of *E. coli* APase.

Enzymatic activity

The optimum condition for enzymatic activity of SIB1 APase, except for the optimum temperature, was determined at 20 °C using *p*NPP as a substrate. The pH dependence of activity was analyzed using 50 mM Tris–HCl (pH 7.6–8.8) and 50 mM Gly–NaOH (pH 7.7–12.4). SIB1 APase exhibited the highest activity (a specific activity of 950 units/mg) at pH 10.5 (Fig. 4). Analysis of the dependence of activity on the concentration of Gly–NaOH (pH 10.5) indicated that it exhibited the highest activity at 50 mM (data not shown). It exhibited roughly 30% of maximal activity at 20 or 200 mM. Analyses of the dependence of activity on the NaCl and KCl concentrations indicated that the enzyme exhibited the highest activity in the presence of 100 mM KCl (data not shown). The enzyme exhibited roughly 50% of maximal activity in the absence of salt or the presence of 200 mM KCl. NaCl was rather inhibitory and enzymatic activity was reduced by 30% in the presence of 200 mM NaCl as compared to that determined in the absence of salt. Analyses for the dependence of activity on metal ions indicated that SIB1 APase activity was greatly enhanced by the simultaneous addition of Mg^{2+} and Zn^{2+} (Fig. 5). The enzyme gave a maximum specific activity of 950 u/mg in the presence of 20 mM $MgCl_2$

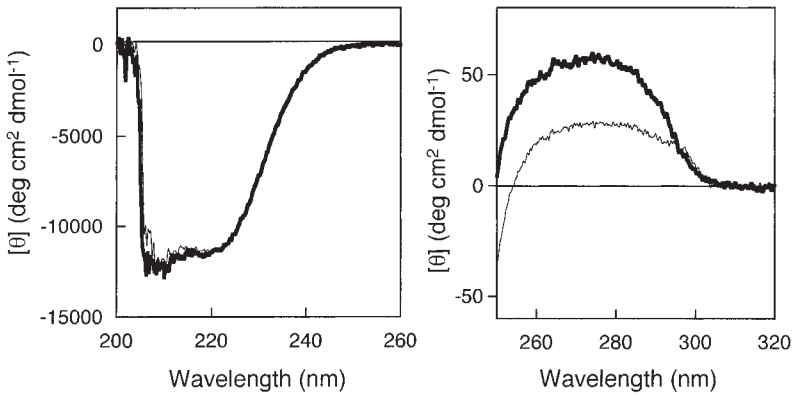

Fig. 3. CD Spectra.
The far-UV (left) and near-UV (right) CD spectra of SIB1 APase (thick line) and *E. coli* APase (thin line) are shown. These spectra were measured as described in "Materials and Methods".

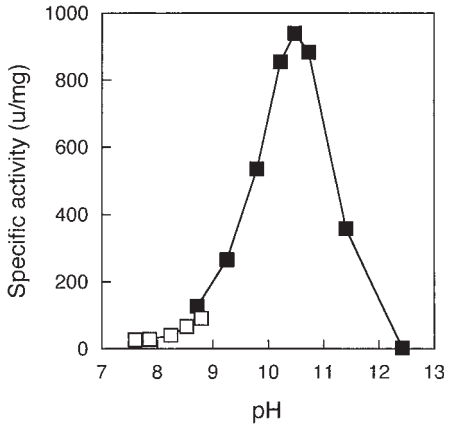

Fig. 4. pH Dependence of the Activity of SIB1 APase.
The enzymatic activity was determined at 20 °C in 50 mM Tris–HCl (pH 7.6–8.8) (□) or 50 mM Gly–NaOH (pH 7.7–12.4) (■) containing 20 mM $MgCl_2$, 5 mM $ZnCl_2$, 100 mM KCl, and 5 mM *p*NPP, as described in "Materials and Methods".

and 5 mM $ZnCl_2$. It exhibited roughly 5% of maximal activity in the absence of these metal ions (a specific activity of 50 u/mg). This activity increased only by at most twice when these metal ions were individually added to the reaction mixture. Other metal ions, such as Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Cu^{2+}, Ca^{2+}, and Sr^{2+}, did not significantly increase SIB1 APase activity.

The temperature dependence of SIB1 APase activity was analyzed by measuring the activity in 50 mM Gly–NaOH (pH 10.5) containing 100 mM KCl, 20 mM $MgCl_2$, 5 mM $ZnCl_2$, and 5 mM *p*NPP at various temperatures and comparing it with that of *E. coli* APase activity. The amounts of *p*NP accumulated on 10 min incubation with the enzyme were compared. SIB1 APase most effectively hydrolyzed the substrate at 50 °C, whereas *E. coli* APase did so at 80 °C (Fig. 6). Thus the optimum temperature for SIB1 APase activity was apparently shifted downward by roughly 30 °C as compared to that for *E. coli* APase activity. The specific activities of SIB1 and *E. coli* APases at various temperatures are summarized in Table 1. The specific activity

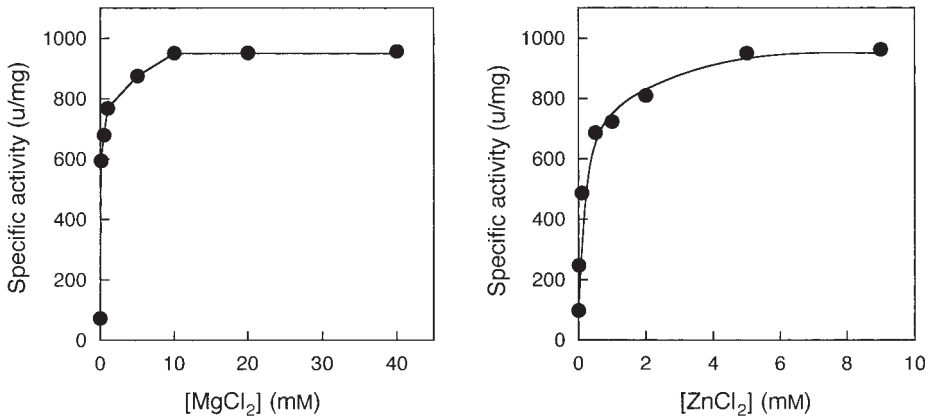

Fig. 5. Dependence of SIB1 APase Activity on Divalent Metal Ion Concentrations.
The enzymatic activities of SIB1 APase were determined at 20 °C in 50 mM Gly–NaOH (pH 10.5) containing 5 mM $ZnCl_2$, 100 mM KCl, 5 mM *p*NPP, and various concentrations of $MgCl_2$ (left), or the same buffer containing 20 mM $MgCl_2$, 100 mM KCl, 5 mM *p*NPP, and various concentrations of $ZnCl_2$ (right), as described in "Materials and Methods".

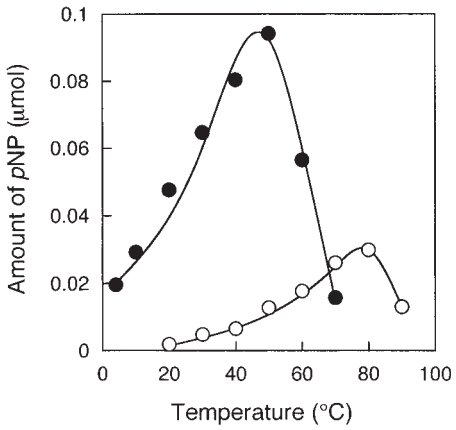

Fig. 6. Temperature Dependence of the Activities of SIB1 and *E. coli* APases.

The enzymatic reaction was carried out at the temperatures indicated in 1 ml of the reaction mixture for 10 min, and the amount of *p*NP accumulated upon reaction was plotted against the temperature. The reaction mixture contained 50 mM Gly–NaOH (pH 10.5), 20 mM $MgCl_2$, 5 mM $ZnCl_2$, 100 mM KCl, 5 mM *p*NPP, and 5 ng of SIB1 APase (●), or 0.1 M CAPS–NaOH (pH 10.0), 0.4 M NaCl, 10 mM $MgCl_2$, 5 mM *p*NPP, and 5 ng of *E. coli* APase (○).

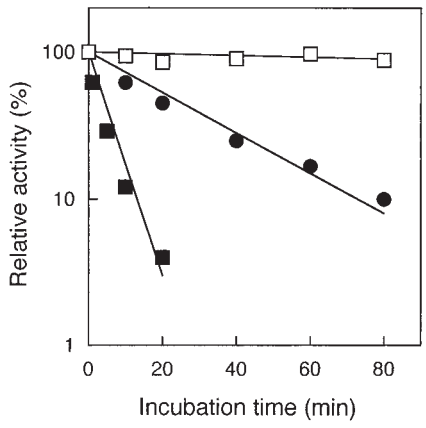

Fig. 7. Thermal Stability of SIB1 and *E. coli* APases.

SIB1 APase was incubated at 60 °C (●) and 70 °C (■), and *E. coli* APase was incubated at 80 °C (□) in 50 mM Tris–HCl (pH 8.0) containing 5 mM $MgCl_2$. At appropriate intervals, an aliquot was withdrawn and determined for residual activity at 40 °C for SIB1 APase and 60 °C for *E. coli* APase, as described in "Materials and Methods".

Table 1. Specific Activities at Various Temperatures

Enzyme	Temperature (°C)	Specific activity (units/mg)
SIB1 APase	4	392
	20	952
	50	1880
E. coli APase	20	37.7
	50	257
	80	600

The specific activities of the enzymes were determined using *p*NPP as a substrate, as described in "Materials and Methods". The substrate concentration was 5 mM. The reaction was carried out for 10 min at the temperatures indicated. Each experiment was carried out in duplicate and the average value is shown. Errors from the average values were within 6%.

of SIB1 APase at 50 °C was 3.1 fold higher than that of *E. coli* APase at 80 °C, suggesting that the maximum hydrolysis rate of SIB1 APase is higher than that of *E. coli* APase. In addition, the specific activities of SIB1 APase were 7.3 and 25.3 fold higher than those of *E. coli* APase at 50 and 20 °C respectively, indicating that SIB1 APase adapts well to low temperatures as compared to *E. coli* APase. The specific activities of SIB1 and *E. coli* APases were not determined at temperatures higher than 55 and 85 °C respectively, because the amounts of *p*NP did not increase in proportion to increases in reaction time for 10 min at these temperatures. These enzymes probably are not fully stable for 10 min at these temperatures.

Stability

The stability of SIB1 APase against thermal inactivation is compared with that of *E. coli* APase in Fig. 7. *E. coli* APase was very stable and lost activity with a half-life of >6 h at 80 °C, whereas SIB1 APase lost ctivity with half-lives of 22 min at 60 °C and 3.9 min at 70 °C. Thus SIB1 APase was considerably less stable than *E. coli* APase.

Discussion

Cold-adaptation mechanism

In this study, the activity and stability of recombinant SIB1 APase were compared with those of *E. coli* APase, which represents mesophilic APases. Because of its decreased stability, downward shift of the optimum temperature for activity, and increased activity at low temperatures, SIB1 APase can be defined as a cold-adapted enzyme. Like other cold-adapted enzymes,[1–3,6] SIB1 APase might acquire a conformational flexibility at low temperatures at a cost in protein stability. An increase in the conformational flexibility of enzymes results in a better accommodation of the substrate and enhancement of the fast conformational changes required for catalysis. To understand the structural basis for the cold-adaptation of enzymes, the crystal structures of several cold-adapted enzymes have been determined and compared with those of mersophilic and thermophilic counterparts.[4] These studies indicate that all structural factors responsible for protein stabilization are attenuated in both strength and number in cold-adapted enzymes.

When the amino acid sequence of SIB1 APase (mature form) is compared with that of *E. coli* APase, the content of the charged residues (Lys+Arg+Glu+Asp) of SIB1 APase (17.5%) is significantly lower than that of *E. coli* APase (20.7%). Computational analysis has suggested that the decrease in content of charged residues causes protein destabilization.[36] Charged residues are usually located at the surface of

protein molecules and stabilize proteins by electrostatic interactions. Therefore, a decrease in electrostatic interactions may be one of the factors responsible for the cold-adaptation of SIB1 APase. In addition, SIB1 APase does not contain cysteine residues, while *E. coli* APase contains four of them. Because these cysteine residues form two disulfide bonds[10] and disulfide bonds usually stabilize proteins,[37] a decrease in the number of disulfide bonds might be another factor responsible for cold-adaptation of SIB1 APase. It has been reported that protein stability decreases as the content of proline residues decreases.[38] Introduction of proline residues into loop regions has been reported to increase protein stability by decreasing the entropy of the unfolded state of proteins.[39] However, the content of proline residues of SIB1 APase (4.9%) is comparable to that of *E. coli* APase (4.7%).

A comparison of the amino acid sequence of SIB1 APase with those of other APases, shown in Fig. 1, indicates that decreases in content of charged residues and number of disulfide bonds are not characteristic features of cold-adapted APases. The content of charged residues of TAB5 APase (20.7%) is comparable to that of *E. coli* APase, and mesophilic MR-1 APase and *B. subtilis* APase III do not contain cysteine residues. However, the content of proline residues of TAB5 APase (2.3%) is considerably lower than that of SIB1 or *E. coli* APase. These results suggest that each protein adopts its own strategy for cold-adaptation. It is difficult to construct a three-dimensional model for the SIB1 APase structure, because *E. coli* APase is the only APase for which the crystal structure is available,[10] and the SIB1 APase sequence has large deletions and insertions at several positions as compared to the *E. coli* APase sequence (Fig. 1). Therefore, it is necessary to determine the crystal structure of SIB1 APase to understand its cold-adaptation mechanism. Availability of the overproduction system for SIB1 APase will facilitate crystallographic studies of it.

It has been suggested that deletion of the α7 heix is responsible for the cold-adaptation of SCAPase.[19] However, this helix is deleted not only in psychrophilic APases but also in mesophilic and thermophilic ones (Fig. 1). Likewise, a large peptide containing the α12 helix of *E. coli* APase is deleted and a large peptide is inserted at the position corresponding to that between the β8 and β9 strands of *E. coli* APase not only in psychrophilic APases but also in mesophilic and thermophilic ones (Fig. 1). These results suggest that these deletions and insertions are not responsible for the cold-adaptation of SIB1 APase.

According to the crystal structure of *E. coli* APase, two monomers interact tightly to form a homodimer.[10] The amino acid residues, which are located mainly in the N-terminal region (α1–α3 helices and β2 strand) and the C-terminal region (β9 and β10 strands and α16 helix), form the interface of a homodimer. When the amino acid sequence of SIB1 APase is compared with that of *E. coli* APase, these interface residues are more variable than those, which are located in the core region (the α4, α6, α13, and α14 helices and the β1, β3, β4, β6, β7, β8 strands) (Fig. 1). This high frequency of amino acid substitutions in the interface may account for the low stability and high flexibility of SIB1 APase as compared to *E. coli* APase, because homodimeric proteins are expected to be destabilized by reducing their monomer-monomer interactions. However, the area of the monomer-monomer interface is too large (20% of the surface area) to identify the amino acid substitutions responsible for destabilization of SIB1 APase by simply comparing the amino acid sequences.

Possible application

APases have been used to remove 5′-terminal phosphate groups from DNA fragments. *E. coli* APase (BAP) has been most widely used for this purpose. However APases should be completely inactivated or eliminated to ligate or phosphorylate the resultant DNA fragments. Because BAP is highly thermostable, phenol/chloroform treatment and ethanol precipitation are necessary to inactivate it. Recombinant SIB1 APase is therefore expected to be more useful than BAP, because a simple heat treatment inactivates the enzyme and might allow subsequent ligation or phosphorylation of DNA fragments successively. Recombinant SIB1 APase has been shown to remove 5′-terminal phosphate groups effectively not only from DNA fragments with 5′-overhangs but also from those with 3′-overhangs and blunt ends (H. Nagahora, personal communication). Note that shrimp APase has often been used as a heat labile APase. SIB1 APase is superior to this APase, however, because of ease of production and purification.

The gene encoding APase (SCAPase) has been cloned from the same genus *Shewanella* and the recombinant protein has been overproduced in *E. coli* and characterized.[19] However, comparison of the amino acid sequence of SCAPase with those of SIB1 and MR-1 APases indicates that the SCAPase sequence lacks the N-terminal 40–50 residues, which contain the signal sequence required for secretion and the α1 helix (Fig. 1). Because the genus *Shewanella* is a Gram-negative bacterium like *E. coli* and APases from Gram-negative bacteria are secreted into the periplasmic space of the cells, SCAPase should have the N-terminal signal sequence. The sequence reported for SCAPase probably represents an incomplete one which lacks the N-terminal 40–50 residues. In addition, recombinant SCAPase is apparently more stable than recombinant SIB1 APase, because its optimum temperature for activity has been reported to be 70 °C. However, the optimum temperature of natural SCAPase for activity has been reported to be 40 °C. Because recombinant SCAPase was overproduced in the *E. coli* strain without the *phoA* mutation and the yield of the purified protein was extremely low (1.7 mg from 30 g of the cells), its activity may represent that of *E. coli* APase derived from the host strain.

Alternatively, deletion of the N-terminal sequence containing the α1 helix might cause an upward shift of the optimum temperature. TAB5 APase, another psychrophilic APase, has been reported to effectively remove 5′-terminal phosphate groups from DNA fragments.[17] However, recombinant TAB5 APase is recovered in membrane fractions and can be dissolved in multimeric form by detergent (0.1% Triton X-100).

Acknowledgments

We thank Dr. A. Nishimura for providing the *phoA* mutant strain *E. coli*, AC109, Dr. H. Nagahora for helpful discussions, and A. Sato and K. Miura for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (C) (no. 14550769) from the Japan Society for the Promotion of Science, and by Grants-in-Aid for Scientific Research (no. 16041229) and National Project on Protein Structure and Functional Analyses from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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