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Comparative Biochemistry and Physiology Part B 129 (2001) 853–861

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Thermolabile alkaline phosphatase from Northern shrimp (*Pandalus borealis*): protein and cDNA sequence analyses

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Received 28 December 2000; received in revised form 16 March 2001; accepted 19 March 2001

Abstract

Sequence analysis of short fragments resulting from trypsin digestion of the thermolabile shrimp alkaline phosphatase (SAP) from Northern shrimp *Pandalus borealis* formed the basis for amplification of its encoding cDNA. The predicted protein sequence was recognized as containing the consensus alkaline phosphatase motif comprising the active site of this protein family. Protein sequence homology searches identified several eukaryote alkaline phosphatases with which the 475-amino acid SAP polypeptide revealed shares 45% amino acid sequence identity. Residues for potential metal binding seem to be conserved in these proteins. The predicted 54-kDa molecular mass of SAP is smaller than previously reported, but is consistent with our recent SDS-PAGE analysis of the native protein. Compared to its homologs, the shrimp enzyme has a surplus of negatively charged amino acids, while the relative number of prolines is lower and the frequency of aromatic residues is higher than in mesophilic counterparts. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Shrimp; Hepatopancreas; Alkaline phosphatase (EC 3.1.3.1); Thermolabile; cDNA; Protein; Sequence alignment

1. Introduction

Alkaline phosphatases (ALPs) (E.C 3.1.3.1) are orthophosphoric-monoester phosphohydrolases usually requiring Zn^{2+} and/or Mg^{2+} for enzyme activity, which is optimal at high pH (Fernley, 1971; McComb et al., 1979). In eukaryotes, the proteins are frequently bound to the cytoplasmic membrane by a glycosyl-phosphatidylinositol (GPI)-modified amino acid in the proximity of the carboxyl terminus (Low and Finean, 1977; Micanovic et al., 1988). The first three-dimensional structure of ALP was revealed from *Escherichia coli* almost two decades ago (Wyckoff et al., 1983), and no ALP crystal structure from eukaryotes has yet been published. Although only partly homologous in sequence (25–35%), ALPs from bacteria and mammals are believed to have similar structures (Kim and Wyckoff, 1990) and their mechanisms of action are understood relatively well (Stec et al., 2000). It has been suggested that these phosphatases belong to a superfamily of

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Abbreviations: ALP, alkaline phosphatase; GPI, glycosyl-phosphatidylinositol; RACE, rapid amplification of cDNA ends; SAP, shrimp alkaline phosphatase

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PII: S1096-4959(01)00391-8

metalloenzymes, also including some sulfatases and phosphate-binding mutases (Galperin et al., 1998). Despite all their similarities, ALP proteins have highly diverged physiochemical properties, and in bacteria, 10-fold variations in monomer sizes (15–160 kDa) have been found (Fitt and Peterkin, 1976; Goldman et al., 1990).

Examples of ALP substrates are DNA, RNA, and ribo-, as well as deoxyribonucleoside triphosphates. In vitro, ALP is exploited in reactions, such as the commonly known dephosphorylation of DNA, to prevent self-ligations after restriction endonuclease cleavage of cloning vectors, or to dephosphorylate dNTPs present in PCR reaction mixtures prior to DNA sequencing reactions (Ruan et al., 1990). Heat-sensitive ALPs may be inactivated by short incubations at temperatures otherwise not harmful to the components present in the reaction mixture (Hoffman and Jendrisak, 1990). Several ALPs of varying temperature-lability have been discovered or engineered from animals (Whitmore and Goldberg, 1972; Ásgeirsson et al., 1995) or bacteria (Kobori et al., 1984; Shandilya and Chatterjee, 1995; de Prada and Brenchley, 1997), but only the heat-sensitive enzyme from an Antarctic bacterium has been published with sequence (Rina et al., 2000). The latter enzyme, in contrast to bacterial ALPs in general, shows specific activities almost as high as its animal counterparts. An irreversible inactivation of the Northern shrimp *Pandalus borealis* alkaline phosphatase (SAP) enzyme is achieved by a short heat-treatment at 65°C. The thermolability of the shrimp enzyme is relatively moderate, and studies on temperature vs. activity in SAP showed a shift of 10°C towards lower temperature compared to the calf enzyme (Olsen et al., 1991). Despite this, SAP is quite stabile during storage or at working conditions, and has an advantageous high specific activity (>2000 U/mg protein). To our knowledge, there are no previous publications on sequences of thermolabile ALP protein or DNA from eukaryotes. Several attempts to sequence SAP have revealed multiple N-terminal ambiguities (not published), and these attempts have thus failed. To circumvent this problem, SAP was cleaved in small peptide fragments and the sequences were used to isolate a SAP cDNA. Sequence features and their context are discussed in view of previous findings on ALPs.

2. Materials and methods

2.1. Protein analyses

Shrimp alkaline phosphatase, purified to apparent homogeneity as previously described (Olsen et al., 1991), was kindly provided by the SAP manufacturer Biotec ASA (Tromsø, Norway). A single protein band was detected by staining with coomassie blue or silver after electrophoresis in 10% NuPAGE Bis–Tris gel system (Novex) using a running buffer of 2-(*N*-morpholino) ethane sulfonic acid containing 0.1% SDS, and the SAP migration length was compared to the Mark12^{TM} protein standard (Novex) for molecular weight determination.

An analysis of total amino acid composition was prepared by dissolving the lyophilized SAP protein in 6 M HCl. The protein suspension was hydrolyzed at 110°C for 24 h followed by evaporation of HCl, before the samples were resuspended in 0.2 M sodium citrate buffer, pH 2.2, and subjected to HPLC for identification and mol.% determination of amino acids in the hydrolyzed product.

Lyophilized SAP protein was also submitted to a commercial sequence analysis service (Innovagen, Sweden), where the protein was fragmented by trypsin and the fragments were separated by reverse-phase HPLC. More than 30 fragments were produced and four of these were selected for further analyses. Mass spectrometry-mediated sequence analyses of the selected fragments H23:18, H23:30, 5ReRP6:26 and 5ReRP6:17 produced 12, 10, eight and five amino acids in sequence, respectively. These amino acid sequences were back-translated by standard codon predictions, and degenerated oligonucleotides of forward and reverse complementary sequences were subsequently custom made (Eurogentec, Belgium).

2.2. Shrimp cDNA synthesis

Freshly collected *P. borealis* shrimps were dissected and the individual hepatopancreas tissues were stored on liquid nitrogen until use. mRNA was isolated from a single hepatopancreas by the use of PolyATract® System 1000 (Promega). Isolated mRNA was used for first-strand cDNA synthesis before second-strand synthesis, and cDNA amplification was performed following instructions given in the Smart™ PCR cDNA synthesis kit (Clontech) and the Advantage cDNA PCR kit (Clontech).

2.3. PCR and DNA sequence analyses

A small aliquot of the synthesized cDNA was used as template in PCRs defined by pair-wise combinations (forward and reverse directions) of the protein-derived oligonucleotide primers. The amplification reactions, having standard mixture compositions, were run for 36 cycles (94°C, 10 s; 51°C, 10 s; 72°C, 1 min) in an Eppendorf gradient thermocycler, and subsequent agarose gel electrophoresis allowed PCR products to be detected. The primer combination of 17F (GCITAYTGGAAYAAR) and 26R (CKYTCICCICCCATDATIAC) gave a distinct amplification product. To confirm the identity of the PCR product, it was sequenced using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham), exploiting the PCR primers for sequencing also.

Based on the DNA sequence found in the PCR product, new, specific forward and reverse primers for 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions were synthesized to fulfill the requirements described for the use of Marathon™ cDNA amplification kit (Clontech). The SAP gene-specific forward primer MalpF (ATTTCGTGGGAAGAATTCGACTTTGC) in combination with the kit AP1 primer, having sequence identity to the ligated Marathon-adaptor, defined one single-amplified 3′-RACE fragment. DNA sequencing analysis confirmed that the RACE products overlapped the internal fragment PCR product sequence.

Sporadically, a 0.5-kb 5′-RACE fragment was amplified using primer AP1 and antisense primer MalpR (GATCTGCCAGCCTCCTGGAACCA). DNA sequencing detected multiple signals for almost the entire sequence positions. New 3′-RACE reactions using alternative gene-specific primers gave similar negative results. Subcloning 5′-RACE product candidates in a vector before sequencing revealed several false positives, but no SAP-related sequences.

A final PCR was performed using primers SapF (AARGCNTAYTGGAAYAARGAT) and SapR (GAAGGTAATCATCTACATCTCA), and the *Pfu* high-fidelity polymerase (Stratagene) for amplification of a SAP cDNA (40 cycles of 94°C, 10 s; 55°C, 15 s; 72°C, 3 min). The resulting 1.5-kb PCR product was analyzed for confirmation of the obtained sequence.

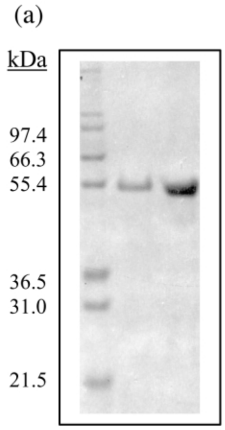
2.4. Computer analysis

DNA and translation product sequence analyses were performed mainly by the programs in the Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, WI, USA). SAP sequence homologs were searched in available databases by FASTA (Pearson, 1990) or BLAST (Altschul et al., 1997), and CLUSTAL W (Thompson et al., 1994) guided the alignment of homologous protein sequences, all programs hosted by the EMBL outstation EBI Internet server. Presentation of multiple sequence alignments was prepared using the GeneDoc program (Nicholas et al., 1997).

3. Results and discussion

3.1. Protein and cDNA analyses

The thermolabile SAP from Northern shrimp hepatopancreas was previously shown to be a dimeric enzyme with catalytically active subunits, and the molecular mass of each subunit was measured as approximately 65 kDa (Olsen et al., 1991). Later product sheets from the commercial distributor United States Biochemicals (USB) operate with a M_r of 59 kDa. When purified SAP was subjected to a SDS-PAGE electrophoresis system of presumably higher resolution than originally used, we found that SAP migrates as a homogenous protein of 54–55 kDa (Fig. 1a). This apparently pure protein was subjected to total amino acid analysis (Fig. 1b), and to trypsin cleavage and subsequent amino acid sequence analyses of four selected fragments (Fig. 1c). Search on homology in published sequences of proteins, or potentially encoding DNA, was limited by the short SAP fragments available for query sequences, and significant protein homologies were not identified.



(b)

Amino acid composition (%)

Ala	9.6	Asp+Asn	13.6
Cys	0.2	Glu +Gln	11.7
Phe	4.1	Met	1.9
Gly	8.4	Pro	3.1
His	2.8	Arg	5.2
Ile	5.4	Ser	4.6
Leu	8.0	Thr	8.5
Lys	4.2	Tyr	3.6
Val	4.9	Trp	-

(c)

Protein fragment sequences

5ReRP6:17
AYWNK
H23:18
DINFRYASAAP(/V)K
5ReRP6:26
VIMGGERR
H23:30
HLITDWLDDK

Fig. 1. Shrimp alkaline phosphatase protein analyses. (a) SDS-PAGE for molecular mass determination of purified shrimp alkaline phosphatase (0.2 and 1 μg in lanes 2 and 3, respectively) by comparison to the standard size proteins (lane 1) after coomassie staining. (b) Summarizes the relative content of amino acids in the purified acid-hydrolyzed protein. (c) Four peptide fragments were produced by trypsin-cleavage of the protein, then the peptides were isolated by RP-HPLC and subsequently sequence-analyzed by MS.

DNA sequences were derived by back-translation of the four analyzed peptides, and degenerated gene-specific PCR primers were accordingly synthesized. The PCR amplification was very sensitive to the annealing temperature, and only one set of primers (17F + 26R) yielded a distinct amplification product. The size of this product was close to 600 bp (not shown), and DNA sequencing revealed an open reading frame of a polypeptide with similarities to known alkaline phosphatases. A 3′-RACE reaction was carried out using a primer sequence identical to a region harbored in the 600-bp PCR product, and the 1.4-kb amplification product contained a long, open reading frame, an in-frame stop codon, and the polyA-tail. A final PCR with primers from the cDNA ends gave a product of 1.5 kb, which was analyzed and found to confirm the contig sequences (Fig. 2). No DNA homologs were detected in available databanks. Specific problems related to the lack of additional 5′ cDNA sequence data are described in Section 2. Possibly, this could reflect a situation of 5′ heterogeneity analogous to the problems previously experienced with the SAP protein N-terminus. The number of amino acids potentially missing in our data is discussed below.

3.2. Deduced protein sequence, substrate and metal binding sites, and protein homologs

An open reading frame of the cDNA encodes a polypeptide of 475 amino acids (Fig. 2) with a theoretical molecular mass of 53 kDa. The derived protein sequence is largely in agreement with the total amino acid composition of the native protein (see Fig. 1b). The four peptide fragments from the purified protein are recognized in the predicted SAP protein sequence, and residues 80–88 (*VTDSAASAT*) encompass a sequence pattern corresponding to the consensus of an alkaline phosphatase active site (PROSITE PS00123). The results of sequence similarity searches in protein databases clearly demonstrated that SAP is a member of the eukaryote ALP family. Three tissue-non-specific (liver/bone/kidney) alkaline phosphatases from human (Weiss et al., 1986), mouse (Terao and Mintz, 1987) and chicken (Crawford et al., 1995), and one tissue-specific (gut) phosphatase from silk moth (Itoh et al., 1991) share significant homology to the SAP protein (45% identity and 60% similarity), as displayed by the multiple sequence alignment (Fig. 3). Marginally lower homology to many other ALPs was detected.

A crystal structure of alkaline phosphatase from *Escherichia coli* has been used to model the core of the three-dimensional structure of mammalian phosphatases by 'considerable circumstantial evidence', and putative amino acid residues involved in active site and metal binding were defined (Kim and Wyckoff, 1990). The extrapolation of data from *E. coli* suggested three binding sites of divalent metal ions (two zinc and one magnesium) in human ALP, and a serine working as a nucleophile in the substrate-interacting cavity (Kim and Wyckoff, 1990; Holtz and Kantrowitz, 1999). These amino acids for the pocket of substrate

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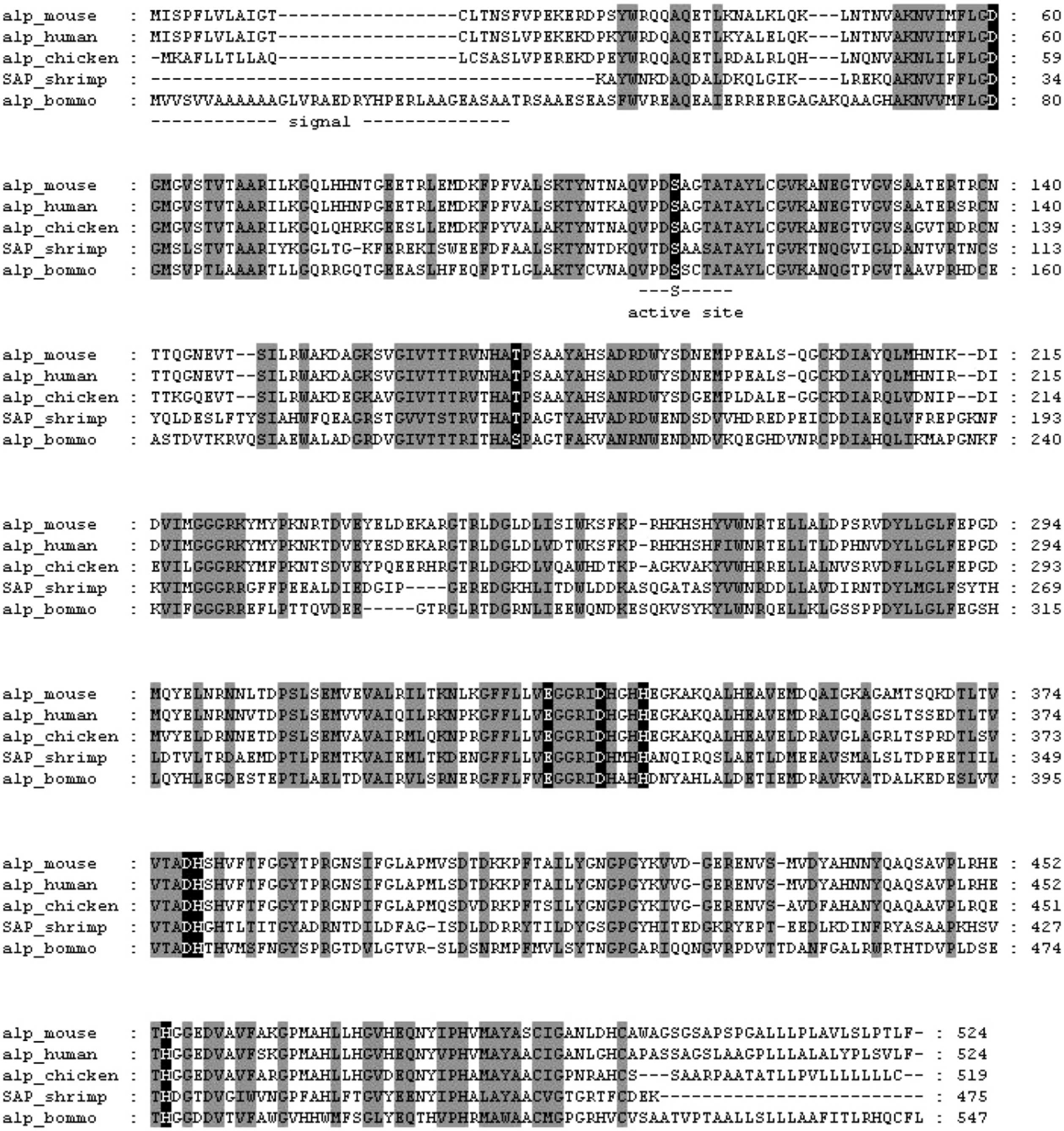
AArGCnTAyTGGAAyAArGATGCCCAAGATGCACTGGATAAACAGCTGGGGATAAAACTG
k  A  Y  W  N  K  D  A  Q  D  A  L  D  K  Q  L  G  I  K  L        20
     5ReRP6:17
CGAGAGAAGCAGGCCAAGAACGTGATCTTCTTCCTAGGAGACGGGATGTCCCTCAGTACA
R  E  K  Q  A  K  N  V  I  F  F  L  G  D  G  M  S  L  S  T        40
GTTACCGCAGCAAGAATCTACAAAGGTGGATTGACCGGGAAATTTGAACGGGAAAAGATT
V  T  A  A  R  I  Y  K  G  G  L  T  G  K  F  E  R  E  K  I        60
TCGTGGGAAGAATTCGACTTTGCTGCTTTGAGTAAGACGTACAATACGGACAAGCAAGTG
S  W  E  E  F  D  F  A  A  L  S  K  T  Y  N  T  D  K  Q  V        80
ACAGATAGTGCTGCCAGTGCCACAGCCTACCTCACTGGGGTGAAGACCAACCAGGGAGTC
T  D  S  A  A  S  A  T  A  Y  L  T  G  V  K  T  N  Q  G  V        100
ATCGGCCTTGATGCCAATACCGTGAGAACAAACTGCTCTTACCAACTTGATGAaTCCCTC
I  G  L  D  A  N  T  V  R  T  N  C  S  Y  Q  L  D  E  S  L        120
TTCACTTACTCCATCGCCCATTGGTTCCAGGAGGCTGGCAGATCAACAGGTGTCGTGACA
F  T  Y  S  I  A  H  W  F  Q  E  A  G  R  S  T  G  V  V  T        140
TCGACCAGGGTAACTCATGCTACTCCTGCAGGAACTTATGCTCACgTGGCTGACAGAGAT
S  T  R  V  T  H  A  T  P  A  G  T  Y  A  H  V  A  D  R  D        160
TGGGAGAACGACAGTGATGTTGTCCACGACAGAGAAGATCCAGAAATATGTGATGACATA
W  E  N  D  S  D  V  V  H  D  R  E  D  P  E  I  C  D  D  I        180
GCTGAACAACTGGTATTCAGAGAACCTGGAAAGAACTTCAAGGTCATCATGGGCGGCGGT
A  E  Q  L  V  F  R  E  P  G  K  N  F  K  V  I  M  G  G  G        200
                                                5ReRP6:26
CGGCGTGGATTCTTTCCTGAGGAAGCGCTTGATATCGAAGATGGCATTCCAGGGGAAAGG
R  R  G  F  F  P  E  E  A  L  D  I  E  D  G  I  P  G  E  R        220
GAAGATGGCAAACACCTCATAACCGATTGGTTGGACGATAAGGCATCTCAGGGAGCCACA
E  D  G  K  H  L  I  T  D  W  L  D  D  K  A  S  Q  G  A  T        240
                        H23:30
GCATCGTATGTCTGGAACCGAGATGATCTATTGGCTGTTGACATTCGAAACACAGACTAC
A  S  Y  V  W  N  R  D  D  L  L  A  V  D  I  R  N  T  D  Y        260
CTTATGGGACTGTTTTCATACACACATCTAGACACAGTTTTAACCAGAGACGCCGAAATG
L  M  G  L  F  S  Y  T  H  L  D  T  V  L  T  R  D  A  E  M        280
GATCCCACCTTgCCGGAAATGACAAAGgTCGCCATCGAAATGCTGACGAAGGATGAAAAT
D  P  T  L  P  E  M  T  K  V  A  I  E  M  L  T  K  D  E  N        300
GGCTTTTTCCTCTTAGTAGAAGGTGGACGGATTGACCATATGCACCACGCCAATCAGATC
G  F  F  L  L  V  E  G  G  R  I  D  H  M  H  H  A  N  Q  I        320
CGTCAGTCGTTAGCGGAGACGCTGGACATGGAGGAAGCTGTATCCATGGCTCTCTCTATG
R  Q  S  L  A  E  T  L  D  M  E  E  A  V  S  M  A  L  S  M        340
ACAGACCCAGAGGAAACAATCATTCTGGTCACAGCTGATCACGGTCACACTCTCACCATC
T  D  P  E  E  T  I  I  L  V  T  A  D  H  G  H  T  L  T  I        360
ACTGGATACGCAGACAGGAACACCGATATTTTGGATTTCGCCGGCATCAGCGACTTGGAT
T  G  Y  A  D  R  N  T  D  I  L  D  F  A  G  I  S  D  L  D        380
GATAGGAGGTACACCATCCTGGACTATGGCAGTGGACCTGGATATCATATCACGGAAGAT
D  R  R  Y  T  I  L  D  Y  G  S  G  P  G  Y  H  I  T  E  D        400
GGCAAACGTTACGAACCTACAGAGGAAGATTTGAAAGACATCAATTTCCGCTACGCATCG
G  K  R  Y  E  P  T  E  E  D  L  K  D  I  N  F  R  Y  A  S        420
                                               H23:18
GCAGCGCCCAAGCATTCAGTTACCCATGATGGCACAGACGTTGGTATTTGGGTCAACGGA
A  A  P  K  H  S  V  T  H  D  G  T  D  V  G  I  W  V  N  G        440
CCTTTCGCTCATCTCTTCACGGGTGTCTATGAAGAGAATTACATTCCCCACGCCTTGGCC
P  F  A  H  L  F  T  G  V  Y  E  E  N  Y  I  P  H  A  L  A        460
TACGCAGCCTGCGTTGGTACAGGACGCACTTTCTGTGACGAAAAATGAGATGTAGATGAT
Y  A  A  C  V  G  T  G  R  T  F  C  D  E  K  *                    475
TACCTTCTTAAGTACATTAAAGATTGC(A)n
```

Fig. 2. cDNA and deduced protein sequence of thermolabile alkaline phosphatase from *Pandalus borealis*. The nucleotide sequence of the amplified cDNA and the encoded 475 amino acid product are shown. Amino acids corresponding to the sequenced peptides of native protein are underlined. The stop codon is marked by an asterisk and the polyA-tail is indicated $(A)_n$. Five nucleotide positions in the 5′-sequence (lower case) are biased by the degenerated PCR primer used for amplification and sequencing, and the positions are thus not specified. The N-terminal lysine residue (lower case) could alternatively be arginine. The *P. borealis* alkaline phosphatase cDNA sequence submitted to GeneBank received the accession number AJ296089.

phosphate groups and for binding of metals are fully conserved in SAP and its homologs (Fig. 3). Two more active site residues (aspartate-153 and lysine-328) have been identified in the *E. coli* enzyme, and they are replaced by histidine in the mammalian enzymes (Holtz and Kantrowitz, 1999). The shrimp enzyme likewise contains histidine in the corresponding positions 146 and 313. A third site occupied by arginine is conserved in all the enzymes (positions 166 and 159 in *E. coli*



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alp_mouse   : MISPFLVLAIGT-----------------CLTNSFVPEKERDPSYWRQQAQETLKNALKLQK---LNTNVAKNVIMFLGD :   60
alp_human   : MISPFLVLAIGT-----------------CLTNSLVPEKEKDPKYWRDQAQETLKYALELQK---LNTNVAKNVIMFLGD :   60
alp_chicken : -MKAFLLTLLAQ-----------------LCSASLVPEREKDPEYWRQQAQETLRDALRLQH---LNQNVAKNLILFLGD :   59
SAP_shrimp  : ------------------------------------------KAYWNKDAQDALDKQLGIK----LREKQAKNVIFFLGD :   34
alp_bommo   : MVVSVVAAAAAAGLVRAEDRYHPERLAAGEASAATRSAAESEASFWVREAQEAIERRERERGAGAKQAAGHAKNVVMFLGD :   80
              ------------ signal --------------

alp_mouse   : GMGVSTVTAARILKGQLHHNTGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTATAYLCGVKANEGTVGVSAATERTRCN : 140
alp_human   : GMGVSTVTAARILKGQLHHNPGEETRLEMDKFPFVALSKTYNTKAQVPDSAGTATAYLCGVKANEGTVGVSAATERSRCN : 140
alp_chicken : GMGVSTVTAARILKGQLQHRKGEESLLEMDKFPYVALAKTYNTNAQVPDSAGTATAYLCGVKANEGTVGVSAGVTRDRCN : 139
SAP_shrimp  : GMSLSTVTAARIYKGGLTG-KFEREKISWEEFDFAALSKTYNTDKQVTDSAASATAYLTGVKTNQGVIGLDANTVRTNCS : 113
alp_bommo   : GMSVPTLAAARTLLGQRRGQTGEEASLHFEQFPTLGLAKTYCVNAQVPDSSCTATAYLCGVKANQGTPGVTAAVPRHDCE : 160
                                                            ---S-----
                                                           active site

alp_mouse   : TTQGNEVT--SILRWAKDAGKSVGIVTTTRVNHATPSAAYAHSADRDWYSDNEMPPEALS-QGCKDIAYQLMHNIK--DI : 215
alp_human   : TTQGNEVT--SILRWAKDAGKSVGIVTTTRVNHATPSAAYAHSADRDWYSDNEMPPEALS-QGCKDIAYQLMHNIR--DI : 215
alp_chicken : TTKGQEVT--SILRWAKDEGKAVGIVTTTRVTHATPSAAYAHSANRDWYSDGEMPLDALE-GGCKDIARQLVDNIP--DI : 214
SAP_shrimp  : YQLDESLFTYSIAHWFQEAGRSTGVVTSTRVTHATPAGTYAHVADRDWENDSDVVHDREDPEICDDIAEQLVFREPGKNF : 193
alp_bommo   : ASTDVTKRVQSIAEWALADGRDVGIVTTTRITHASPAGTFAKVANRNWENDNDVKQEGHDVNRCPDIAHQLIKMAPGNKF : 240

alp_mouse   : DVIMGGGRKYMYPKNRTDVEYELDEKARGTRLDGLDLISIWKSFKP-RHKHSHYVWNRTELLALDPSRVDYLLGLFEPGD : 294
alp_human   : DVIMGGGRKYMYPKNKTDVEYESDEKARGTRLDGLDLVDTWKSFKP-RHKHSHFIWNRTELLTLDPHNVDYLLGLFEPGD : 294
alp_chicken : EVILGGGRKYMFPKNTSDVEYPQEERHRGTRLDGKDLVQAWHDTKP-AGKVAKYVWHRRELLALNVSRVDFLLGLFEPGD : 293
SAP_shrimp  : KVIMGGGRRGFFPEEALDIEDGIP----GEREDGKHLITDWLDDKASQGATASYVWNRDDLLAVDIRNTDYLMGLFSYTH : 269
alp_bommo   : KVIFGGGRREFLPTTQVDEE-----GTRGLRTDGRNLIEEWQNDKESQKVSYKYLWNRQELLKLGSSPPDYLLGLFEGSH : 315

alp_mouse   : MQYELNRNNLTDPSLSEMVEVALRILTKNLKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDQAIGKAGAMTSQKDTLTV : 374
alp_human   : MQYELNRNNVTDPSLSEMVVVAIQILRKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGQAGSLTSSEDTLTV : 374
alp_chicken : MVYELDRNNETDPSLSEMVAVAIRMLQKNPRGFFLLVEGGRIDHGHHEGKAKQALHEAVELDRAVGLAGRLTSPRDTLSV : 373
SAP_shrimp  : LDTVLTRDAEMDPTLPEMTKVAIEMLTKDENGFFLLVEGGRIDHMHHANQIRQSLAETLDMEEAVSMALSLTDPEETIIL : 349
alp_bommo   : LQYHLEGDESTEPTLAELTDVAIRVLSRNERGFFLFVEGGRIDHAHHDNYAHLALDETIEMDRAVKVATDALKEDESLVV : 395

alp_mouse   : VTADHSHVFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAILYGNGPGYKVVD-GERENVS-MVDYAHNNYQAQSAVPLRHE : 452
alp_human   : VTADHSHVFTFGGYTPRGNSIFGLAPMLSDTDKKPFTAILYGNGPGYKVVG-GERENVS-MVDYAHNNYQAQSAVPLRHE : 452
alp_chicken : VTADHSHVFTFGGYTPRGNPIFGLAPMQSDVDRKPFTSILYGNGPGYKIVG-GERENVS-AVDFAHANYQAQAAVPLRQE : 451
SAP_shrimp  : VTADHGHTLTITGYADRNTDILDFAG-ISDLDDRRYTILDYGSGPGYHITEDGKRYEPT-EEDLKDINFRYASAAPKHSV : 427
alp_bommo   : VTADHTHVMSFNGYSPRGTDVLGTVR-SLDSNRMPFMVLSYTNGPGARIQQNGVRPDVTTDANFGALRWRTHTDVPLDSE : 474

alp_mouse   : THGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYASCIGANLDHCAWAGSGSAPSPGALLLPLAVLSLPTLF- : 524
alp_human   : THGGEDVAVFSKGPMAHLLHGVHEQNYVPHVMAYAACIGANLGHCAPASSAGSLAAGPLLLALALYPLSVLF- : 524
alp_chicken : THGGEDVAVFARGPMAHLLHGVDEQNYIPHAMAYAACIGPNRAHCS---SAARPAATATLLPVLLLLLLLC-- : 519
SAP_shrimp  : THDGTDVGIWVNGPFAHLFTGVYEENYIPHALAYAACVGTGRTFCDEK------------------------- : 475
alp_bommo   : THGGDDVTVFAWGVHHWMFSGLYEQTHVPHRMAWAACMGPGRHVCVSAATVPTAALLSLLLAAFITLRHQCFL : 547
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Fig. 3. Sequence alignments of the shrimp alkaline phosphatase and homologous proteins. Proteins of high homology to SAP were identified by BLAST and the sequences were aligned using CLUSTAL W. Identical amino acids are shaded (gray) and proposed metal binding sites are highlighted (black background). The PROSITE PS00123 alkaline phosphatase active site motif including the serine nucleophile is specified below its appearance, and predicted signal sequences in the SAP homologs are indicated. The homologous ALPs are tissue-non-specific from mouse (accession no. P09242), human (P05186), chicken (Q92058), and tissue-specific from silkworm gut (P29523).

and *P. borealis*, respectively), and was found to be important for phosphate/substrate binding in the *E. coli* ALP (Chaidaroglou et al., 1988).

The four aligned homologs of SAP have individually been assigned as membrane-bound. However, prediction tests did not indicate a membrane GPI-anchor in SAP. The alignment shows that SAP C-terminus ends 25–30 amino acids earlier than the other ALPs. This could indicate that the negative GPI-anchor prediction is correct, and that SAP is not membrane-bound. Additionally, various prediction tools failed to identify transmembrane regions in the shrimp protein. The theoretical calculated size of cDNA-derived SAP is close to the $\leq$ 55-kDa native protein mass determined by SDS-PAGE. The minor difference may suggest that 5–10 amino acids are missing in our protein sequence, and from the alignment in Fig. 3, it appears that the ALPs have an extension of seven–nine N-terminal residues, in addition to their signal peptide, compared to the SAP sequence. Thus, it is likely that the native mature form of the shrimp enzyme contains approximately 482 amino acids, consistent with the molecular mass as determined by the SDS-PAGE analysis.

3.3. Cold-adaption, negative charge, and structural flexibility

An interesting feature of SAP is its high negative charge compared to the homologs. This is due to a considerable surplus of acidic residues (17.2% Asp and Glu in SAP and 10.8–12.6% in the other ALPs), while the levels of positively charged residues are similar to the other ALPs (9.5% Lys and Arg in SAP and 9.5–10.6% in the homologs). The result is a pI of 4.5 in SAP, whereas the homolog pI values range from 6.2 to 7.0. The highly temperature-sensitive phoA from the psycrophilic bacterium TAB5 (Rina et al., 2000) contains 10.7 and 9% negatively and positively charged amino acids, respectively, giving a predicted pI of 5.5 which is notably higher than the pI of the less temperature-sensitive SAP. Thus, the frequent appearance of acidic residues should not per se explain the moderate cold-adaption of SAP. We would nevertheless like to refer to our recent report of an antibacterial and cold-active lysozyme-like protein from Icelandic scallops (Nilsen et al., 1999). The protein was found to have a considerably lower pI than its primary structure homologs in marine, as well as terrestrial, invertebrates, in that particular case also due to a higher ratio of Asp and Glu with no reduction in numbers of Lys and Arg. Although isolated examples, possibly by chance, these two examples of thermolabile or cold-active enzymes in cold-adapted organisms may suggest evolutionary traits in (sub-)Arctic marine invertebrates. Available sequence information from such organisms is unfortunately very limited.

Cold-adapted enzymes are commonly accepted to require structural flexibility (Gerday et al., 1997), and this view was supported by the crystal structure studies on the psycrophilic citrate synthase from an Antarctic bacterium (Russell et al., 1998). A locally fixed protein structure is often a result of the rigid amino acid proline, which is found to be less abundant in psycrophilic than in mesophilic enzymes (Chessa et al., 1999; Georlette et al., 2000), and SAP has a consistently low proportion of Pro residues (2.7%) compared to the homologous proteins (3.8–4.8%). Even lower levels of Pro (2.0%) were found in phoA of the psycrophilic TAB5 bacterium. The sequences aligned in Fig. 3 and a previous alignment of bacterial and yeast ALP sequences (Rina et al., 2000) show that several of the Pro replacements in SAP are not unique to the shrimp protein. However, SAP is distinguished from the other ALPs by the absence of Pro in the sequence up to position 149. Finally, the relative amount of aromatic residues is almost 40% higher in SAP than in the eukaryote proteins compared (relative frequency of 9.4 and 7%, respectively), and in this respect SAP resembles the TAB5 phoA.

The lack of direct structural data from eukaryote ALPs makes their structure predictions reasonably tentative. Even more uncertainties are added when an attempt is made to explain thermal stability variations by 3-D structure differences. Determination of the crystal structure of SAP by a collaborating group is now approaching the final refinement stage. This will undoubtedly provide much valuable information about the thermolabile shrimp enzyme. Efforts to produce recombinant SAP have been initiated and structure–function relationships can hopefully be studied in the near future.

Acknowledgements

This work was financed by The Regional Authority of Northern Norway. In appreciation, we thank Dr Sean McSweeney (ESRF, Grenoble, France) and Dr Egil Olsen (Norwegian Institute of Fisheries & Aquacultures, Tromsø, Norway) for their help in reviewing the manuscript.

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