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Critical Active-Site Residues Identified by Site-Directed Mutagenesis in *Pseudomonas aeruginosa* Phosphorylcholine Phosphatase, A New Member of the Haloacid Dehalogenases Hydrolase Superfamily

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Abstract. *Pseudomonas aeruginosa* phosphorylcholine phosphatase (PChP), the product of the PA5292 gene, is synthesized when the bacteria are grown with choline, betaine, dimethylglycine, or carnitine. In the presence of Mg^{2+}, PChP catalyzes the hydrolysis of both phosphorylcholine (PCh) and *p*-nitrophenylphosphate (*p*-NPP). PCh saturation curve analysis of the enzyme with or without the signal peptide indicated that the peptide was the fundamental factor responsible for decreasing the affinity of the second site of PChP for PCh, either at pH 5.0 or pH 7.4. PChP contained three conserved motifs characteristic of the haloacid dehalogenases superfamily. In the PChP without the signal peptide, motifs I, II, and III correspond to the residues ^{31}DMDNT^{35}, ^{166}SAA^{168}, and K^{242}/^{261}GDTPDSD^{267}, respectively. To determine the catalytic importance of the D31, D33, T35, S166, K242, D262, D265, and D267 on the enzyme activity, site-directed mutagenesis was performed. D31, D33, D262, and D267 were identified as the more important residues for catalysis. D265 and D267 may be involved in the stabilization of motif III, or might contribute to substrate specificity. The substitution of T35 by S35 resulted in an enzyme with a low PChP activity, but conserves the catalytic sites involved in the hydrolysis of PCh (K_{m1} 0.03 mM, K_{m2} 0.5 mM) or *p*-NPP (K_m 2.1 mM). Mutating either S166 or K242 revealed that these residues are also important to catalyze the hydrolysis of both substrates. The substitution of lysine by arginine or by glutamine revealed the importance of the positive charged group, either from the amino or guanidinium groups, because K242Q was inactive, whereas K242R was a functional enzyme.

In previous work, we provided evidence that the PA5292 gene, located in the *Pseudomonas aeruginosa* PAO1 genome, encodes phosphorylcholine phosphatase (PChP) [14], an enzyme that catalyzes the hydrolysis of phosphorylcholine (PCh), phosphorylethanolamine, and the nonphysiological substrate *p*-nitrophenylphosphate (*p*-NPP) [11, 12, 16]. This enzyme is specifically synthesized when the bacteria are grown with choline, betaine, dimethylglycine, or carnitine as the sole carbon, nitrogen, or carbon and nitrogen source in isoosmolar or hyperosmolar conditions in culture media containing a high or low phosphate concentration [11–13]. Molecular and biochemical experiments with the recombinant enzymes indicated that the *pchP* gene encodes a PChP with a molecular mass slightly less than 40 kDa that is exported to the bacterial periplasmic space [14]. PCh saturation curves that demonstrated changes in the enzyme's K_{m2} led us to propose that the presence of the signal peptide may be the fundamental factor responsible for altering the low-affinity site of recombinant PChP expressed in *Escherichia coli* [14]. To confirm this hypothesis, the first objective of this work was therefore to elucidate the catalytic properties of recombinant enzymes with and without the signal peptide. Bioinformatic studies [14] indicated that PChP contained three conserved motifs characteristic of the haloacid dehalogenases (HAD) superfamily [20, 21]: motif I, DXDX[T/V], in the N-terminal region of the protein, and two less well-known conserved motifs,

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Table 1. Oligonucleotide primers for site-directed mutagenesis

Mutant	Sequence of mutagenic primers[a]
^{31}EMENS^{35}	5′-GCCTATGCCGTGTTC**GAG**ATG**GAG**AAC**TCC**AGCTATCGCTACGAC-3′
^{31}AMANA^{35}	5′-GCCTATGCCGTGTTC**GCC**ATG**GCC**AAC**GCC**AGCTATCGCTACGAC-3′
D31E	5′-GCCTATGCCGTGTTC**GAG**ATGGACAACACCAGC-3′
D33E	5′-GCCGTGTTCGACATG**GAG**AACACCAGCTATCGC-3′
T35S	5'-TTCGACATGGACAAC**TCC**AGCTATCGCTACGAC-3′
S166T	5′-GAGGTCTACGTGAA**CA**GCGCGGCCCATG-3′
K242R	5′-GGATGGCCGGC**AGG**CAGGCTGCGATCC-3′
K242Q	5′-GGATGGCCGGC**CAG**CAGGCTGCGATCC-3′
G261A	5′-CCTGGTCGCC**GCC**GATACCCCGG- 3′
D262E	5′-GGTCGCCGGC**GAG**ACCCCGGACAGC-3′
D265E	5′-GGCGATACCCCG**GAG**AGCGACGGTTAC-3′
D267E	5′-CCCCGGACAGC**GAG**GGTTACATGCTG-3′

[a] A pair of complementary primers was used to create each mutant; however, only the sequence of the sense strand is shown. Changes in the sequences are shown in boldface type and underlined.

motif II, [S/T]XX, and motif III, K-[G/S]DXXX[D/N], which were originally described by Koonin and Tatusov [10]. The second objective of this work was thus to confirm the catalytic importance of the residues located in motifs I, II, and III in recombinant PChP without the signal peptide.

Materials and Methods

Materials. Oligonucleotide primers were produced by BioSynthesis Inc. (Lewisville, TX). Isopropyl-beta-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, *p*-NPP, and PCh were purchased from Sigma (St. Louis, MO).

Bacterial Strains and Growth Conditions. *E. coli* XL10 and *E. coli* ER2566 were routinely grown at 37°C in Luria Bertani (LB) medium supplemented with 150 μg mL^{-1} ampicillin. *P. aeruginosa* PAO1 was routinely grown in LB medium at 37°C.

DNA Methodology. Genomic or plasmid DNA isolation was performed using commercial kits (Bio-Rad and Promega, respectively). Restriction enzymes and T4 ligase were used according to the manufacturer's instructions (Promega). DNA fragments were purified from agarose gel with a QIAquick kit (Qiagen). Using polymerase chain reaction (PCR), a 1-kb fragment was amplified from *Pseudomonas aeruginosa* PAO1 wild-type chromosomal DNA using primers JA1 (acgtggtgcgctgagggatgc) [14] and 92sps (c**catatg**accgaactcgagcactggc). The latter primer contained the first codon after the putative signal peptide, as predicted by the SignalP server [3], and incorporates a unique *Nde*I site (shown above in bold and underlined). The PCR-amplified DNA fragment encoding the reading frame for the 327 amino acids of the mature wild-type enzyme without the signal peptide was cloned into the pGEM®-T Easy vector to generate pGEMT-*pchP*. pGEMT-*pchP* was transformed into *E. coli* XL10 Gold (Stratagene) in accordance with the manufacturer's protocol, with selection for ampicillin-resistant transformants; the insertion was verified by restriction analysis.

For expression of PChP$_{327}$, a 1-kb *Eco*RI-*Nde*I DNA fragment was excised from pGEMT-*pchP* (*pchP$_{327}$* gene), gel purified, and subcloned in pTYB12 (New England Biolabs) as an N-terminal fusion to the intein, giving rise to pTYB-*pchP$_{327}$* as in [14]. pTYB-*pchP$_{327}$* was transformed into *E. coli* XL10 Gold, for maintenance, or into *E. coli* ER2566 (New England Biolabs) for expression and purification, with selection for ampicillin-resistant transformants. For expression of PChP with signal peptide (PChP$_{349}$), pTYB-*pchP$_{349}$* was constructed as in [14]. The inserts of pGEMT-*pchP* and pTYB-*pchP$_{327}$* were sequenced by Macrogen, Inc. (Kumchun-Ku, Seoul, Korea) to ensure that no errors were introduced due to PCR or subcloning procedures.

Site-Directed Mutagenesis. pTYB-*pchP$_{327}$* was used as template to introduce specific mutations using Quick Change XL site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer's instructions. The sense primers used for targeted mutagenesis are listed in Table 1. The successful introduction of mutations was confirmed by sequencing at least three different plasmids for each mutant; sequencing was performed at Macrogen, Inc. (Kumchung-Ku, Seoul, Korea).

Prediction of the Signal Peptide Cleavage Site. For this purpose, we utilized the updated SignalP 3 server at CBS Prediction Servers [3]. The most likely cleavage site for PA5292 was found between position 22 and 23: ^{1}MTFAKGILAALALAAAVGQASA^{22}–^{23}T. In this way, the original complete protein containing 349 aminoacyl residues after the elimination of the signal peptide contained 327 aminoacyl residues.

Expression and Purification of PChP and Mutant Enzymes Without Signal Peptide. To overexpress the wild-type and mutant enzymes, *E. coli* ER2566 was transformed with pTY-*pchP$_{327}$*, pTY-*pchP$_{349}$*, or the mutated expression plasmids. The resulting transformants were grown in LB liquid medium supplemented with 150 μg mL^{-1} ampicillin and 0.5% glucose at 37°C until the OD$_{600}$ reached 0.6. Protein expression was induced by the addition of 0.4 mM IPTG to cultures, followed by incubation for 16 h at 18°C. Purification of the enzymes was performed using the IMPACT-CN system (New England Biolabs) according to the manufacturer's instructions. Purified enzymes were dialyzed against 10 mM Tris-HCl, pH 8, with 30% glycerol and subsequently stored at −10°C.

PChP Activity, Enzyme Kinetics, and Protein Assay. PChP and acid phosphatase activities were measured as described in [16]. The standard assay contained 100 mM sodium acetate buffer (pH 5.0) or HEPES buffer (pH 7.4), 2 mM $MgCl_2$, and 5 mM *p*-NPP or 0.5 mM PCh. The *p*-nitrophenol levels formed during the reaction were measured at

Table 2. Kinetic constants of PChP expressed in *Escherichia coli* with and without the signal peptide

Kinetic constants obtained with:	PChP		
	+ signal peptide	− signal peptide	− vs. + signal peptide ratio
p-NPP as substrate, pH 5.0			
$K_{m\ app}$, mM	2.0	2.0	1
k_{cat} s^{-1}	1.4×10^4	1.1×10^6	79
k_{cat}/K_m, s^{-1} mM^{-1}	7×10^3	5.5×10^5	79
PCh as substrate, pH 5.0			
K_{m1}, mM	0.05	0.03	0.6
k_{cat} s^{-1}	3.5×10^3	3.7×10^5	106
k_{cat}/K_{m1}, s^{-1} mM^{-1}	7×10^4	1.2×10^7	171
K_{m2}, mM	3.6	0.5	0.14
k_{cat} s^{-1}	6.3×10^4	1.7×10^6	27
k_{cat}/K_{m2}, s^{-1} mM^{-1}	1.7×10^4	3.4×10^6	200
PCh as substrate, pH 7.4			
K_{m1}, mM	0.05	0.03	0.6
k_{cat} s^{-1}	1.7×10^3	3.5×10^5	205
k_{cat} /K_{m1}, s^{-1} mM^{-1}	3.4×10^4	1.2×10^7	353
K_{m2}, mM	3.5	0.5	0.14
k_{cat} s^{-1}	5.2×10^4	2.2×10^6	42
k_{cat} /K_{m2}, s^{-1} mM^{-1}	1.5×10^4	4.4×10^6	293

410 nm. Inorganic phosphate (Pi) was determined as in [6]. One unit of acid phosphatase or PChP was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from *p*-NPP or 1 μmol of Pi from PCh per minute at 37°C. Kinetic studies were performed using 0.3–0.6 μg of purified enzyme. Saturation curves for different *p*-NPP or PCh concentrations using purified *E. coli*-expressed PChP with and without the signal peptide were determined as in [14, 16]; results were analyzed as in previous reports [14, 16]. K_m and V_{max} values were estimated by fitting initial rate data according to the Eadie-Hofstee method, as in [14]. Protein concentration was determined according to Bradford [4], using bovine serum albumin as the standard.

Sequence Analysis and Modeling. DNA and protein sequence analyses were performed by consulting the databases of the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk), European Molecular Biology Laboratory (EMBL) (http://www.embl-heidelberg.de), National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), and the Structural Bioinformatics Group from Imperial College, UK (SBG) (http://www.sbg.bio.ic.ac.uk) using BLASTp [2]. Alignment of highly similar sequences was performed with ClustalX [19], using default parameters. The Biomolecular Interaction Network Database (BIND) (http://bind.ca/) and the Small Molecule Interaction Database (SMID) (http://smid.blueprint.org) [1] were used to investigate known molecular interactions. Protein fold recognition was performed using the 3D-PSSM [9] program of SBG (UK); the structural models of PChP were constructed with Swiss PDB Viewer [7] using the crystal coordinates of *Methanococcus jannaschii* phosphoserine phosphatase (PSP) [21], an enzyme with 19.4% sequence identity with the human phosphatase PHOSPHO1 [18] and 17% sequence/structure with PChP as predicted by 3D-PSSM.

Results and Discussion

To examine the effect of the signal peptide on enzyme kinetics, apparent K_m and k_{cat} values were determined using *p*-NPP or PCh as substrates (Table 2). Saturation curves for PChP (with or without signal peptide), obtained using differing substrate concentrations indicated that in the absence of the signal peptide, the enzyme's catalytic efficiency increased notably with both substrates (Table 2). With *p*-NPP as substrate, the increase in the catalytic efficiency was produced by an increased k_{cat} value without modification of the K_m value. As was shown with purified enzyme isolated from the periplasmic space of *P. aeruginosa* [16], the recombinant enzyme with or without signal peptide exhibited two affinity sites for PCh (Table 2). At pH 5.0 or 7.4, in the absence of the signal peptide, the catalytic efficiency corresponding to the high-affinity site increased between two and three orders of magnitude, with little change in the K_{m1} values (Table 2). The signal peptide, however, notably decreased the affinity of the PChP's second PCh binding site, either at pH 5.0 or pH 7.4 (Table 2). In these cases, the decreased catalytic efficiency was caused by a diminished k_{cat} and principally by the high increase of the K_{m2} from 0.5 to 3.5 mM, observed in the absence or in the presence of the signal peptide, respectively (Table 2). Considering an in vivo situation, where the synthesized protein is exported to the periplasmic space without its signal peptide, the above results indicated the importance of this secretion pathway in producing an enzyme with a higher catalytic efficiency than the complete protein containing the signal peptide.

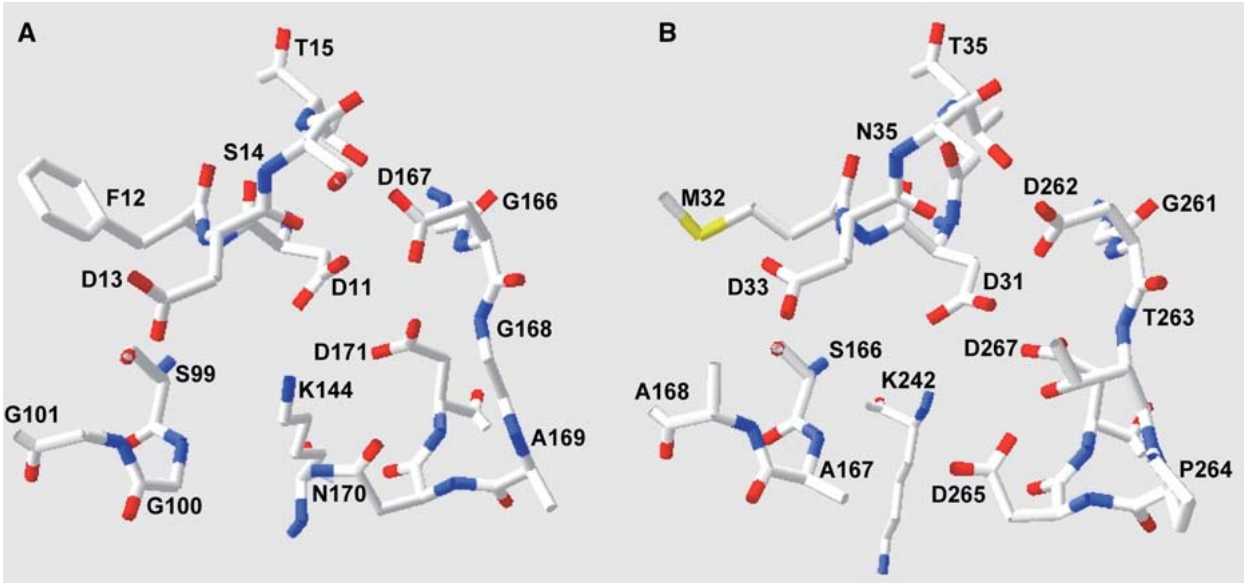

Fig. 1. Structural models showing the aminoacyl residues from motifs I, II, and III from *Methanococcus janaschii* phosphoserine phosphatase (A) taken from [21], and *Pseudomonas aeruginosa* phosphorylcholine phosphatase (B).

In the complete PChP protein sequence predicted in the database as PA5292 (gi15600485), the motifs I, II, and III are ^{53}DMDNT^{57}, ^{188}SAA^{190}, and ^{283}GDTPDSD^{289} because the signal peptide was included [14]. Because the mature enzyme that *P. aeruginosa* secretes into the periplasmic space lacks the 22 residues of the signal peptide, the motifs I, II, and III are indicated in this work as ^{31}DMDNT^{35}, ^{166}SAA^{168}, and ^{261}GDTPDSD^{267}, respectively. Additionally, in this work we have included lysyl residue K242 as a residue of motif III; K242 may be considered equivalent to K144 of the *M. jannaschii* PSP (Fig. 1). To determine the catalytic importance of the aminoacyl residues on these motifs, site-directed mutagenesis was performed on the aspartyl and threonyl residues located in motif I, on the seryl residue of motif II, and on the lysyl, glycyl, and aspartyl residues of motif III (Fig. 1).

The first conserved aspartyl residue (D31 in PChP; D11 in PSP) is the residue that is phosphorylated during phosphoester hydrolysis [17, 21]. As proposed in [21], the oxygen atom of the carboxyl group may be involved in a nucleophilic attack on the phosphorus atom. The second aspartyl residue (D33 in PChP; D13 in PSP) is also important for catalysis; it participates in the phosphorylation of the first aspartyl residue, D31. Simultaneous nonconservative substitutions affected by changing D31, D33, and T35 to alanyl residues (resulting in the mutant ^{31}AMANA^{35}), and conservative substitutions obtained by simultaneously replacing D31, D33, and T35 by E31, E33, and S35 (mutant ^{31}EMENS^{35}), respectively, in motif I, produced proteins without PChP activity, as measured using either PCh (at pH 5.0 or 7.4) or *p*-NPP (at pH 5.0) as substrate (Table 3).

After these observations, specific site-directed conserved mutagenesis was conducted, substituting individual amino acid residues D31 by E31 (mutant D31E), D33 by E33 (mutant D33E), and T35 by S35 (mutant T35S). Although normal protein expression was observed in all three cases, the individual mutants, D31E and D33E, produced proteins possessing practically no enzyme activity; in T35S, a low PChP activity was detected (Table 3). This mutant, like the wild-type enzyme, appears to conserve identical catalytic sites involved in the hydrolysis of *p*-NPP (K_m 2.1 mM) or with PCh (K_{m1} 0.03 mM, K_{m2} 0.5 mM). A clear function has not been assigned to T15 in PSP (T35 in PChP), a residue that is absolutely conserved between members of the HAD superfamily [21]. The substitution of threonyl by a seryl residue in PChP probably causes structural perturbations beyond the immediate vicinity of the side chain, possibly impeding protein folding, resulting in a protein with a very low enzymatic activity compared to the wild-type enzyme. The seryl residue from motif II, S99, and K144, a remote lysyl residue that is part of motif III in PSP, are equivalent to PChP residues S166 and K242, respectively (Fig. 1).

According to [21], these residues form hydrogen bonds with phosphate. Mutating residues S166 and K242 revealed that these residues are also important to catalyze the hydrolysis of PCh or *p*-NPP by *P. aeruginosa* PChP (Table 3). The substitution of lysine by

Table 3. Specific activity of wild-type PChP and percentage of specific activity found in mutated enzymes in motifs I, II, or III

Enzyme	Motifs	Specific activity with *p*-NPP and PCh as substrates		
		p-NPP	PCh	
	I	pH 5.0	pH 5.0	pH 7.4
Wild type	^{31}DMDNT^{35}	74.5 (100)	88.4 (100)	86.9 (100)
		Relative values		
Mutant	^{31}EMENS^{35}	—	—	—
Mutant	^{31}AMANA^{35}	—	—	—
Mutant	D31E	—	—	—
Mutant	D33E	—	—	—
Mutant	T35S	5.9	3.0	3.6
	II			
Wild type	^{166}S	100	100	100
Mutant	S166T	7.0	20.3	26.2
	III			
Wild type	^{242}K [a]	100	100	100
Mutant	K242R	106.7	56.9	74.6
Mutant	K242Q	—	—	—
Wild type	^{261}GDTPDSD^{267}	100	100	100
Mutant	G261A	63.4	38.8	64.8
Mutant	D262E	1.3	6.5	11.1
Mutant	D265E	64.0	71.0	70.0
Mutant	D267E	1.5	2.4	2.5

Enzyme activity of wild type or each recombinant enzyme was measured in the presence of 2 mm $MgCl_2$ with 5 mM *p*-NPP at pH 5 in acetate buffer or with 0.5 mM PCh at pH 5 in acetate buffer or pH 7.4 with HEPES buffer. Values of specific activity, Units.(mg protein)$^{-1}$, are the average of three independent experiments. Relative values shown in different mutated enzymes are the average of at least two independent experiments. (—) Indicates that the enzyme activity measured with respect to the wild-type enzyme was less than 0.05%.

[a]Located 19 aminoacyl residues separated left from motif III.

arginine (K242R) or by glutamine (K242Q) revealed the importance of the positive charged group, either from the amino or guanidinium groups from lysine or arginine, because K242Q was completely inactive; whereas K242R was a functional enzyme (Table 3). The motif III in PChP, ^{261}GDTPDSD^{267}, may be equivalent to the ^{166}GDGAND^{171} sequence present in PSP (Fig. 1). According to the model shown in Fig. 1, the D262 from PChP is the aspartyl residue equivalent to the D167 in PSP. Site-directed mutagenesis of the two other aspartyl residues in motif III, D265 and D267, indicated that the aspartyl residue essential for the catalytic activity was the D267, because in this case the enzyme activity measured with *p*-NPP or PChP decreased more than 97% compared to the wild-type enzyme (Table 3). After the substitution of D265 by E265, the enzyme activity, measured with the same substrates, had lost 30–35% of wild-type activity (Table 3).

These results are in very good agreement with the model predicted for PChP because in this model D265 is far away from the catalytic pocket formed by motifs I, II, and III. Therefore, according to results shown in Table 3 and Fig. 1, the two aspartyl residues more important for the catalysis in PChP are D262 and D267, because the conservative substitutions by glutamyl residues produced proteins with a very low enzyme activity. Probably, as is the case for *Methanococcus janaschii* PSP [21], D262 from PChP (D167 in PSP) may form a coordination bond to Mg^{2+}, and D267 may be the aspartyl residue equivalent to the D171 in PSP. In addition, as has occurred in PSP, D267 may be the aspartyl residue that stabilizes motif III that forms the Mg^{2+} binding group [21], as shown for the model proposed in Fig. 1. The human phosphatase PHOSPHO1 (3.1.3.75) was also identified as a member of the HAD superfamily. In this enzyme, the catalytic motifs I, II, and III are ^{32}DFDET^{36}, ^{122}SDA, and ^{177}K/^{202}GDGAND^{207}, respectively [18]. PHOSPHO1, working in synchronization with phospholipase C, is involved in the generation of Pi for matrix mineralization in human, mice, and chicken [8, 18]. In *P. aeruginosa*, PChP and hemolytic phospholipase C (PlcH) are specifically and simultaneously synthesized when the bacteria are grown in the presence of choline or other derivatives alkylammonium compounds [11, 13]. Through the synchronized action of these enzymes, the bacteria might break down the host cell's membrane

phospholipids, to produce choline plus Pi [11, 12, 16]. Therefore, although choline and Pi are the final products of these enzymes, in the presence of high Pi concentration, *P. aeruginosa* utilizes PlcH and PChP to cover its metabolic requirements for choline [12].

Although both PChP and PHOSPHO1 carry out very different functions, both are activated by divalent cations and use phosphorylethanolamine and PCh as substrates [5, 15, 15, 16]. Like all members of the HAD superfamily, no significant sequence homology was found between PHOSPHO1 and PChP; yet both enzymes share the characteristics of this family of proteins and contain the conserved motifs I, II, and III; ^{31}DMDNT^{35}, ^{166}SAA^{168}, and ^{242}K/^{261}GDTPDSD^{267} in PChP, and ^{32}DFDET^{36}, ^{122}SDA^{124}, ^{177}K/^{202}GDGAND^{207} in PHOSPHO1. Both enzymes also share the two aspartyl residues in motif I, the seryl residue in motif II, and two aspartyl residues in motif III. These two enzymes differ, however, in two respects: (1) in an extra residue in motif II (D123) that contributes to substrate specificity of PHOSPHO1 [18], and which is not present in PChP; and (2) in the presence of an extra D265 residue in motif III of PChP, which is absent in PHOSPHO1. Because in both enzymes the same catalytic pocket is formed by the three motifs, it is possible that PChP D265 may be also involved in the stabilization of motif III, joining together with D267, or it might contribute to substrate specificity in a similar manner, as does aspartyl residue D123 of the PHOSPHO1 phosphatase.

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