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Transition from octahedral to tetrahedral geometry causes the activation or inhibition by Zn^{2+} of *Pseudomonas aeruginosa* phosphorylcholine phosphatase

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Abstract *Pseudomonas aeruginosa* phosphorylcholine phosphatase (PchP) catalyzes the hydrolysis of phosphorylcholine, which is produced by the action of hemolytic phospholipase C on phosphatidylcholine or sphyngomielin, to generate choline and inorganic phosphate. Among divalent cations, its activity is dependent on Mg^{2+} or Zn^{2+}. Mg^{2+} produced identical activation at pH 5.0 and 7.4, but Zn^{2+} was an activator at pH 5.0 and became an inhibitor at pH 7.4. At this higher pH, very low concentrations of Zn^{2+} inhibited enzymatic activity even in the presence of saturating Mg^{2+} concentrations. Considering experimental and theoretical physicochemical calculations performed by different authors, we conclude that at pH 5.0, Mg^{2+} and Zn^{2+} are hexacoordinated in an octahedral arrangement in the PchP active site. At pH 7.4, Mg^{2+} conserves the octahedral coordination maintaining enzymatic activity. The inhibition produced by Zn^{2+} at 7.4 is interpreted as a change from octahedral to tetrahedral coordination geometry which is produced by hydrolysis of the $\left[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2\right]$ complex.

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Introduction

Pseudomonas aeruginosa phosphorylcholine phosphatase (PchP) is synthesized when bacteria utilize choline, betaine, dimethylglycine, or carnitine as sources of energy. PchP catalyzes the hydrolysis of phosphorylcholine (or phosphocholine) to choline and inorganic phosphate (Pi) (Lisa et al. 1994, 2007). PchP is a member of the haloacid dehalogenase (HAD) superfamily (Massimelli et al. 2005; Beassoni et al. 2006) as it contains the three characteristic conserved sequence motifs (I, II, and III) of this superfamily. In mature PchP, which contains 327 amino acids because it has lost the 22 amino acid signal peptide, motifs I, II, and III are the following: ^{31}**D**M**D**NT^{35}, ^{166}**S**, and ^{242}**K**/^{261}G**D**TPDS**D**^{267}, respectively. Considering the molecular model (Beassoni et al. 2008) based on the crystal structure of *Methanococcus jannaschii* phosphoserine phosphatase (PSP) (Wang et al. 2001), the coordination of Mg^{2+} occurs with a pair of electrons contributed by oxygen atoms from the $-COO^-$ of ($O\delta_1$)D31 and ($O\delta_2$)D262, the backbone C=O of D33, and one oxygen of the phosphate moiety (O_1–P) of the substrate, thereby forming a monodentate complex M–O_1–P–$(O)_3$ (Beassoni et al. 2008). Kinetic experiments performed at pH 5.0 with *p*-nitrophenylphosphate (*p*-NPP) as a substrate

indicated that either the native periplasmic enzyme (Domenech et al. 1992) or the recombinant PchP (Beassoni et al. 2008) had a greater affinity for Zn^{2+} and Cu^{2+} than for Mg^{2+}. In terms of catalytic efficiency, Zn^{2+} and Cu^{2+} also were better than Mg^{2+} (Beassoni et al. 2008). These results were explained by considering the concept of chemical hardness and softness which states that hard acids prefer to coordinate to hard bases and soft acids to soft bases (Parr and Pearson 1983). Considering our previous results and following our studies with PchP, we investigated how Mg^{2+} and Zn^{2+} could affect PchP activity with phosphorylcholine at pH 5.0 and 7.4. These pH values were selected because with this substrate and Mg^{2+}, the optimal pH was a plateau between 5.0 and 8.0 (Salvano and Domenech 1999).

Materials and methods

Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG), restriction enzymes, molecular biology regents, and HisLink™ resin were purchased from Promega. *p*-NPP and phosphorylcholine were purchased from Sigma (St. Louis, MO). All other chemicals were of pro-analysis quality.

Bacterial strains and growth conditions

E. coli strains were routinely grown at 37°C in Luria–Bertani (LB) medium supplemented with 150 μg mL^{-1} ampicillin. *E. coli* XL10 Gold strain (Stratagene) was used for plasmid maintenance and *E. coli* BL21 CodonPlus (Stratagene) was used for protein overexpression.

Cloning and enzyme purification

$pchP_{327}$ was subcloned in pET15b (Novagen) as an *Eco*R I-*Nde* I fragment from pTYB-$pchP_{327}$ (Beassoni et al. 2006), and the pET15-*pchP* vector was obtained to express PchP as the N-terminal fusion to a $MGSS(H)_6SSGLVPRGSH$ tag. The PchP His-tag fusion protein was expressed in *E. coli* BL21 Codonplus (Stratagene) as follows. A 1-L culture was grown in LB medium containing 150 μg mL^{-1} of ampicillin and 0.5% glucose until $OD_{550} = 0.6$–0.8, and then protein expression was induced with 0.4 mM IPTG for 18 h at 18°C. The culture was harvested at 5,000 rpm for 10 min. The pellet was suspended in 33 mM HCl–Tris, pH 8.0, 40×. An equal volume of shock solution (40% sucrose, 33 mM Tris–HCl, pH 8.0, 1.5 mM EDTA plus ≅100 μg of lysozyme) was added, and the mixture was incubated at room temperature for 15 min with soft agitation. The mixture was centrifuged at 5,000 rpm for 10 min, the supernatant was discarded, and an equal volume of ice cold 1 mM $MgCl_2$ was quickly added to the pellet. After 10 min incubation at 0°C, the mixture was centrifuged at 6,000 rpm for 10 min, the supernatant containing the periplasmic fraction was discarded, the pellet containing the spheroplasts was suspended in equal proportions of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the extract was disrupted by sonication. Finally, the extract was clarified by centrifugation for 15 min at 14,000 rpm. The supernatant was incubated with 2–3 mL of HisLink™ Protein Purification Resin overnight at 4°C. After this incubation, the resin was loaded onto a column, and purification was performed with an imidazole gradient in column buffer (50 mM NaH_2PO_4, 300 mM NaCl) according to the manufacturer's instructions. Purified enzymes were dialyzed against 10 mM Tris–HCl, pH 8.0, with 30% glycerol and stored at −20°C. The protein yield was 15–20 mg L^{-1} with a specific activity, measured with 10 mM *p*-NPP and 2 mM Mg^{2+} of 110 μmol *p*-nitrophenol min^{-1} (mg protein)$^{-1}$. The specific activity of the same preparation, measured with 0.05 mM phosphorylcholine and 2 mM Mg^{2+}, was 54.5 μmol inorganic phosphate min^{-1} (mg protein)$^{-1}$.

Molecular modeling and molecular dynamics

PchP was modeled based on the atomic coordinates of 1f5s using a combination or threading server Protein Fold Recognition Server (Phyre) and comparative modeling software MODELLER 9v6 (Sali and Blundell 1993) while secondary structure restraints were added according to PSI-PRED predictions. The phosphate, Mg^{2+} and two water molecules were added by superimposing the final model with 1f5s.

Protein determination, enzyme activity, and kinetic data analysis

Protein concentration was determined by spectrophotometric measurement at 280 nm using the theoretical molar extinction coefficient ($\varepsilon = 69{,}915\ M^{-1}\ cm^{-1}$) calculated with the "ProtParam" tool for physicochemical parameter prediction, which is available at the Expasy server (Gasteiger et al. 2005). The standard assay to measure acid phosphatase activity was performed with 0.06 mM phosphorylcholine as substrate, 10 mM Mg^{2+} or 0.1 mM Zn^{2+} as activators, buffer at pH 5.0, 7.4, or as indicated, and 0.01–0.05 μg of enzyme. The buffers utilized were 100 mM NaAc/HAc (pH 5.0), 100 mM KAc/HAc (pH 6.0), and 50 mM Hepes adjusted with NaOH to pH 6.8 or 7.4. The Pi formed after incubation was determined by the addition of ammonium molybdate, Tween 20, and malachite green according to (Baykov et al. 1988). The $\varepsilon_{630\ nm}$ utilized was $53{,}100\ M^{-1}\ cm^{-1}$. One unit of PchP was defined as the amount of enzyme that released 1 μmol of inorganic phosphate (Pi) from phosphorylcholine per minute at 37°C. Kinetic data were analyzed with the computer program DYNAFIT (http://www.biokin.com/dynafit) (Kuzmic 1996) by assuming rapid equilibrium kinetics (Segel 1975, Cornish Bowden 1995).

p*K*a values for ionization of *O*-phosphocholine was kindle provided by ACD, Inc (www.acdlabs.com) who utilized *ACD/pKa v8.02* for Windows. The calculated pKa_1 and pKa_2 values were 1.59 ± 0.10 and 6.05 ± 0.30, respectively. Considering the pKa_1 and pKa_2 values for the ionization of *O*-phosphocholine and the equation of Henderson-Hasselbalch, at pH 5.0 and at 7.4 the concentration of O=P(O–CHOLINE) $(OH)O^-$ and $O=P(O–CHOLINE)(O^-)_2$ are approximately 250 times greater than the respective acid conjugated. Under the assay conditions used in this work, the anionic forms of *O*-phosphocholine are neutralized by monovalent cations, Na^+ or K^+ or are forming bonds with water molecules or protein groups, one of them belonging to the D33 residue. Therefore, the relationship $–COO^-$/–COOH from D33 is altered at pH 5.0 and 7.4, and consequently may be interfering, in an indirect way, with the coordination of D33 –C=O group with the metal ion. Since this possibility is true either when the enzyme activity is measured in the presence of Mg^{2+} or Zn^{2+} the protonation/deprotonation of *O*-phosphocholine at different pH do not change by the presence of Mg^{2+} or Zn^{2+}. This concept was reinforced by the below results showing, that the PchP activity measured in the presence of Mg^{2+} was not modified at pH 7.4. Therefore, it was assumed that the activation at pH 5.0 or inhibition at pH 7.4 produced by Zn^{2+} was caused by an intrinsic property of this ion. The charges of $O=P(O–CHOLINE)(OH)O^-$ and $O=P(O–CHOLINE)(O)_2^{=}$ were not considered because $–(O–CHOLINE)(OH)O^-$ or $–(O–CHOLINE)(O)_2^{=}$ are not included in the coordination sphere of the metal ion (Beassoni et al. 2008, Fig. 4).

Results

The amino acids from motifs I, II, and III plus the water molecules involved in coordination of the metal ion and the hydrophobic zone formed by the amino acids previous to motifs I, II, and III are shown in Fig. 1. The Mg^{2+} binding site involves two carboxylic groups corresponding to residues D31 and D262, the backbone of the D33 carbonyl group, and the oxygen bound to the phosphate moiety ($O=PO_3$). The rest of the octahedral coordination sphere is completed by two water molecules (Fig. 1), one of which is associated with D267 from motif III and the same oxygen atom of the phosphate moiety bound to D31. The second water molecule is bound to the carboxylic groups from D262 and E42, since it is closer with respect to D40 or E43 (Fig. 1, D40 in PchP corresponds to E20 in *M. janaschii*), via a bridge with a water molecule to the central metal. This model also shows the amino acid residues previous to motifs I (^{27}YAVF^{30}) and III (^{257}ILVA^{260}) that are responsible for creating the environment of low dielectric constant (ε) necessary for coordination of the metal ion (Dudev and Lim 2000). The amino acid residues ^{162}VYVI^{166} previous to the motif II ^{166}S are not involved with the cation coordination but do form the hydrophobic structure which is related to the phosphate moiety of the substrate.

Data obtained with phosphorylcholine at pH 5.0 indicated that when PchP activity was measured under optimal conditions of substrate and cation activator concentrations, a higher specific activity was obtained with Zn^{2+} compared to Mg^{2+} (Fig. 2). At pH 7.4, the enzyme activity measured with Mg^{2+} was practically identical to the activity measured at

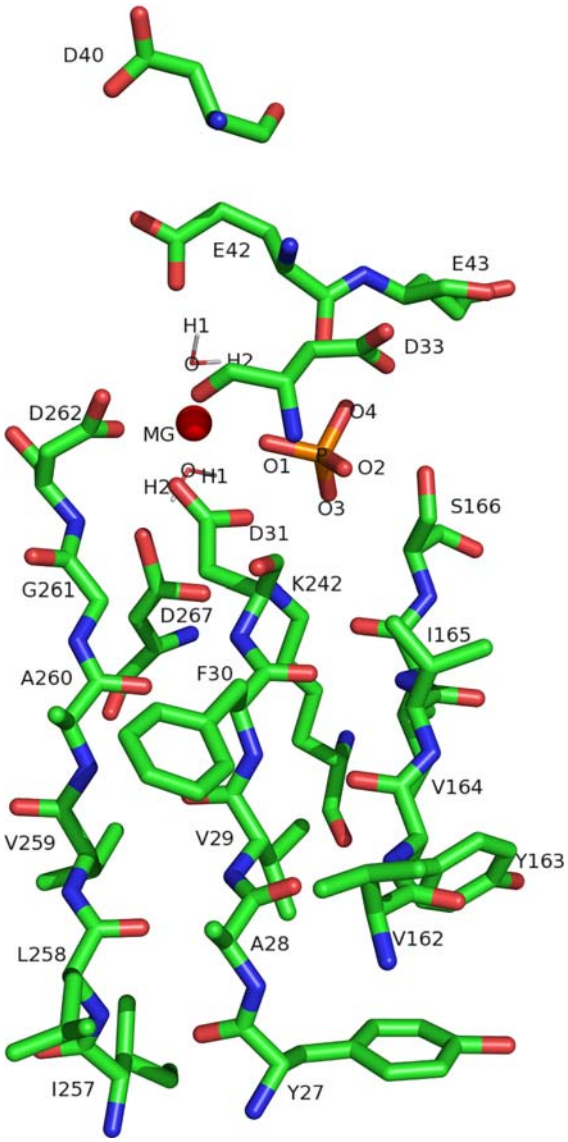

Fig. 1 Molecular model of PchP active site plus the hydrophobic pocket surrounding the active site. By alignment, Asp 40 in PchP is equivalent to Glu 20 of *Methanococus janaschii* phosphoserine phosphatase

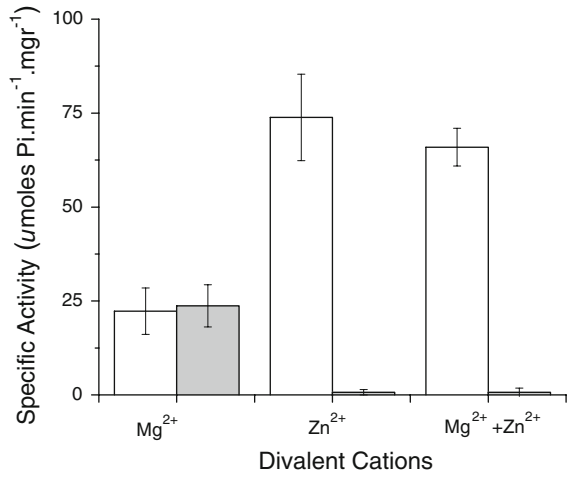

Fig. 2 Specific activity of PchP measured at pH 5.0 (*open rectangle*) or 7.4 (*filled rectangle*). The assay conditions were: 0.06 mM phosphorylcholine plus 10 mM Mg^{2+}, 0.1 mM Zn^{2+} in 100 mM HAc/NaAc at pH 5.0, or 50 mM HEPES at pH 7.4. Values ± SD are the average of three or more independent experiments

pH 5.0. However Zn^{2+} did not produce enzyme activation (Fig. 2). At pH 7.4, with saturating Mg^{2+} concentrations, very low Zn^{2+} concentrations inhibited enzymatic activity (Fig. 2). Inhibition curves produced by crescent concentrations of Zn^{2+}, performed at pH 7.4, are shown in Fig. 3A. According to data analyzed with the simulator Dynafit®, the inhibition produced by Zn^{2+} indicated that this cation may bind to the free enzyme (E) or the PchP-Mg^{2+} ($E.Mg^{2+}$) complex with the same affinity (K_i1 = 0.35 μM), thereby forming the nonproductive complex E-Zn^{2+} (Fig. 3B). Determination of PchP activity at different pH values with phosphocholine and Mg^{2+} revealed that in the range of pH 5.0–7.4, the activity dependent on Mg^{2+} was not affected (Fig. 4A). Figure 4A also shows that after pH 5.0, the activation by Zn^{2+} decreased to 60% at pH 6.8 and was practically zero at pH 7.4. Incubation of the enzyme with Zn^{2+} at pH 7.4, with or without Mg^{2+}, produced an inactive complex unable to catalyze the hydrolysis of phosphorylcholine. However, this inhibition was reversible because when the same tube was adjusted from pH 7.4 to pH 5.0, nearly full activity was recovered (Fig. 4B).

Discussion

To explain the above experiments we considered fundamentally theoretical calculations related to the coordination of metal ion with water and their hydrolysis at different pH values, the interaction of carboxylic and carbonyl groups, the substitution of metal ions in the active site, and the hydrophobic cavity, with a low ε, proximal to the active site of PchP. In aqueous solutions, Mg^{2+} and Zn^{2+} are hexahydrate complexes, and $[Mg(H_2O)_6]^{2+}$ (Katz

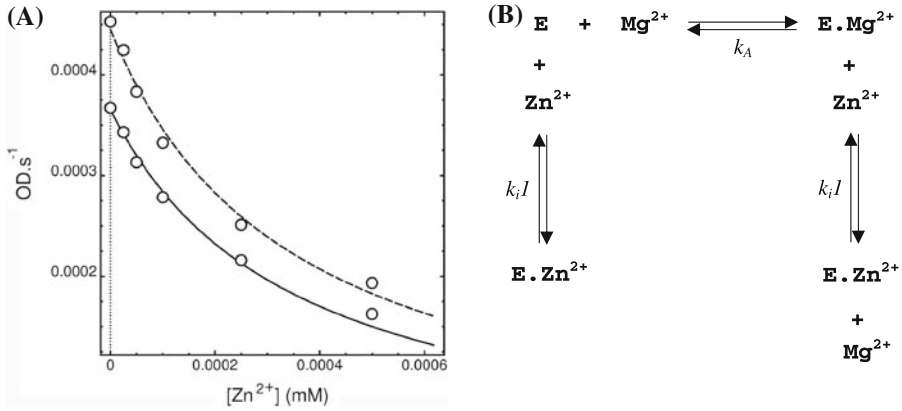

Fig. 3 Effect of variable Zn^{2+} concentrations on PchP activity measured in the presence of Mg^{2+} ions. Experiments were performed at pH 7.4 with 0.06 mM phosphorylcholine and 0.5 mM Mg^{2+} (*dotted line*) or 10 mM Mg^{2+} (*solid line*) (**A**). Proposed model of inhibition following DYNAFIT analysis of the same data (**B**)

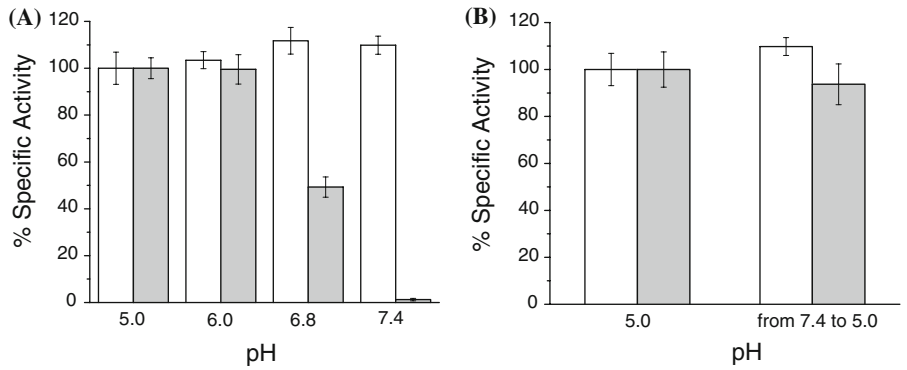

Fig. 4 Effect of pH on PchP activity measured as in Fig. 2 with 10 mM Mg^{2+} (*open rectangle*) or 0.06 mM Zn^{2+} (*filled rectangle*). The buffers used were: 100 mM HAc/NaAc, pH 5.0, 100 mM HAc/KAc, pH 6.0, 50 mM Hepes/NaOH, pH 6.8, and 50 mM Hepes/NaOH, pH 7.4 (**A**). Reversibility of inhibition by Zn^{2+} (**B**). Initially, the enzyme was incubated with 0.06 mM Zn^{2+} in 100 mM HAc/NaAc buffer at pH 5.0 or 50 mM HEPES buffer at pH 7.4. After incubations, the pH of the enzyme incubated at pH 7.4 was adjusted to pH 5.0. The enzyme concentration was adjusted by dilution. Therefore, the enzyme activity in both preparations was measured at pH 5.0 with the same enzyme concentration

et al. 1998; Pavlov et al. 1998; Lightstone et al. 2001) and $[Zn(H_2O)_6]^{2+}$ (Bochatay and Pearson 2000; Li et al. 2004; Zhu and Pan 2005) are in an octahedral arrangement. Metal ions are Lewis acids, and in aqueous solution the hydrated ions behave as acids in terms of Brønsted-Lowry acid–base theory. The first hydrolysis step is generically given as $[Me(H_2O)_n]^{q+} + H_2O \rightleftharpoons [Me(H_2O)_{n-1}(OH^-)]^{(q-1)+} + H_3O^+$. The dissociation constant, p*K*a, for this reaction is more or less linearly related to the charge-to-size ratio of the metal ion (Tanaka and Osaki 1967). Thermodynamic parameters calculated for deprotonation reactions in $[Mg(H_2O)_6]^{2+}$ support the assertion that deprotonation occurs only for the first water molecule (Kluge and Weston 2005). The energy required to deprotonate one of the water molecules of the $[Mg(H_2O)_6]^{2+}$ complex is less than that required to deprotonate an isolated water molecule, and removal of a proton from one of the water molecules in $[Mg(H_2O)_6]^{2+}$ yields a complex that maintains its octahedral structure (Katz et al. 1998). According to Bochatay and Pearson (2000) and Zhu and Pan c(2005), this is not the case for $[Zn(H_2O)_6]^{2+}$ hydrolysis since the geometry of coordination changes from octahedral to tetrahedral when the pH is near neutral. If in the $[Mg(H_2O)_6]^{2+}$ complex one of the six water molecules is replaced with a carboxylate group to form $[Mg(H_2O)_5\,(HCOO)^-]^+$, a large amount of energy is required to deprotonate the remaining magnesium-bound water molecules. This effect may be interpreted as an increase in p*K*a value, making proton-transfer in the reaction $[Mg(H_2O)_5(COO)^-]^+ + H_2O \rightarrow [Mg(H_2O)_4(OH)^-(COO)^-] + H_3O^+$ "an unlikely mechanism for the formation of a magnesium-bound

hydroxide group in biological systems" (Katz et al. 1998). The simultaneous presence of enzyme and metal ions in solution leads to the formation of the enzyme-metal ion complex, $E + Me^{2+} \rightarrow EMe^{2+}$. In PchP, this implies that substitution of water molecules bound to the metal ion by O-ligands found in the carboxylic group of D31 and D262 and the carbonyl group of D33. This conclusion is supported because water-ligand exchange is thermodynamically preferable (Dudev and Lim 2001, 2003; Kluge and Weston 2005) and occurs in a solvent-inaccessible enzyme cavity with low ε (Dudev and Lim 2000, 2003). The PchP model shows that there was a hydrophobic cavity in the vicinity of the catalytic site of PchP, allowing the hexahydrate of Mg^{2+} or Zn^{2+} to exchange its first shell water for carboxylic and carbonyl groups. Based on theoretical calculations of changes of free energies (ΔG_{ex}) for the reaction $[Mg(H_2O)_6]^{2+} + L^z \rightarrow [Mg(H_2O)_5L]^{2+z}$ (L for ligand), Dudev and Lim (2001) showed that, in a low ε environment, $[Mg(H_2O)_6]^{2+}$ will more easily exchange formate (equivalent to deprotonated aspartic) than formamide (equivalent to the carbonyl group of the backbone peptide). Therefore, the negatively charged aspartic residues D31 and D262 substitute for the first two water molecules. The preference for both negatively charged ligands is useful for neutralizing the positive charge of Mg^{2+} to form a complex capable of coordinating with the oxygen atom from the carbonyl group and the oxygen atom O=P(O–CHOLINE)$(O^-)_2$/O=P(O–CHOLINE)(OH)O^-/O=P(O–CHOLINE)$(OH)_2$ from the phosphate moiety of phosphocholine. This forms the $\left[Mg^{2+}L_2^{-1}L_2^0(H_2O)_2\right]$ complex maintaining octahedral coordination geometry. Similarly, it can also occur when PchP catalyzes the reaction at pH 5.0 with Zn^{2+} as an activator. This possibility is supported by Bochatay and Pearson (2000) and Zhu and Pan (2005), which indicate that hydrolysis of Zn^{2+} will change the coordination geometry from octahedral to tetrahedral when the pH is increased from acidic to alkaline levels. Considering the high affinity of Zn^{2+} for PchP [5], (Fig. 3b) and that Zn^{2+} is also octahedrally coordinated, the loss of catalytic activity produced at pH 7.4 may be caused by a change in the general charge of the $\left[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2\right]$ complex from neutral to -1 or -2, of the type $\left[Zn^{2+}L_2^{-1}L_2^0(HO)^{-1}(H_2O)\right]^{-1}$ or $\left[Me^{2+}L_2^{-1}L_2^0(HO)_2^{-1}\right]^{-2}$, respectively. These changes in the charge of the complex ion would produce changes in the coordination geometry in the metal binding site of PchP from an octahedral (active enzyme) to a tetrahedral (inactive enzyme) arrangement. The final point to be considered is the selectivity of the metal ion in the PchP catalytic pocket. The K_A values obtained for $Zn^{2+} \ll Mg^{2+}$ (Beassoni et al. 2008) implies that in the PchP active site, the substitution reactions occur as follows: $[MgL_4(H_2O)_2] + [Zn(H_2O)_6]^{2+} \rightarrow [ZnL_4(H_2O)_2] + [Mg(H_2O)_6]^{2+}$. Exchange in this direction, but not the reverse, is energetically favorable. It has been shown that the Mg^{2+} binding sites are not specific for Mg^{2+} and that other divalent cations, especially Zn^{2+}, may replace Mg^{2+} in its binding site (Dudev and Lim 2001). Therefore, another hexaaquo metal ion, at a determined pH, can substitute for Mg^{2+} in maintaining the catalytic activity with a similar global conformation of the protein. Thus, the change to PchP activity (higher activation produced by Zn^{2+} vs. Mg^{2+}) depends on characteristics of the metal complex and its steric and electronic properties. Our above results are in perfect accordance with this possibility since Zn^{2+} was able to form the E-Zn^{2+} complex through substitution of Mg^{2+} in the E-Mg^{2+} complex. We believe that the PchP inhibition produced by Zn^{2+} at pH 7.4, even in the presence of Mg^{2+}, is due to the substitution of Mg^{2+} by Zn^{2+} and that at moderately alkaline pH values this ion prefers a tetrahedral geometry, making it incapable of maintaining the correct coordination geometry in the active site.

Considering that PchP mutants D31E and D33E did not have enzymatic activity when measured with *p*-NPP or phosphocholine in the presence of Mg^{2+} or Zn^{2+} (Beassoni et al. 2006, 2008), and that hydrolysis of the substrate involves a nucleophilic attack on the phosphorus atom achieved by the participation of D31 and D33 (Beassoni et al. 2008), it is possible that the change of octahedral to tetrahedral coordination might fundamentally affect the coordination of the D31 and D33 carboxyl groups, thus producing an inactive enzyme while in a neutral or mildly alkaline environment.

From a physiological perspective, the differential dependence of PchP on Mg^{2+} or Zn^{2+} may indicate a regulatory pathway controlled by the environment of the hydrolytic sequence produced by hemolytic phospholipase C (acting coordinately on phosphatidylcholine or

sphyngomielin) to produce phosphocholine, which is transformed by PchP into choline and Pi. Mg^{2+} is one of the most important cations, participating in all pathways necessary to sustain life in all living organisms. Zn^{2+}, though to a lesser extent than Mg^{2+}, is a cation that is quantitatively important for developing structural and catalytic functions. Frequently, this ion develops its function as a tetrahedral complex (http://tanna.bch.ed.ac.uk, Harding 2000, 2001). We believe that *P. aeruginosa* PchP prefers to use Mg^{2+} as a cofactor (despite the very low affinity with respect to Zn^{2+}) due to the abundance of this cation and its ability to work in a broad range of pH conditions. When bacteria are found in environments below pH 6.0 with Mg^{2+} or Zn^{2+}, PchP activity is largely increased and this activity may more easily provide choline and Pi for the settlement and infection of *P. aeruginosa*. The increase of other ionic species of bacterial periplasmic Zn^{2+} subjected to neutral or alkaline pH conditions can trigger a strong inhibitory effect on PchP activity, thus stopping the production of choline and Pi, and in this way forcing the bacteria to consume other nutrients in the environment.

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