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Structure-based rationalization of urease inhibition by phosphate: novel insights into the enzyme mechanism

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Abstract The structure of Bacillus pasteurii urease (BPU) inhibited with phosphate was solved and refined using synchrotron X-ray diffraction data from a vitrified crystal (1.85 Å resolution, 99.3% completeness, data redundancy 4.6, R-factor 17.3%, PDB code 6UBP). A distance of 3.5 Å separates the two Ni ions in the active site. The binding mode of the inhibitor involves the formation of four coordination bonds with the two Ni ions: one phosphate oxygen atom symmetrically bridges the two metal ions (1.9–2.0 Å), while two of the remaining phosphate oxygen atoms bind to the Ni atoms at 2.4 Å. The fourth phosphate oxygen is directed into the active site channel. Analysis of the H-bonding network around the bound inhibitor indicates that phosphate is bound as the H₂PO₄⁻ anion, and that an additional proton is present on the Oδ2 atom of Asp³⁶³, an active site residue involved in Ni coordination through Oδ1. The flexible flap flanking the active site cavity is in the open conformation. Analysis of the complex reveals why phosphate is a relatively weak inhibitor and why sulfate does not bind to the nickels in the active site. The implications of the results for the understanding of the urease catalytic mechanism are reviewed. A novel alternative for the proton donor is presented.

Keywords Urease · Bacillus pasteurii · X-ray diffraction · Nickel · Phosphate

Abbreviations AHA: acetohydroxamic acid · BME: β-mercaptoethanol · BPU: Bacillus pasteurii urease · DAP: diamidophosphate · DFT: density functional theory · JBU: jack bean urease · KAU: Klebsiella aerogenes urease · NAT: native enzyme · PHO: phosphate · PPD: phenyl phosphorodiamidate

Introduction

Competitive phosphate inhibition of urease (urea amidohydrolase, E.C. 3.5.1.5), a nickel-containing metalloenzyme, was first described in 1934 [1] and later confirmed by several authors [2, 3, 4, 5, 6]. The elucidation of the urease-phosphate interaction at the molecular level should lead to a deeper understanding of the mechanism of catalysis. Two detailed investigations have been reported, dealing with the kinetic characterization of the interaction between phosphate and Klebsiella aerogenes urease (KAU) [5] and jack bean (Canavalia ensiformis) urease (JBU) [7]. JBU consists of six subunits, each made of 840 amino acids [8, 9], while KAU has an (αβγ)₃ quaternary structure, with 101, 106, and 567 residues in the α, β, and γ subunits, respectively [10]. The αβγ fragment of KAU is highly homologous to the single subunit of JBU [11].

Phosphate competitively inhibits KAU in the pH range 5.0–7.0. The inhibition is weak and not purely competitive at pH > 7.0, while the enzyme is labile at
pH < 5.0. Within the pH range 5.0–7.0 the inhibition is pH dependent, with values of $K_i$ increasing from ca. 0.1 mM at pH 5 to ca. 50 mM at pH 7. Within this pH range, $pK_i$ exhibits a slope of −1 from pH 5.0 to 6.3, and a slope of −2 from pH 6.3 to 7.0. These results suggest that phosphate inhibition of urease requires protonation of two ionizable groups with $pK_{a1} = 6.3$ and $pK_{a2} < 5$ [5].

Similar results were more recently obtained for JBU [7], where the pH dependence of phosphate inhibition was investigated in the range 5.8–8.1. It was shown that phosphate is a competitive inhibitor in the pH range 5.8–7.5, with $K_i$ values increasing from 0.53 mM at pH 5.8 to 123 mM at pH 7.5. Similar to KAU, the slope of $pK_i$ versus pH was −1 in the pH range 5.8–6.5 and −2 in the range 6.5–7.5. These results revealed the existence of two ionizable groups having dissociation constants corresponding to $pK_{a1} = 6.5$ (similar to the value of 6.3 observed for KAU) and $pK_{a2} = 7.2$. At pH values higher than 7.6 there was no competitive inhibition. The interpretation of the kinetic data led the authors to suggest that $pK_{a3}$ corresponds to the deprotonation of the true inhibitor, the $H_2PO_4^−$ anion [7].

Comprehensive analysis of these data, together with the established conservation of the active site residues in KAU and JBU [11], leads to the conclusion that phosphate inhibition of urease involves three protonation sites, with $pK_i$ values of ca. 7.2, 6.5, and <5. Several different inhibition mechanisms can therefore be proposed, depending on whether the three values of $pK_i$ are associated with the inhibitor or with active site residues.

In the absence of structural details for a phosphate-urease complex, the following interpretation of the overall kinetic data were inferred [5, 7]. The enzyme active site was known to contain a group with $pK_i = 6.5$, assigned to a histidine residue [12, 13], suggesting the assignment of $pK_{a1}$ to this residue. Moreover, the kinetic data on JBU strongly supported the view that the $pK_{a3}$ of 7.2 corresponds to the deprotonation of the $H_2PO_4^−$ anion. This would leave the lowest $pK_{a2} (<5)$ assigned to either the first deprotonation of $H_2PO_4^−$ ($pK_{a1} = 6.5$) or to a deprotonation of an active site residue (possibly a carboxylate group of Asp or Glu).

The determination of the native (NAT) structures of two microbial ureases, from K. aerogenes [14] (PDB code 1FWJ, resolution 2.0 A) and Bacillus pasteurii [15] (PDB code 2UBP, resolution 2.0 A), provides a reliable background for a better understanding of the inhibition mechanism by phosphate. These two ureases are essentially identical in terms of backbone structure. In particular, the three $x$ subunits, constituting the core of the $(xβ)3$ trimer, consist of an $xβ$ barrel domain and a $β$-type domain. In turn, the $β$ subunits, located on the surface of the trimer, feature a predominantly $β$ structure. Finally, the $γ$ subunits consist of $xβ$ domains located on top of each pair of $x$ subunits, favoring their association to form the trimers of trimers. The structures also reveal a highly conserved position of the metal ions and the amino acid residues in the active site [16].

In the structure of native B. pasteurii urease (BPU), two Ni ions are bridged by the carboxylate group of the carbamylated Lys220, bound to Ni(1) through O81 and to Ni(2) through O62. Ni(1) is further coordinated by His1238 Nδ and His1275 Nε, while Ni(2) is bound to His1237 Nε, His1239 Nε, and Asp1263 Oδ1 (Fig. 1A). The Ni-Ni distance is 3.7 Å. The coordination geometry is pseudo square pyramidal for the penta-coordinated Ni(1) and pseudo octahedral for the hexa-coordinated Ni(2), in agreement with EXAFS data on native BPU [17]. Four water/hydroxide molecules constitute a tetrahedral cluster in the active site; one of these ($W_4$) symmetrically bridges the two Ni ions, and was proposed to be in the hydroxo form, while two additional water molecules, $W_1$ and $W_2$, bind Ni(1) and Ni(2), respectively. The fourth water ($W_3$) is not involved in any coordination bonds with Ni, but is H-bonded to the first three solvent molecules [15, 16].

The structures of BPU complexed with several inhibitors have also been determined. In BPU inhibited with β-mercaptoethanol (BME) (PDB code 1UBP, resolution 1.65 Å) the thiolate atom symmetrically bridges the binuclear Ni center, with a Ni-Ni distance of 3.1 Å. BME further chelates Ni(1) via its terminal OH [16, 18] (Fig. 1B), resulting in both Ni ions being penta-coordinate. The structure of BPU inhibited with acetoxy-oxamic acid (AHA) (PDB code 4UBP, resolution 1.55 Å) shows the inhibitor symmetrically bridging the two penta-coordinate Ni ions in the active site through the hydroxamate oxygen. The Ni ions are separated by 3.53 Å. The inhibitor further chelates one Ni ion through the carbonyl oxygen [19] (Fig. 1C). In crystals of BPU obtained from solutions containing phenyl phosphorodiamidate (PPD, PDB code 3UBP), a molecule of its enzymatic hydrolysis product, diaminophosphoric acid (DAP) [20], is coordinated to Ni(1) and to Ni(2) using three of the four atoms potentially available for coordination (Fig. 1D). One oxygen atom of DAP, originally identified as a P-OH group [15] and later proposed to be the anionic P-O' moiety [21], bridges the two Ni ions, while one oxygen and one nitrogen atom bind to Ni(1) and Ni(2), respectively. The second nitrogen atom of DAP points away towards the cavity opening [15, 16]. The Ni-Ni distance is 3.8 Å. The binding of DAP does not change the coordination geometry of the Ni ions with respect to the native enzyme, consistent with EXAFS studies reported for PPD-inhibited BPU [17].

The present report describes the structure determination of BPU crystallized in the presence of phosphate (PHO). This is the first structure of a urease complexed with phosphate, and reveals the unexpected tridentate binding mode of this inhibitor. The structure of the PHO-BPU complex provides a rationale for the kinetic data on phosphate inhibition, and suggests a mechanism for the formation of the phosphate-urease complex, allowing some deeper insights into the enzymatic catalysis of urea hydrolysis.