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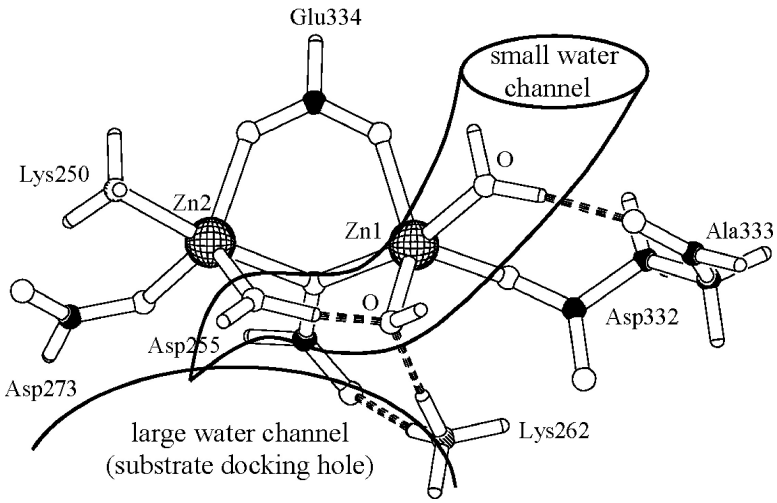

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A Water Sluice Is Generated in the Active Site of Bovine Lens Leucine Aminopeptidase

Stefan Erhardt,[†] Elke Jaime,[‡] and Jennie Weston*,[‡]

Institute of Organic and Macromolecular Chemistry, Friedrich Schiller University, Humboldtstrasse 10, D-07743 Jena, Germany and Fachbereich Chemie, Phillips Universität, Hans Meerwein Strasse, D-35032 Marburg, Germany

Received November 30, 2004; E-mail: c9weje@uni-jena.de

One of the major problems in interpreting the mode of action of zinc-based bimetalloenzymes is that, in most cases, the structure of the reactive species is not known. The hypersurface of zinc is intrinsically quite flat (easy change in coordination number and geometry[1]), and in the presence of water, the possibility of specific hydration (to an unknown extent) must be considered.

Bovine lens leucine aminopeptidase (*bl*LAP) is a widespread bizinc hydrolase whose function is to remove the N-terminal residue in a peptide chain.[2] It plays a key role in the degradation and modification of proteins in the metabolism of mammals[3] and has even been implicated in human HIV pathophysiology.[4] *bl*LAP has a hexameric structure[5] in which each monomer functions independently of the others.[6] The active site (one per monomer) is sitting on the edge of two water channels: a large one for the substrate[7] located below the two metal ions and a much smaller one (function unknown) located above Zn1 (Figure 1).[5,8]

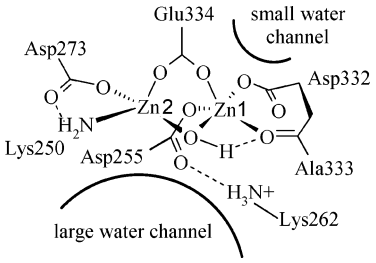

Figure 1. Solid-state structure of the active site of *bl*LAP.

We have modified simple DFT models for the active site of *bl*LAP developed by us[9] and Karplus et al.[10] to include part of the backbone connecting Ala333 to Asp332. We found that this linkage is necessary for modulating the coordination of the carbonyl group to Zn1. In addition, we used an NH_4^+ ion to model the side chain of Lys262.[11] Lys262 is involved in the hydrolysis mechanism[12] and provides direct hydrogen-bond stabilization in inhibitor complexes.[13] Using this extended model for the resting state of the enzyme (species **2**), we have examined the effect of specific water molecules to investigate the role of the second smaller water channel as well as to identify likely candidates for the nucleophilic species in the mode of action of *bl*LAP. Our calculations show that at least five intermediates **1**−**5**, all in equilibrium with each other, have to be considered.

X-ray structural analyses of *bl*LAP show that the solid-state form of the active site contains a bridging μ-OH unit,[8] which has been postulated to be the nucleophilic species.[7] The position of the NH_4^+ group in the Lys262 side chain is flexible and has a ca. 2.5 Å radius of allowed motion below Zn1. There is no direct hydrogen bond between N_{lys262} and Oμ in the solid state (the distance between these atoms is 4.7 Å; pdb file 1LAM). However, in most of the inhibitor complexes, N_{lys262} has moved in ca. 2 Å to help stabilize the tetrahedral structure of the complex. We find that, if we constrain the NH_4^+ ion in our model to remain within this radius of allowed motion, it spontaneously moves in to form a hydrogen bond to Oμ. Except for the N_{lys262}−Oμ distance, the individual calculated parameters for complex **2** match the solid-state structure quite satisfactorily. The free electron pair on Oμ is purely p in character ($\epsilon_{lpr} = -9.0$ eV) and is well-positioned for nucleophilic attack on a substrate approaching Zn2.

As we have discussed earlier,[9] Zn1 exhibits an unusual trigonal bipyramidal coordination. The carbonyl (amide bond to Ala333) is only loosely coordinated. Zn1 easily undergoes a pseudorotation that pulls the μ-OH unit to the Zn1 side of the active site. A localized, Zn1-bound hydroxide is formed in a slightly endothermic process (+3.3 kcal/mol). An AM1/MM study has postulated that such a monodentate Zn1-bound hydroxide is the active species in the mode of action of *bl*LAP.[14] Judging by the energy of the p orbital ($\epsilon_{lpr} = -8.1$ eV), species **1** is indeed slightly more nucleophilic than **2**.

Placement of a water in the small water channel (located just above Ala333 in the active site) and optimization resulted in complex **3**. The water *spontaneously* inserted itself (quasi barrierless process) into the relatively weak Zn1−O carbonyl bond. Intermediate **3** is only slightly less stable (+0.9 kcal/mol) than the corresponding μ-OH species **2** and a free water. The inserted water in **3** cannot function as a nucleophile since it is located too far away from the substrate docking channel.

Species **3** then undergoes a slightly exothermic (−1.6 kcal/mol) conversion to **4**. A pseudorotation on Zn1 lowers its coordination to four by breaking the μ-OH contact. A metal-bound H_3O_2 functionality results. The hypersurface is quite flat, and after extensive calculations, we conclude that the interconversion barrier is less than 3 kcal/mol. Similar "loosely bridged" H_3O_2 zinc species have been considered in DFT and MD calculations on β-lactamase isolated from *Bacteroides fragilis*.[15] Experimental support for **4** is independently offered by several solid-state structures of biomimetic zinc complexes.[16] Intermediate **4** contains a lone pair on the Zn2-bound water that is optimally oriented to attack a substrate. Similar to **1**, this orbital is activated for nucleophilic attack ($\epsilon_{lpr} = -8.7$ eV) as compared to **2** or **3**. Kinetic investigations on binuclear zinc complexes have also reported that H_3O_2 species are intrinsically more reactive than μ-OH units.[16,17] Table 1 shows hybridization and NBO energies of the nucleophilic lone electron pair on oxygen in **1**−**5**.

Complex **4** is capable of adding a second water molecule to form **5**, which we describe as a "water sluice". Species **5** can be expected to be every bit as nucleophilic as **4** ($\epsilon_{lpr} = -8.6$ eV) and is only slightly (3.5 kcal/mol) more unstable. Although this activated Zn2-

[†] Phillips University.
[‡] Friedrich Schiller University.

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Table 1. Hybridization (%p) and NBO Energies (eV) of the Nucleophilic Lone Electron Pair on Oxygen in **1**−**5**

		%p	ϵ_{lpr} (eV)
1	O_{Zn1}	96	−8.1
2	O_{μ}	100	−9.0
3	O_{μ}	99	−9.1
4	O_{Zn1}	87	−10.2
	O_{Zn2}	100	−8.7
5	O_{Zn1}	88	−9.9
	O_{Zn2}	100	−8.6

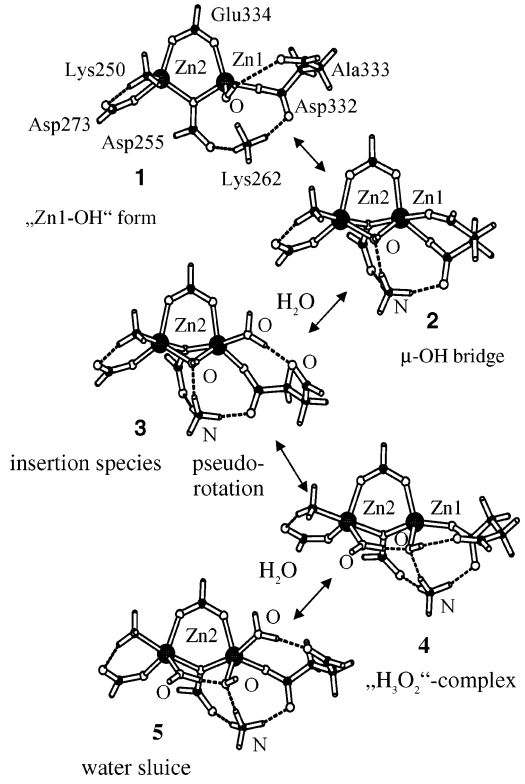

Figure 2. Hydrated intermediates found for the active site of *bl*LAP.

bound oxygen is actually a "water", it can very easily be converted into the OH form by a hydrogen transfer to the Zn1-bound "OH" when needed ($\Delta E_{H-transfer} < 0.8$ kcal/mol). The "H_3O_2" units in both **4** and **5** are stabilized by a strong hydrogen bridge between the NH_4^+ (Lys262) and the Zn1-bound oxygen, very similar to the stabilization observed in most known inhibitor complexes of *bl*LAP.

These calculations provide a plausible mechanism for continually regenerating a series of species capable of acting as active nucleophiles in the *bl*LAP mediated hydrolysis of peptides. A second water channel coupled with an easily reversible change in the coordination number (via pseudorotation) on Zn1 makes this possible. A key feature is the role of the weakly bound carbonyl ligand: it functions as a "traffic cop" to direct water coming from the small channel into the heart of the active site. In view of these new mechanistic possibilities, we are currently investigating the mechanism of substrate docking and subsequent nucleophilic attack in the mode of action of *bl*LAP.

All calculations were performed at the B3LYP/lanl2dz level using Gaussian98.[18] Cleanly isolating the transition structures for the interconversions **1** ⇌ ⇌ **5** proved to be quite difficult due to the extremely flat hypersurface that is directly coupled with pseudorotation on Zn1. We therefore performed explicit IRC calculations to verify that these interconversions are extremely facile processes. More reliable energies were obtained by B3LYP/aug-cc-pVTZ (Zn; SSD+2f1g[19]) single-point calculations on the B3LYP/lanl2dz geometries. NBO analyses of the bonding orbitals in **1**−**5** were performed using B3LYP/[D95(first row atoms) + 6-311+G(d) (Zn)]//B3LYP/lanl2dz wave functions. The B3LYP method has been reported to prefer bidentate coordination modes for zinc-bound carboxylates (models for Asp and Glu) with the smaller lanl2dz basis and monodentate modes with the larger 6-311+G(d,p) basis.[10] In our case, the H-bond network provided by Lys262 avoids this problem, and we find that B3LYP/6-311+G(d,p) geometries (calculated for selected structures) are very similar to their B3LYP/lanl2dz counterparts. Basis set effects will be discussed in a forthcoming full article on the mode of action of *bl*LAP. Table 2 shows equilibrium positions for the interconversions **1** ⇌ ⇌ **5**.

Table 2. Equilibrium Positions (Gibb's Free Energies in kcal/mol) for the Interconversions **1** ⇌ ⇌ **5**

	Δ*G* (kcal/mol)		Δ*G* (kcal/mol)
1 ⇌ **2**	+3.3	**3** ⇌ **4**	−1.6
2 + H_2O ⇌ **3**	+0.9	**4** + H_2O ⇌ **5**	+3.5

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