

## Molecular Details of Urease Inhibition by Boric Acid: Insights into the Catalytic Mechanism

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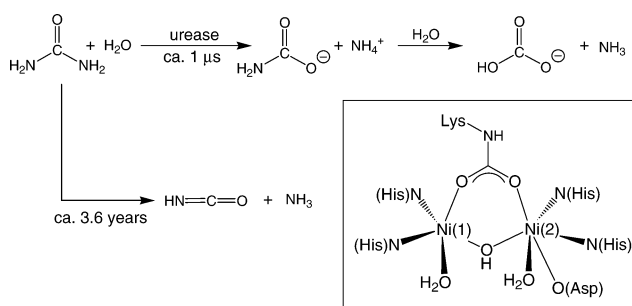
Urea, formed in large amounts as catabolic end-product for nitrogen-containing compounds, has a half-life of ca. 3.6 years at 38 °C in aqueous solution.<sup>1</sup> Environmental problems would occur were it not for the production, by plants, fungi, and bacteria, of the enzyme urease,<sup>2,3</sup> able to increase the rate of urea hydrolysis 10<sup>14</sup> times (Scheme 1).<sup>4</sup> The reaction results in a pH increase, responsible for negative effects of urease activity in human health and agriculture. Urease is a virulence factor in infections of urinary and gastrointestinal tracts,<sup>5</sup> possibly causing severe diseases such as peptic ulcers and stomach cancer as in the case of *Helicobacter pylori*,<sup>6</sup> while the efficiency of soil nitrogen fertilization with urea (the most used fertilizer worldwide) decreases due to ammonia volatilization and root damage caused by soil pH increase.<sup>7</sup> Control of the activity of urease through the use of inhibitors could counteract these negative effects.

Boric acid, B(OH)<sub>3</sub>, acts as a competitive inhibitor of ureases from jack bean,<sup>8</sup> *Proteus mirabilis*,<sup>9</sup> and *Klebsiella aerogenes*,<sup>10</sup> with a K<sub>i</sub> of 0.12, 0.1, and 0.33 mM, respectively. The inhibition is maximal between pH 6.2 and 9.3, suggesting that only the neutral trigonal B(OH)<sub>3</sub>, and not the B(OH)<sub>4</sub><sup>-</sup> anion, is an inhibitor of urease. The detailed mechanism of bacterial urease inhibition by boric acid could not be established by kinetic studies alone. Here we report on the structural determination of a urease–boric acid complex, which clarifies the molecular details of the inhibition, revealing a unique binding mode for this inhibitor while providing insights into the role of the nickel ions in enzymatic urea hydrolysis.

Urease (urea amidohydrolase, E.C. 3.5.1.5) is a large heteropolymeric metalloprotein characterized by the presence of a dinuclear Ni<sup>2+</sup> center in the three independent active sites. The structures of three bacterial ureases, from *K. aerogenes*,<sup>11</sup> *Bacillus pasteurii*,<sup>12</sup> and *H. pylori*,<sup>13</sup> indicate the conservation of the main structural features of the metal center (Scheme 1), with the Ni ions bridged by the carboxylate group of a carbamylated Lys and a hydroxide ion. The two Ni ions differ in their coordination environment, with Ni(1) bound to two His N and a terminal water molecule (NiN<sub>2</sub>O<sub>3</sub>) and Ni(2) bound to two His N, a terminal water molecule, and an additional carboxylate Asp O (NiN<sub>2</sub>O<sub>4</sub>). Several structures of inhibitor complexes with *B. pasteurii* urease (BPU) are available, all of them indicating a substantial lability of the terminal water molecules and the bridging hydroxide, while the protein framework involving the ligands and the metal ions is rigidly maintained, except for a mechanistically significant rotational freedom for the side chain of the Ni(2)-bound Asp residue.<sup>12,14–16</sup>

The 2.10 Å structure of BPU, crystallized in the presence of B(OH)<sub>3</sub>, confirms the rigidity of the protein scaffold, with an overall

Scheme 1



backbone RMSD of only 0.2 Å from the structure of the native enzyme. The helix–loop–helix flap flanking the active site channel is in the open conformation, as found in the native enzyme.<sup>12</sup> The 2F<sub>o</sub> – F<sub>c</sub> difference map indicates that the arrangement of the protein ligands around the Ni ions is essentially identical to that observed in native BPU. The difference map shows an additional trigonal piece of electron density in the vicinity of the Ni ions, toward the open side of the active site channel. The density suggests the presence of a planar trigonal moiety bridging the dinuclear Ni center through two atoms. The trigonal molecule has almost exactly replaced the two water molecules terminally bound to Ni(1) and Ni(2), as well as a third water molecule, present in native BPU and completing a tetrahedral topology of solvent molecules in the native enzyme. The trigonal volume of electron density can be satisfactorily modeled using the trigonal form of boric acid, as shown also in the omit maps in Figures 1a and 1b.

B(OH)<sub>3</sub> is symmetrically placed between the Ni ions (nonbonded B–Ni distances of 2.9 and 3.0 Å), leaving in place the bridging hydroxide and not perturbing the Ni–Ni distance (3.7 and 3.6 Å in the native and B(OH)<sub>3</sub>-inhibited forms, respectively). Two inhibitor oxygen atoms are bound to the Ni ions (at 2.2 and 2.1 Å), while the third oxygen points toward the cavity opening, away from the Ni ions. As a result of boric acid binding, the geometry and coordination number of the Ni ions do not change significantly.

The Ni(1)-bound inhibitor oxygen atom receives an H-bond from His<sup>α222</sup> Nε (at 2.6 Å), the latter being protonated because of the interaction of His<sup>α222</sup> Nδ with the peptide NH group of Asp<sup>α224</sup>. The Ni(2)-bound inhibitor oxygen atom forms an H-bond with Ala<sup>α170</sup> O (at 2.8 Å), while the distal inhibitor OH group is H-bonded to a water molecule involved in an H-bonding network with an additional solvent molecule and Ala<sup>α366</sup> O.

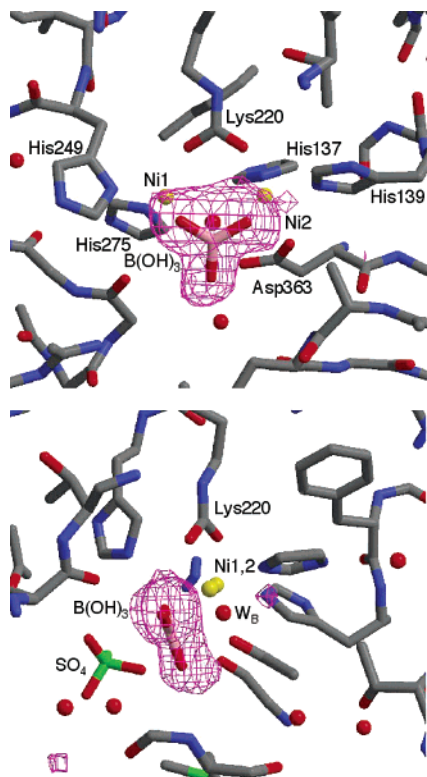
The H-bonding network between the inhibitor atoms and the active site residues has in the past provided a clear definition of the protonation state of the bound inhibitors, in most cases clarifying and complementing the pH dependence of the inhibition kinetics. In the present case, the protonation state of the inhibitor is well

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**Figure 1.** (a) Omit map of the boric acid molecule calculated with Fourier coefficients  $F_o - F_c$  and phases derived from the final model from which the boric acid atoms have been removed (purple map, contoured at  $4.0\sigma$ ). (b) Omit map as in panel a, but rotated  $90^\circ$  along a vertical axis and showing the topology of the Ni ions (lined up and almost overlapping), the bridging hydroxide, and the boric acid inhibitor.

established as the neutral  $B(OH)_3$  form, and the H-bonding network simply highlights the role of neighboring residues in stabilizing an unprecedented binding mode of  $B(OH)_3$  to a dinuclear center. The present structure provides important clues for drug design of more potent urease inhibitors based on the boric acid functionality.

The isoelectronic structure of  $B(OH)_3$  and urea, the bridging-chelating binding mode of boric acid, the topology of the H-bonding network, the replacement of the labile water molecules in the active site by a neutral trigonal molecule, and the presence of the nonsubstituted Ni-bridging hydroxide in the complex of urease with  $B(OH)_3$  all provide evidence that gives further support to the proposal of a mechanism involving a topologically similar binding of the substrate urea to the Ni ions in the active site and a key role for the bridging hydroxide as the nucleophile in the reaction.<sup>12</sup> In the present structure,  $B(OH)_3$  can be considered a *substrate analogue*, revealing the molecular details of the step of substrate

binding. This evidence, coupled with the structure of diamidophosphate-bound BPU,<sup>12</sup> a *transition-state analogue*, completes a molecular puzzle for the mechanism of the reaction. The lack of reactivity of  $B(OH)_3$  with the bridging hydroxide (placed at  $2.1 \text{ \AA}$  from the B atom, in a direction almost perpendicular to the plane of the molecule, Figure 1c) could be due to unfavorable symmetry and energy of the highest energy orbital carrying the two electrons necessary for the bond formation (the HOMO) on the nucleophile and the lowest energy empty orbital (the LUMO) on the inhibitor. Detailed calculations, involving not only the immediate coordination environment of the Ni ions<sup>17</sup> but also second shell ligands, are necessary to understand fully the electronic parameters guiding the enzymatic reaction, possibly also revealing the reason why Ni is preferred over Zn for this key reaction in biology.

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**Supporting Information Available:** Experimental details (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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