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Kinetic and structural studies reveal a unique binding mode of sulfite to the nickel center in urease

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ABSTRACT

Urease is the most efficient enzyme known to date, and catalyzes the hydrolysis of urea using two Ni(II) ions in the active site. Urease is a virulence factor in several human pathogens, while causing severe environmental and agronomic problems. *Sporosarcina pasteurii* urease has been used extensively in the structural characterization of the enzyme. Sodium sulfite has been widely used as a preservative in urease solutions to prevent oxygen-induced oxidation, but its role as an inhibitor has also been suggested. In the present study, isothermal titration microcalorimetry was used to establish sulfite as a competitive inhibitor for *S. pasteurii* urease, with an inhibition constant of 0.19 mM at pH 7. The structure of the urease–sulfite complex, determined at 1.65 Å resolution, shows the inhibitor bound to the dinuclear Ni(II) center of urease in a tridentate mode involving bonds between the two Ni(II) ions in the active site and all three oxygen atoms of the inhibitor, supporting the observed competitive inhibition kinetics. This coordination mode of sulfite has never been observed, either in proteins or in small molecule complexes, and could inspire synthetic coordination chemists as well as biochemists to develop urease inhibitors based on this chemical moiety.

1. Introduction

Urease (urea aminohydrolase, EC 3.5.1.5) is a nickel-dependent non-redox enzyme whose catalytic function is the hydrolysis of urea to yield ammonia and carbamate at a rate 10^{15} times higher than the uncatalyzed reaction, making it the most efficient enzyme known to date [1]. Carbamate then spontaneously evolves to produce another molecule of ammonia and carbon dioxide. Urease catalyzes this last step of organic nitrogen mineralization in bacteria, fungi, plants, algae and invertebrates [2–5]. The overall hydrolysis of the products generated by urease activity determines an increase in pH of the surrounding milieu, causing negative consequences in medical and agricultural settings [2–7]. This enzyme represents the main virulence factor for a large variety of ureolytic human pathogens such as *Helicobacter pylori* [8], *Mycobacterium tuberculosis* [9], *Yersinia enterocolitica* [10], *Cryptococcus neoformans* [11], and *Proteus mirabilis* [12]. Furthermore, ureolytic bacteria expressing urease are widespread in soils that are treated with urea, a nitrogen fertilizer used worldwide [13], and their activity contributes to a number of significant environmental and economic problems such as loss of nitrogen from soil and release of ammonia in the atmosphere, ammonia toxicity for plants, and seedlings damage [14]. In all these instances, a tight control of urease activity is required to counteract its deleterious effects. For this purpose, several classes of molecules have been proposed and tested, both in medicine and agriculture, as urease inhibitors [4,15–18].

Knowledge about the structure of the nickel-containing active site cavity has been derived through studies on native ureases isolated from several sources [19–22], which revealed a conservation in the coordination environment around the two Ni(II) ions (Fig. 1A).

On the basis of structural information obtained from the crystal structures of native *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii*) urease (SPU) [18,20] and of its complexes with a range of other ligands [18,20,23–27], a general scheme of the catalytic mechanism of ureases was proposed [1,5,20,28–31]. In this mechanism, the nickel-bridging hydroxide acts as the nucleophilic group that attacks the urea molecule chelating the bimetallic nickel cluster using an oxygen atom and a nitrogen atom. An extended network of second shell hydrogen bonds appears to stabilize the substrate binding during the catalytic process. In addition, a flexible flap changes the active site channel from an open to a closed conformation (Fig. 1B).

Sulfite has been extensively used as a preservative in solutions of jack bean (*Canavalia ensiformis* urease, JBU) [32,33] and SPU [18,20,23–27,34,35]. The role of sulfite as a stabilizer of the urease activity has been interpreted in the past as due to the maintenance of the redox state of the conserved cysteine residue on the enzyme active site flap, which is essential for enzyme activity in its reduced thiol form (Fig. 1B, C). Indeed, it is known that the thiol groups of cysteines

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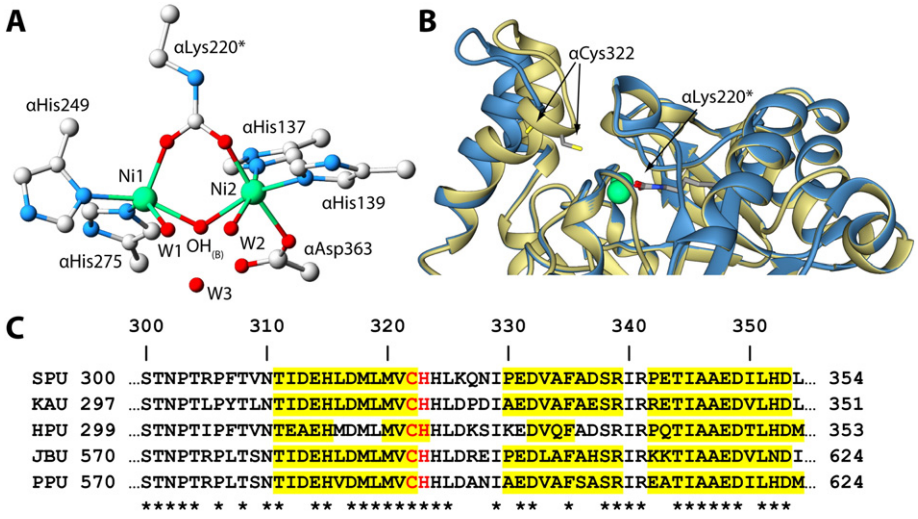
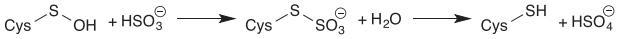


Fig. 1. (A) Details of the active site of native SPU. Atoms are colored according to the atom type. (B) Superimposition of the open (blue ribbons, 4CEU) and closed (yellow ribbons, 3UBP) conformation of the flexible flap. Ni(II) ions are shown as green spheres. The αCys322 side chain is reported as "sticks". (C) Multiple sequence alignment of the flap region of the ureases for which a crystal structure is available: SPU, *Klebsiella aerogenes* urease (KAU), *Helicobacter pylori* urease (HPU), jack bean urease (JBU), and pigeon pea urease (PPU). The position of SPU αCys322 and αHis323 are in red, while the α-helices are highlighted in yellow. The asterisks indicate the fully conserved amino acids.

can form oxygen derivatives such as sulfenic (Cys-SOH), sulfinic (Cys-SO_2H), and sulfonic (Cys-SO_3H) functionalities [36], while stable sulfenic acids may be produced by mild oxidation of sterically hindered thiols [37]. In the case of the sulfenic functional group, the role of sulfite could be the intermediate formation of an enzymatically inactive S-sulfocysteine (Cys-S-SO_3^-), in turn undergoing hydrolysis to sulfate and back to Cys-SH:



In addition, it has been shown that the enzymatic activity of JBU solutions, stored in the presence of β-mercaptoethanol (BME) but in contact with air oxygen, decreases [32] because of the formation of a mixed disulfide bond involving BME and the conserved active site flap Cys592 [22]. This process is reverted by treatment of inactivated urease with sulfite [32] possibly through the reduction of this disulfide bond [38]:

The opposite action of BME and sulfite on JBU was also suggested on the basis of an increase in the anodic electrophoretic mobility of native urease treated with sodium sulfite caused by an increased negative charge of the protein molecule; this process could be reversed by treatment with BME and was explained by the formation of S-sulfocysteines involving the several cysteine residues on the surface of JBU [39].

In addition to a protective action on the urease active site cysteine, sulfite has been reported to be an inhibitor of JBU, with a competitive inhibition mechanism deduced from the temperature dependence of the activation energy of the urea enzymatic hydrolysis as a function of sulfite concentration [40]. A pH-dependent kinetic study further suggested that the bisulfite mono-anion, and not the sulfite di-anion, is the actual inhibitor of JBU with inhibition constants in the milli-molar range, and that this process does not entail an interaction of bisulfite with the sulfhydryl group of essential cysteines, but rather an addition to the active site, whose nature was still very obscure at the time [41]. These early reports were later supported by a study that indicated sulfite acting as a competitive inhibitor for JBU with an inhibition constant $K_i = 2.23 \pm 0.45$ mM at pH 7.0 [42]. This inhibition role of sulfite on urease was then proposed to involve a direct interaction with the Ni(II) ions in the active site on the basis of an apparent increase of the nickel affinity of JBU in the presence of this anion [38].

In this study, we report a molecular characterization of the inhibition of SPU by sulfite. In particular, we describe and discuss the results of pH-dependent kinetic measurements carried out using a calorimetry-based assay, indicating that sulfite acts as a competitive inhibitor of SPU. This conclusion is supported by the crystal structure of the SPU–sulfite complex at 1.65 Å resolution, showing an unprecedented binding of the inhibitor to the active site Ni(II) ions through its three oxygen atoms.

2. Materials and methods

2.1. Protein purification

S. pasteurii DSM 33 cells were obtained using a modification of a previously described procedure [18], and SPU was isolated in a pure form to a specific activity of about 2500 units per milligram using the following protocol. *S. pasteurii* cells, resuspended in a buffer containing 50 mM phosphate pH 7.5, 50 mM Na_2SO_3, 1 mM EDTA (buffer A), additionally containing 10 mM $MgCl_2$ and 20 μg/mL DNAse I, were disrupted by three passages through a French® pressure cell press (SLM Aminco) at 20,000 psi. The soluble fraction was obtained by differential centrifugation, first at 30,000 ×*g* for 30 min and then at 150,000 ×*g* for 2 h, in order to remove cell debris and aggregates. The crude extract was dialyzed overnight against buffer A and then loaded onto a Q Sepharose XK 50/20 (GE Healthcare) anionic exchange column, previously equilibrated with the same buffer. A step gradient procedure was used to elute the protein with increasing ionic strength (NaCl in buffer A was used at concentrations of 150, 350, 450 mM) at a flow rate of 5 mL min^{-1}. The active fractions, eluted and detected at a concentration of 350 mM NaCl, were pooled and the ionic strength raised to 1 M using $(NH_4)_2SO_4$. After centrifugation at 30,000 ×*g* for 15 min to remove the precipitate, the solution was loaded onto a Phenyl Sepharose XK 26/20 (GE Healthcare) hydrophobic interaction column equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$ and eluted with a linear gradient from 1 M to 0 M at a flow rate of 3 mL min^{-1}. Urease, eluted with 400 mM $(NH_4)_2SO_4$, was concentrated using an Amicon ultrafiltration cell equipped with a membrane of 100,000 Dalton molecular weight cut-off. The resulting enzyme solution was loaded onto a Superdex 200 XK 16/60 (GE Healthcare) gel filtration column equilibrated with buffer A, containing 150 mM NaCl in order to prevent non-specific