

Inhibition of urease by bismuth(III): Implications for the mechanism of action of bismuth drugs

Li Zhang¹, Scott B. Mulrooney², Andy F.K. Leung¹, Yibo Zeng¹, Ben B.C. Ko¹, Robert P. Hausinger^{2,*} & Hongzhe Sun^{1,*}

¹Department of Chemistry and Open Laboratory of Chemical Biology, The University of Hong Kong, Pokfulam, Hong Kong, P.R. China; ²Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, 48824-4320, USA; *Author for correspondence (Tel: +517-355-6463 Ext. 1610; Fax: +517-353-8957; E-mail: hausinger@msu.edu; Tel: +852-2859-8974; Fax: +852-2857-1586; E-mail: hsun@hkucc.hku.hk)

Received 18 October 2005; Accepted 24 November 2005

Key words: bismuth, inhibition, nuclear magnetic resonance spectroscopy, urease, UV-vis spectroscopy

Abstract

Bismuth compounds are widely used for the treatment of peptic ulcers and *Helicobacter pylori* infections. It has been suggested that enzyme inhibition plays an important role in the antibacterial activity of bismuth towards this bacterium. Urease, an enzyme that converts urea into ammonia and carbonic acid, is crucial for colonization of the acidic environment of the stomach by *H. pylori*. Here, we show that three bismuth complexes exhibit distinct mechanisms of urease inhibition, with some differences dependent on the source of the enzyme. Bi(EDTA) and Bi(Cys)₃ are competitive inhibitors of jack bean urease with K_i values of 1.74 ± 0.14 and 1.84 ± 0.15 mM, while the anti-ulcer drug, ranitidine bismuth citrate (RBC) is a non-competitive inhibitor with a K_i value of 1.17 ± 0.09 mM. A ¹³C NMR study showed that Bi(Cys)₃ reacts with jack bean urease during a 30 min incubation, releasing free cysteines from the metal complex. Upon incubation with Bi(EDTA) and RBC, the number of accessible cysteine residues in the homohexameric plant enzyme decreased by 5.80 ± 0.17 and 11.94 ± 0.13 , respectively, after 3 h of reaction with dithio-bis(2-nitrobenzoic acid). Kinetic analysis showed that Bi(EDTA) is both a competitive inhibitor and a time-dependent inactivator of the recombinant *Klebsiella aerogenes* urease. The active C319A mutant of the bacterial enzyme displays a significantly reduced sensitivity toward inactivation by Bi(EDTA) compared with the wild-type enzyme, consistent with binding of Bi³⁺ to the active site cysteine (Cys³¹⁹) as the mechanism of enzyme inactivation.

Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme found in a wide variety of plants and a broad range of bacterial species where it catalyzes the hydrolysis of urea to form carbonic acid and two molecules of ammonia (Mobley *et al.* 1995; Sirko & Brodzik 2000; Hausinger & Karplus 2001). Jack bean urease exists as a homo-hexamer, with each subunit (~91 kDa) containing two nickel ions (Riddles *et al.* 1991;

Sheridan *et al.* 2002), whereas bacterial ureases are heteropolymers with subunits that resemble portions of the eukaryotic sequence. For example, *Klebsiella aerogenes* and *Bacillus pasteurii* ureases are comprised of three distinct subunits ($\alpha\beta\gamma$) and form a heterotrimer ($(\alpha\beta\gamma)_3$) with three-fold symmetry (Jabri *et al.* 1995; Pearson *et al.* 1997; Benini *et al.* 1999). In contrast, *Helicobacter pylori* urease contains only two types of subunits ($\alpha\beta$), with the heterodimer forming a trimer ($(\alpha\beta)_3$) which further assembles to form a supramolecular

dodecameric assembly $((\alpha\beta)_3)_4$ (Ha *et al.* 2001). The X-ray crystal structures of urease from *K. aerogenes* (Jabri *et al.* 1995; Sheridan *et al.* 2002), *B. pasteurii* (Pearson *et al.* 1997), *H. pylori* (Ha *et al.* 2001) and jack bean (Sheridan *et al.* 2002) reveal a conserved coordination of Ni^{2+} in the dinuclear nickel centers; thus, insights derived from studies of urease from one source are generally applicable to those of other sources. The detailed mechanism of urea hydrolysis (Suarez *et al.* 2003) involves urea coordination to the metallocenter and either hydrolysis by a metal-bound hydroxide with release of ammonia and carbamate or elimination to form cyanate followed by hydrolysis.

H. pylori, a Gram-negative pathogenic bacterium which specifically colonizes the human gastric mucosa, has been regarded as a primary causative agent of chronic gastritis and peptic ulcer diseases including mucosa-associated lymphoid tissue lymphoma (Wotherspoon *et al.* 1991; Blaser 1993). Although *H. pylori* is acid sensitive and only replicates at pH of 7–8, it survives in the stomach under highly acidic conditions (Stingl *et al.* 2001, 2002a, b; Stingl & De Reuse 2005). Urease activity is believed to be essential for the colonization of the gastric mucus by *H. pylori* (Lee 1996) and for the survival of *H. pylori* at very acidic pH (Mobley *et al.* 1991; Bauerfeind *et al.* 1997). It is required for acid survival *in vitro* and for infection of the normal acid-secreting stomach (Eaton *et al.* 1991; Perez-Perez *et al.* 1992; Akada *et al.* 2000; Scott *et al.* 2002). The enzyme leads to the production of ammonia, which maintains the periplasmic pH value at 6.2 (Rektorschek *et al.* 1998). Synthesis of this enzyme accounts for as much as 10% of the total protein in *H. pylori* (Bauerfeind *et al.* 1997; Davis & Mobley 2005).

Bismuth compounds have been used as therapeutic agents for more than two centuries. Bismuth subsalicylate (Pepto-Bismol®), colloidal bismuth subcitrate (De-Nol®) and ranitidine bismuth citrate (RBC, Pylorid®) have been widely used for the treatment of diarrhea, dyspepsia and peptic ulcers all over the world (Lambert & Midolo 1997; Briand & Burford 1999; Sun *et al.* 2004). Various studies have shown that the effectiveness of bismuth-containing drugs is due to their bactericidal action against *H. pylori*, although the detailed mechanism of action remains unclear (Butcher *et al.* 1992; Prewett *et al.* 1992). Enzyme inhibition has long

been thought to play an important role in the effectiveness of bismuth drugs. A brief report indicated that urease inhibition by a bismuth β -mercaptoethanol complex was ca. 1000-fold more effective than β -mercaptoethanol alone (Asato *et al.* 1997). Given both the importance of urease for infection by *H. pylori* and the enhanced inhibition of the bismuth β -mercaptoethanol complex, we hypothesized that bismuth drugs may exert their antibacterial action through the inhibition of urease.

In the present study, the inhibitory effects of several bismuth complexes on urease were examined. Because urease is very highly conserved in sequence and three-dimensional structure, we expected that the source of enzyme to study would not be critical; nevertheless, we chose to examine both plant and bacterial enzyme representatives by focusing on jack bean and *K. aerogenes* ureases. Three different bismuth complexes were characterized for their mechanisms of reversible inhibition of jack bean urease. NMR and chemical modification studies were used to provide evidence that urease Cys residues bind bismuth. By using the recombinant *K. aerogenes* urease and its Cys319A variant, we demonstrated for the first time that binding of Bi^{3+} to an active site cysteine residue (Cys³¹⁹) contributes to bismuth inhibition of urease.

Materials and methods

Materials

Jack bean urease samples, with specific activities of 928 and 21.3 units per mg protein, were purchased from Sigma (Catalogue No: U-0251 and U1500, respectively). One unit of the plant enzyme activity liberates 1.0 μmol of NH_3 from urea per min at pH 7.0 and 25 °C. Urea (Sigma, ultrapure), phenol red, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma), 2-[4-(2-hydroxyethyl)-1-piperazino]-ethanesulphonic acid (HEPES, Acros), 2-(N-morpholino) ethanesulphonic acid (MES, Acros), bismuth subnitrate ($\text{Bi}(\text{NO}_3)_3$, Aldrich), sodium hypochlorite (13% active chlorine, Acros), sodium nitroprusside (ABCR), ^{13}C -labeled-L-cysteine (^{13}C , >98%, Cambridge Isotope Laboratories) were all used as received. RBC (Batch No. AWS347B) was kindly provided by GlaxoWell-

come China Ltd. (Hong Kong). All reagents were of analytical grade. $\text{Bi}(\text{Cys})_3$ was prepared by addition of 3 mol equivalents of reduced cysteine to $\text{Bi}(\text{NO}_3)_3$, followed by the adjustment of pH to ~ 5.0 . Crystalline $\text{Bi}(\text{EDTA})$ was synthesized as described previously (Summers *et al.* 1994).

Kinetic study of jack bean urease

The concentration of jack bean urease was determined spectrophotometrically from the absorbance at 280 nm by using an extinction coefficient for a 1% solution ($\epsilon_{1\%}$) of 6.2 cm^{-1} (Dixon *et al.* 1980). All UV-visible spectroscopic experiments were performed with 1-cm cuvettes on a computer-controlled HP8453 spectrometer at room temperature. Urease activity was measured spectrophotometrically at 560 nm as described previously (Mobley *et al.* 1988). Briefly, jack bean urease was added to 3 mM sodium phosphate buffer containing 7 $\mu\text{g}/\text{ml}$ phenol red and 3–24 mM urea at pH 7.0. Reactions were carried out at room temperature and rates of urea hydrolysis were calculated from linear portions of the curves. The effects of fixed concentrations of bismuth complexes were examined as described previously (Houimel *et al.* 1999).

Time-dependent inhibition of jack bean urease by bismuth complexes

Jack bean urease (U-0251) was incubated with appropriate amounts of bismuth complexes in 3 mM phosphate buffer, pH 7.0, at 25 °C. At different time intervals, aliquots were removed and added to a solution containing 7 $\mu\text{g}/\text{ml}$ phenol red and 24 mM urea in 3 mM phosphate buffer, pH 7.0. The initial rates were calculated from the maximum linear portions of the curves obtained by monitoring the reaction spectrophotometrically at 560 nm.

Thiolate group analysis of jack bean urease

The reactive thiolate content ($-\text{SH}$) of urease was measured by using the colorimetric reagent dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman 1959). Urease (0.39 mg/ml) was added to a 50 mM Tris-HCl buffer at pH 7.5 in the presence of 0.02 mM DTNB, and the reaction was monitored at 412 nm for 3 h ($\epsilon_{412} = 1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$, pH 7.5). Similar experiments were carried out for the enzyme

that had been incubated with an inhibitor (bismuth complex) at room temperature. Excess low molecular mass ligands including the inhibitor were removed by using a Centricon YM-50 and the reaction was initiated via addition of DTNB.

Release of cysteine from $\text{Bi}(\text{Cys})_3$ during incubation with jack bean urease

$\text{Bi}(\text{Cys})_3$ prepared with ^{13}C -labeled cysteine was incubated with various molar ratios of urease (U1500, $\sim 0.1 \text{ mM}$ hexamer) in 3 mM phosphate buffer at pH 7.0 in D_2O . NMR spectra were acquired on a Bruker 500 spectrometer for 20,000–30,000 transients using a 45° pulse width (5 μs), a relaxation delay of 2 s, and 16 K data points. The spectra were processed using an exponential function (8–10 Hz) before Fourier transformation.

*Purification and assay of *K. aerogenes* urease*

Wild-type *K. aerogenes* urease and its C319A mutant were expressed in *E. coli* DH5 using either pKAU17 or the site-directed mutant of pKAU17, and purified to >95% purity by a method described previously (Martin & Hausinger 1992). Urease was identified in column fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels (Laemmli 1970). Protein concentrations were assayed by the method of Lowry *et al.* (Lowry *et al.* 1951) or by using the BioRad protein assay. The enzyme was assayed at 37 °C in 50 mM HEPES, pH 7.8, in the presence of 50 mM urea. The C319A mutant, previously shown to exhibit a lower pH optimum (Martin & Hausinger 1992), was assayed at 37 °C in 50 mM MES buffer, pH 6.5, in the presence of 50 mM urea. The amount of released ammonia was assessed in aliquots sampled at time intervals by conversion to indophenol (Weatherburn 1967), and the rates were determined by linear regression analysis.

*Time-dependence of *K. aerogenes* urease inhibition by $\text{Bi}(\text{EDTA})$*

Two millimolar $\text{Bi}(\text{EDTA})$ was incubated with purified wild-type and variant ureases in 50 mM HEPES buffer at pH 7.0, 37 °C. Aliquots were taken at different time intervals and assayed for urease activity as described previously (Todd &

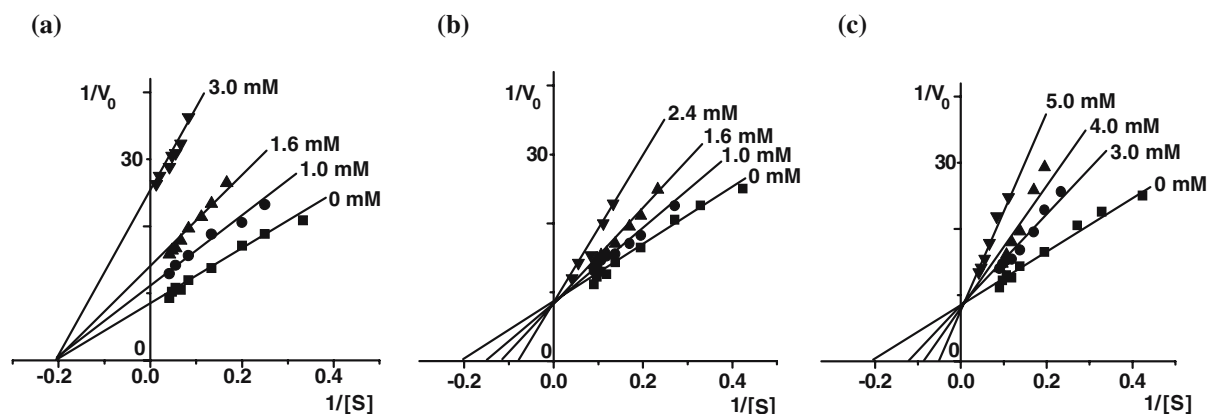


Figure 1. Inhibition of jack bean urease by RBC, Bi(EDTA) or Bi(Cys)₃. Double-reciprocal plots of $1/v_0$ versus $1/[S]$ for urea hydrolysis in the presence of the indicated concentrations of (a) ranitidine bismuth citrate (RBC), (b) Bi(EDTA) and (c) Bi(Cys)₃, respectively.

Hausinger 1991). Initial velocities were determined in the presence of different inhibitor concentrations. The K_i value was determined from re-plots of the slopes or apparent K_m values obtained in double-reciprocal plots versus inhibitor concentration (Dixon & Webb 1979).

Results

Jack bean urease inhibition by bismuth compounds

Incubation of jack bean urease (U-0251, 0.95 µg/ml) in the presence of 1.6 mM RBC, 1.6 mM Bi(EDTA), or 4.0 mM Bi(Cys)₃ led to the immediate inhibition of urease activity, with no further time-dependent changes in the reaction velocities. This result suggested that each of these compounds is a fast-binding, reversible inhibitor of the plant enzyme.

The effects of varied concentrations of RBC, Bi(EDTA), or Bi(Cys)₃ on the kinetics of jack bean urease were investigated (Figure 1). The kinetic parameters of the uninhibited reactions yielded a urea K_m of 4.85 ± 0.26 mM and V_{max} of 133.7 ± 7.33 µmol NH₃/min/mg. The addition of RBC resulted in a decrease of the apparent V_{max} while the K_m value remained almost unchanged, indicative of non-competitive inhibition. A plot of the inverse of the apparent maximal rates versus the concentration of the inhibitor allowed estimation of the inhibition constant (K_i) of 1.17 ± 0.09 mM for this compound.

In contrast to the situation for RBC, Bi(EDTA) and Bi(Cys)₃ were shown to inhibit the plant urease by a competitive mechanism (Figure 1), as shown by the absence of any effect on V_{max} while altering the apparent K_m values. On the basis of re-plots of the apparent K_m versus inhibitor concentrations the K_i values were calculated to be 1.74 ± 0.14 and 1.84 ± 0.15 mM, respectively. EDTA and cysteine were also studied as potential inhibitors at concentrations of 1.6 mM and showed no apparent inhibition to jack bean urease (data not shown).

Protection of thiol groups of jack bean urease by bismuth

In order to investigate whether thiolate groups from cysteine residues of the enzyme are involved in the binding of Bi³⁺, the number of free thiols (–SH) of the high specific activity sample of jack bean urease were assayed in the absence or presence of bismuth complexes by using DTNB. The enzyme in the absence of inhibitor was found to contain 17.79 ± 0.17 mol of ‘accessible’ thiol groups per mol of hexameric enzyme after 3 h of reaction with DTNB. After incubating the enzyme (0.39 mg/ml) with RBC (1.60 mM) or Bi(EDTA) (1.60 mM) in 50 mM Tris–HCl buffer at pH 7.5 for >45 min, the thiol contents dramatically decreased to 5.85 ± 0.09 and 11.99 ± 0.13 mol of thiol per mol of the hexamer, respectively. The decrease in the number of free thiol groups suggested that bismuth

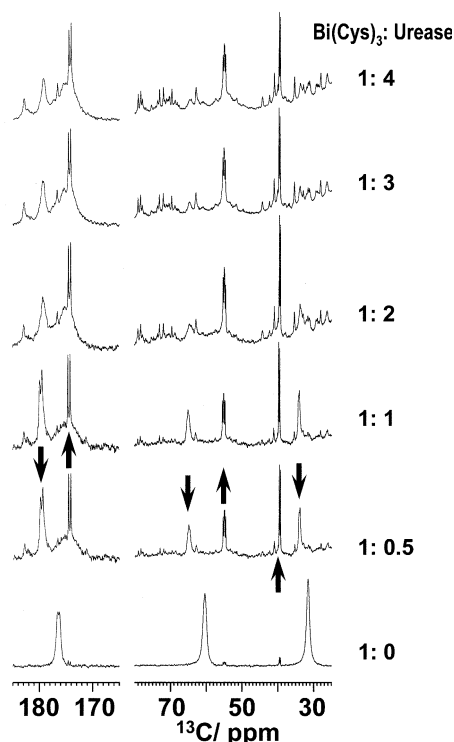


Figure 2. $\text{Bi}(\text{Cys})_3$ reacts with jack bean urease to release free cysteine: 125 MHz ^{13}C NMR spectra of $\text{Bi}(\text{Cys})_3$ after 30 min incubation with different mole equivalents of jack bean urease. The peaks labeled with upward arrows indicate the ^{13}C signals of free ^{13}C -labeled cysteine, whereas those labeled with downward arrows indicate the remaining $\text{Bi}(\text{Cys})_3$ inhibitor (shown without addition of the enzyme in the lower panel).

binds to thiol groups of the enzyme (Riddles *et al.* 1983; Sun *et al.* 1999; Busenlehner *et al.* 2002).

NMR studies of urease reactivity with $\text{Bi}(\text{Cys})_3$

^{13}C NMR spectroscopy was used to further investigate the mode of inhibition of $\text{Bi}(\text{Cys})_3$ on jack bean urease. Figure 2 shows the aliphatic region and carboxyl region of ^{13}C NMR spectra of $\text{Bi}(\text{Cys})_3$ complex prepared from ^{13}C -labeled cysteine in the presence of 0, 0.5, 1.0, 2.0, 3.0 and 4.0 mol equiv of urease (hexamer). In the absence of urease, three broad signals were observed (31.1, 60.0 and 176.2 ppm) and assigned to the bound β , α and carboxylate carbons of bismuth-bound cysteine, while three very weak signals (39.5, 55.0 and 173.9 ppm) were assigned to the corresponding signals of the free amino acid. Upon the addition of 0.5 mol equivalents of urease, the free cysteine features increased at the expense of the bismuth-

bound cysteine signals. The free-Cys features increased in intensity upon addition of 1 mol equiv of urease and reached their maximum after the addition of 2 mol equiv of urease. In contrast, the broad signals corresponding to the bismuth-bound ^{13}C -cysteine further decreased in intensity and almost disappeared upon further addition of urease. No precipitation was observed after the reaction.

Inhibition of *K. aerogenes* urease by $\text{Bi}(\text{EDTA})$ and identification of an inhibitory bismuth-binding site

To study whether a cysteine residue known to be located at the entrance of the urease active site is the binding site for Bi^{3+} , the wild-type and C319A mutant of the *K. aerogenes* enzyme were examined (Figure 3). The K_m and $V_{\max}$ of uninhibited wild-type *K. aerogenes* urease were determined to be 1.04 ± 0.08 mM and 200.0 ± 10.0 $\mu\text{mol NH}_3/\text{min}/\text{mg}$, respectively. During short time period assays where the extent of enzyme inactivation was insignificant (*vide infra*), nearly the same $V_{\max}$ values were obtained while the K_m values increased upon incubation of the enzyme with $\text{Bi}(\text{EDTA})$ (2 and 5 mM). These results suggest that $\text{Bi}(\text{EDTA})$ inhibits *K. aerogenes* urease in a reversible, competitive manner with a K_i of 2.46 ± 0.59 mM.

In addition to the reversible inhibition described above, incubation of the purified wild-type urease with 2 mM $\text{Bi}(\text{EDTA})$ led to first-order decreases in enzyme activity (Figure 3b). Significantly, the apparent first-order rate constants for inactivation of the C319A mutant and wild-type enzymes are 5.04×10^{-4} and $9.95 \times 10^{-3} \text{ min}^{-1}$, respectively. The 19-fold decrease in the rate of inactivation of the C319A mutant compared to the wild-type enzyme strongly suggests that Cys^{319} is an inhibitory binding site for Bi^{3+} .

Discussion

Despite the wide use of bismuth complexes as anti-ulcer drugs, the mechanism of their antibacterial actions against *H. pylori* remains unclear. Urease has long been thought to play a crucial role for the acid resistance of *H. pylori*, neutralizing the gastric acidity and resulting in a neutral microenvironment surrounding the bacterium within the gastric lumen, and is regarded as a valid target for anti-

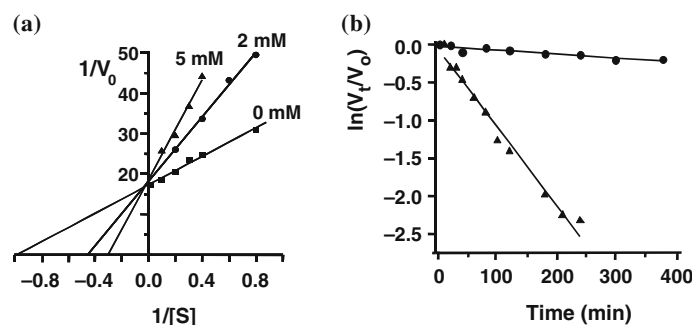


Figure 3. Inhibition of *K. aerogenes* urease by Bi(EDTA): (a) Double reciprocal plots of urea hydrolysis inhibition of the wild-type *K. aerogenes* urease by Bi(EDTA). (b) Kinetics of urease inactivation upon addition of Bi(EDTA). The wild-type and the C319A mutant urease were treated with 2 mM Bi(EDTA) in 50 mM HEPES buffer at pH 7.0. v_t/v_0 (where v_t is the velocity at time t and v_0 is the initial velocity) is shown as a function of time for wild-type ($\blacktriangle$) and C319A ($\bullet$) enzymes.

ulcer drug design. Thus, urease inhibition is a reasonable mechanism to explain the bismuth antibacterial activities.

We investigated the kinetics of inhibition of jack bean urease by three bismuth compounds. No significant time-dependent inactivation was detected, whereas all three bismuth complexes were found to be reversible inhibitors of the jack bean enzyme. The clinically used anti-ulcer drug, RBC, is a non-competitive inhibitor of the enzyme with K_i value of 1.17 mM, while Bi(EDTA) and Bi(Cys)₃ are competitive inhibitors with K_i values of 1.74 and 1.84 mM, respectively. The enzyme inhibition is clearly due to bismuth since our data show no apparent inhibition from either EDTA or cysteine at these concentrations. The non-competitive inhibition mechanism for RBC is probably due to the polymeric structure of this drug (Sun *et al.* 2004). On the basis of the measured K_i values, the affinities of jack bean urease for the bismuth complexes range as follows (strong to weak): RBC > Bi(EDTA) ≥ Bi(Cys)₃.

As a borderline metal ion, Bi(III) is known to bind strongly to thiolate sulfur and to form stable complexes with thiolate-containing biomolecules such as the tripeptide glutathione or cysteine-rich proteins such as metallothionein or *Staphylococcus aureus* pI258 CadC (Sun *et al.* 1999; Busenlehner *et al.* 2002). Titration of jack bean urease with DTNB showed that there are around 18 'accessible' thiolate groups present per molecule of enzyme, consistent with values previously reported (Blakeley & Zerner 1984; Srivastava & Kayastha 2000). After incubation of the jack bean urease with RBC and Bi(EDTA), the DTNB-accessible

thiols decreased by about 12 and 6 per hexamer (i.e., 2 and 1 per monomer), respectively, confirming the possible binding of bismuth to exposed thiolate groups of jack bean urease. The decrease in the bismuth-bound ¹³C-cysteine signals of the ¹³C-labeled Bi(Cys)₃ complex together with the increase in free cysteine signals (Figure 2), when incubated with enzyme, implied that Bi(III) binds to one or more cysteine residues of the enzyme via thiolate exchange. Such a thiolate exchange for metal trafficking between different molecules (*e.g.*, proteins and enzymes) has been demonstrated for Cu(I) and As(III)/Sb(III) (Wernimont *et al.* 2000; Li *et al.* 2002; Yan *et al.* 2003).

In addition to the rapid-binding competitive inhibition as seen with jack bean urease, Bi(EDTA) also is a slow-binding inactivator of *K. aerogenes* urease. This difference is likely to arise from substitutions of amino acids on the characteristic flap at the entrance to the active site cavity (Figure 4) (Takishima *et al.* 1988; Mulrooney & Hausinger 1990; Labigne *et al.* 1991; Ha *et al.* 2001). The flexibility of this flap is consistent with its distinct positions found in different ureases, as observed in the X-ray crystal structures (Ha *et al.* 2001). Significantly, Cys³¹⁹ is located on this flap in the *K. aerogenes* enzyme.

In order to test whether Cys³¹⁹ functions as the bismuth-binding site for Bi(EDTA) in *K. aerogenes* urease, the wild-type and C319A mutant enzymes were compared for their rates of inactivation by bismuth. Inactivation of the C319A enzyme occurred approximately 19-fold slower than for the wild-type urease under identical conditions, clearly indicating that urease inactivation is mainly attrib-

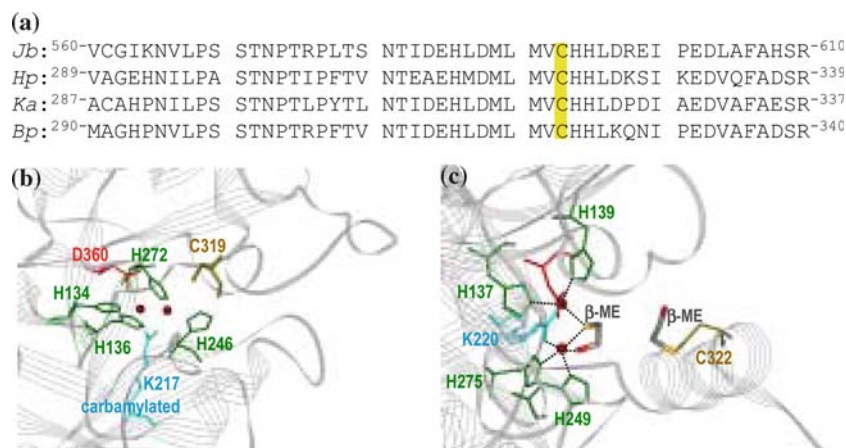


Figure 4. (a) Comparison of urease sequences surrounding the active site cysteine residue in the α -subunits of ureases. The conserved cysteine is highlighted in yellow. Jb, jack bean (Takishiam *et al.* 1988); Hp, *H. pylori* (Labigne *et al.* 1991); Ka, *K. aerogenes* (Mulrooney & Hausinger 1990); Bp, *B. pasteurii* (Benini *et al.* 1998). The active sites of (b) *K. aerogenes* urease (1FWJ) and (c) β -mercaptoethanol-bound *B. pasteurii* urease (1UBP) as determined by X-ray crystallography. The latter structure shows one inhibitory thiol bound to the dinuclear active site and a second β -ME bound as a mixed disulfide to Cys322.

uted to the interaction of bismuth with Cys³¹⁹. When comparing urease sequences from different species, it was found that the critical cysteine residue is conserved in several species (Figure 4a). Prior studies have shown that chemical modification of *K. aerogenes* Cys³¹⁹ or substitution of this residue by larger amino acids leads to the reduction or elimination of enzyme activity, although it is not essential for catalysis as shown by the activity of the C319A variant (Todd & Hausinger 1989; Martin & Hausinger 1992). Similarly, structural studies of urease from *B. pasteurii* reveal that Cys³²², equivalent to the Cys³¹⁹ in *K. aerogenes* urease, participates in catalysis, but again is not essential (Benini *et al.* 1998, 1999). β -Mercaptoethanol was found to inhibit the plant enzyme directly by bridging the two Ni(II) ions (via the sulfur atom of the inhibitor) in the active site and indirectly through formation of a disulfide bond with Cys³²², hence sealing the entrance to the active site cavity (Figure 4c). Our ICP-MS data (not provided) on the jack bean urease after incubation with bismuth complexes showed that nickel was retained by the bismuth-inhibited enzyme, again indirectly confirming the targeting of Bi(III) to Cys³¹⁹ instead of replacing the metal.

In summary, bismuth complexes inhibit urease activity effectively by several different inhibition modes. Bismuth binds to a cysteine residue of the enzyme (Cys³¹⁹ in *K. aerogenes* and probably Cys⁵⁹² in jack bean urease) at the entrance of the active site, as shown by protection against the thiol-specific reagent DTNB, the release of Cys from Bi(Cys)₃

upon binding urease as monitored by ¹³C NMR, and the decreased rate of inactivation observed in the C319A mutant urease. Direct displacement of nickel by bismuth was not observed. The observed interactions with urease shed light on the mechanism of antimicrobial effects of bismuth drugs.

Acknowledgements

This work is supported by the Hong Kong Research Grants Council (HKU703904P to HZS), Livzon Pharmaceutical Ltd. and National Institutes of Health Grant DK45686 (to RPH).

References

- Akada JK, Shirai M, Takeuchi H, Tsuda M, Nakazawa T. 2000 Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Mol Microbiol* **36**, 1071–1084.
- Asato E, Kamamuta K, Akamine Y, Fukami T, Nukada R, Mikuriya M, Deguchi S, Yokota Y. 1997 Bismuth(III) complexes of 2-mercaptoethanol: preparation, structural and spectroscopic characterization, antibactericidal activity toward *Helicobacter pylori*, and inhibitory effect toward *H. pylori*-produced urease. *Bull Chem Soc Jpn* **70**, 639–648.
- Bauerfeind P, Garner R, Dunn BE, Mobley HL. 1997 Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. *Gut* **40**, 25–30.
- Benini S, Rypniewski WR, Wilson KS, Ciurli S, Mangani S. 1998 The complex of *Bacillus pasteurii* urease with β -mercaptoethanol from X-ray data at 1.65 Å resolution. *J Biol Inorg Chem* **3**, 268–273.
- Benini S, Rypniewski WR, Wilson KS, Miletto S, Ciurli S, Mangani S. 1999 A new proposal for urease mechanism

- based on the crystal structures of the native and inhibited enzyme from *Bacillus pasteurii*: why urea hydrolysis costs two nickels. *Struct Fold Des* **7**, 205–216.
- Blakeley R, Zerner B. 1984 Jack bean urease: the first nickel enzyme. *J Mol Catal* **23**, 263–292.
- Blaser MJ. 1993 *Helicobacter pylori*: microbiology of a 'slow' bacterial infection. *Trends Microbiol* **1**, 255–260.
- Briand GG, Burford N. 1999 Bismuth compounds and preparations with biological or medicinal relevance. *Chem Rev* **99**, 2601–2658.
- Busenlehner LS, Apuy JL, Giedroc DP. 2002 Characterization of a metalloregulatory bismuth (III) site in *Staphylococcus aureus* p1258 CadC repressor. *J Biol Inorg Chem* **7**, 551–559.
- Butcher GP, Ryder SD, Hughes SJ, Stewart M, Bird N, Haqqani MT, Rhodes JM. 1992 Use of an ammonia electrode for rapid quantification of *Helicobacter pylori* urease: its use in the endoscopy room and in the assessment of urease inhibition by bismuth subsalicylate. *Digestion* **53**, 142–148.
- Davis GS, Mobley HL. 2005 Contribution of dppA to urease activity in *Helicobacter pylori* 26695. *Helicobacter* **10**, 416–423.
- Dixon M, Webb EC. 1979 Enzymes. (Third edition). New York: Academic Press, 138–164.
- Dixon NE, Hinds JA, Fihelly AK, Gazzola C, Winzon DJ, Blakeley RL, Zerner B. 1980 Jack bean urease (EC 3.5.1.5). IV. The molecular size and the mechanism of inhibition by hydroxamic acids. Spectrophotometric titration of enzymes with reversible inhibitors. *Can J Biochem* **58**, 1323–1334.
- Eaton KA, Brooks CL, Morgan DR, Krakowka S. 1991 Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun* **59**, 2470–2475.
- Ellman GL. 1959 Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**, 70–77.
- Faraci WS, Yang BV, O'Rourke D, Spencer RW. 1995 Inhibition of *Helicobacter pylori* urease by phenyl phosphorodiamidates: mechanism of action. *Bioorg Med Chem* **3**, 605–610.
- Ha NC, Oh ST, Sung JY, Cha KA, Lee MH, Oh BH. 2001 Supramolecular assembly and acid resistance of *Helicobacter pylori* urease. *Nature Struct Biol* **8**, 505–509.
- Hausinger RP, Karplus PA. 2001 Urease. In: Messerschmidt A, Huber R, Poulos TL, Wieghardt K eds. *Handbook of Metalloproteins*. John Wiley & Sons, Ltd.; 867–879.
- Houimel M, Mach JP, Cortesby-Theulaz I, Cortesby B, Fisch I. 1999 New inhibitors of *Helicobacter pylori* urease holoenzyme selected from phage-displayed peptide libraries. *Eur J Biochem* **262**, 774–780.
- Jabri E, Carr MB, Hausinger RP, Karplus PA. 1995 The crystal structure of urease from *Klebsiella aerogenes*. *Science* **268**, 998–1004.
- Labigne A, Cussac V, Courcoux P. 1991 Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J Bacteriol* **173**, 1920–1931.
- Laemmli UK. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lambert JR, Midolo P. 1997 The actions of bismuth in the treatment of *Helicobacter pylori* infection. *Aliment Pharmacol Ther Suppl* **11**, 27–33.
- Lee A. 1996 The nature of *Helicobacter pylori*. *Scand J Gastroenterol Suppl* **214**, 5–8; discussion 9–12.
- Li S, Rosen BP, Borges-Walmsley MI, Walmsley AR. 2002 Evidence for cooperativity between the four binding sites of dimeric ArsD, an As (III)-responsive transcriptional regulator. *J Biol Chem* **277**, 25992–26002.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Martin PR, Hausinger RP. 1992 Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. *J Biol Chem* **267**, 20024–20027.
- Mobley HL, Cortesia MJ, Rosenthal LE, Jones BD. 1988 Characterization of urease from *Campylobacter pylori*. *J Clin Microbiol* **26**, 831–836.
- Mobley HL, Hu LT, Foxal PA. 1991 *Helicobacter pylori* urease: properties and role in pathogenesis. *Scand J Gastroenterol* **187**, 39–46.
- Mobley HL, Island MD, Hausinger RP. 1995 Molecular biology of microbial ureases. *Microbiol Rev* **59**, 451–480.
- Mulrooney SB, Hausinger RP. 1990 Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J Bacteriol* **172**, 5837–5843.
- Pearson MA, Michel LO, Hausinger RP, Karplus PA. 1997 Structures of Cys319 variants and acetohydroxamate-inhibited *Klebsiella aerogenes* urease. *Biochemistry* **36**, 8164–8172.
- Perez-Perez GI, Olivares AZ, Cover TJ, Blaser MJ. 1992 Characteristics of *Helicobacter pylori* variants selected for urease deficiency. *Infect Immun* **60**, 3658–3663.
- Prewett EJ, Luk YW, Fraser AG, Lam WM, Pounder RE. 1992 Comparison of one-day oral dosing with three bismuth compounds for the suppression of *Helicobacter pylori* assessed by the ¹³C-urea breath test. *Aliment Pharmacol Ther* **6**, 97–102.
- Rektorschek M, Weeks D, Sachs G, Melchers K. 1998 Influence of pH on metabolism and urease activity of *Helicobacter pylori*. *Gastroenterology* **115**, 628–641.
- Riddles PW, Andrews RK, Blakeley RP, Zerner B. 1983 Jack bean urease. 6. Determination of thiol and disulphide content – reversible inactivation of the enzyme by the blocking of the unique cysteine residue. *Biochim Biophys Acta* **743**, 115–120.
- Riddles PW, Whan W, Blakeley RL, Zerner B. 1991 Cloning and sequencing of a jack bean urease-encoding cDNA. *Gene* **108**, 265–267.
- Scott DR, Marcus EA, Weeks DL, Sachs G. 2002 Mechanisms of acid resistance due to the urease system of *Helicobacter pylori*. *Gastroenterology* **123**, 187–195.
- Sheridan L, Wilmot CM, Cromie KD, van der Logt P, Phillips SE. 2002 Crystallization and preliminary X-ray structure determination of jack bean urease with a bound antibody fragment. *Acta Crystallogr D Biol Crystallogr* **58**, 374–376.
- Sirko A, Brodzik R. 2000 Plant ureases: roles and regulation. *Acta Biochim Pol* **47**, 1189–1195.
- Srivastava PK, Kayastha AM. 2000 Significance of sulfhydryl groups in the activity of urease from pigeonpea (*Cajanus cajan* L.) seeds. *Plant Sci* **159**, 149–158.
- Stingl K, Altendorf K, Bakker EP. 2002a Acid survival of *Helicobacter pylori*: how does urease activity trigger cytoplasmic pH homeostasis? *Trend Microbiol* **10**, 70–74.
- Stingl K, De Reuse H. 2005 Staying alive overdosed: how does *Helicobacter pylori* control urease activity? *Int J Med Microbiol* **295**, 307–315.
- Stingl K, Uhlemann EM, Deckers-hebestreit G, Schmid R, Bakker EP, Altendorf K. 2001 Prolonged survival and

- cytoplasmic pH homeostasis of *Helicobacter pylori* at pH 1. *Infect Immun* **69**, 1178–1180.
- Stingl K, Uhlemann EM, Schmid R, Altendorf K, Bakker EP. 2002b Energetics of *Helicobacter pylori* and its implications for the mechanism of urease-dependent acid tolerance at pH 1. *J Bacteriol* **184**, 3053–3060.
- Suarez D, Diaz N, Merz KM Jr. 2003 Ureases: quantum chemical calculations on cluster models. *J Am Chem Soc* **125**, 15324–15337.
- Summers SP, Abboud KA, Farrah SR, Palenik GJ. 1994 Syntheses and structures of bismuth (III) complexes with nitrilotriacetic acid, ethylenediaminetetraacetic acid, and diethylenetriaminepentaacetic acid. *Inorg Chem* **33**, 88–92.
- Sun H, Li H, Harvey I, Sadler PJ. 1999 Interactions of bismuth complexes with metallothionein (II). *J Biol Chem* **274**, 29094–29101.
- Sun H, Zhang L, Szeto KY. 2004 Bismuth in medicine. *Metal Ions Biol Syst* **41**, 333–378.
- Takishima K, Suga T, Mamiya G. 1988 The structure of jack bean urease. The complete amino acid sequence, limited proteolysis and reactive cysteine residues. *Eur J Biochem* **175**, 151–165.
- Todd MJ, Hausinger RP. 1989 Competitive inhibitors of *Klebsiella aerogenes* urease. Mechanisms of interaction with the nickel active site. *J Biol Chem* **264**, 15835–15842.
- Todd MJ, Hausinger RP. 1991 Reactivity of the essential thiol of *Klebsiella aerogenes* urease. Effect of pH and ligands on thiol modification. *J Biol Chem* **266**, 10260–10267.
- Weatherburn MW. 1967 Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem* **39**, 971–974.
- Wernimont AK, Huffman DL, Lamb AL, O'Halloran TV, Rosenzweig AC. 2000 Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. *Nature Struct Biol* **7**, 766–771.
- Wotherspoon AC, Ortiz-Hidalgo C, Falzon MR, Isaacson PG. 1991 *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* **338**, 1175–1176.
- Yan S, Li F, Ding K, Sun H. 2003 Reduction of pentavalent antimony by trypanothione and formation of a binary and ternary complex of antimony(III) and trypanothione. *J Biol Inorg Chem* **8**, 689–697.