*Journal of Enzyme Inhibition and Medicinal Chemistry*, August 2008; 23(4): 535–542

informa
healthcare

# Mono- (Ag, Hg) and di- (Cu, Hg) valent metal ions effects on the activity of jack bean urease. Probing the modes of metal binding to the enzyme

BARBARA KRAJEWSKA

*Jagiellonian University, Faculty of Chemistry, 30-060, Kraków, Ingardena 3, Poland*

*(Received 18 August 2007; accepted 18 September 2007)*

**Abstract**
The inhibition of urease by heavy metal ions has been habitually ascribed to the reaction of the ions with enzyme thiol groups, resulting in the formation of mercaptides. To probe the modes of metal binding to the enzyme, in this work the reaction of mono- (Ag, Hg) and di- (Cu, Hg) valent metal ions with jack bean urease was studied. The enzyme was reacted with different concentrations of the metal ions for different periods of times, when its residual activitiy was assayed and thiol content titrated. The titration carried out with DTNB was done to examine the involvement of urease thiol groups in metal ion binding. The binding was further probed by reactivation of the metal ion-enzyme complexes with DTT, EDTA and dilution. The results are discussed in terms of the HSAB concept. In inhibiting urease the metal ions showed a common feature in that they inhibited the enzyme within a comparable micromolar range, and also in that their inhibition was multisite. By contrast, the main distinguishing feature in their action consisted of the involvement of enzyme thiol groups in the reaction. $Hg^{2+}$ and $Hg_2^{2+}$ inhibition was found thoroughly governed by the reaction with the enzyme thiols, and the complete loss of enzyme activity involved all thiols available in the enzyme under non-denaturating conditions. In contrast, $Ag^{+}$ and $Cu^{2+}$ ions for the complete inactivation of the enzyme required 53 and 60% of thiols, respectively. Accordingly, $Ag^{+}$ and $Cu^{2+}$ binding to functional groups in urease other than thiols, i.e. N- and O-containing groups, cannot be excluded. Based on the reactivation experiments this seems particularly likely for $Cu^{2+}$, whose concurrent binding to thiols and other groups might distort the architecture of the active site (the mechanism of which remains to be elucidated) resulting in the observed inhibitory effects.

**Keywords:** *Urease, heavy metal ions, inhibition, metal-binding sites, enzyme thiols*

## Introduction

Urease (EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbon dioxide [1]. The structure of the enzyme and its molecular weight depend on the enzyme origin, be it bacterial, plant, fungal or algal. Plant and fungal ureases, among them plant urease from *Canavalia ensiformis* (jack bean) [2], mostly are homohexamers $\alpha_6$, whereas bacterial ureases, such as the ones from *Klebsiella aerogenes* [3] and *Bacillus pasteurii* [4], typically are heterotrimers $(\alpha\beta\gamma)_3$. A notable exception is *Helicobacter pylori* urease having a composition $((\alpha\beta)_3)_4$ [5]. The active site of the enzyme, always located in the $\alpha$ subunits, contains a binuclear nickel centre, and its amino acid sequence has been found conserved principally in all known ureases. Accordingly, the catalytic mechanism is believed to be the same for all ureases regardless of origin.

Ureases have long been known for their sensitivity to the inhibition by heavy metal ions [6–10]. In addition to a better understanding of urease action, studies of this inhibition have been carried out for inhibition-based metal detection that could be exploited in the construction of biosensors and biosensing systems [11–15].

The relative effectiveness of the heavy metal ions as inhibitors of jack been urease has been reported to decrease in the following approximate order: $Hg^{2+} > Ag^{+} > Cu^{2+} > > Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$[16,17], with $Hg^{2+}$, $Ag^{+}$ and $Cu^{2+}$ ions nearly always listed as the most

Correspondence: B. Krajewska, Jagiellonian University, Faculty of Chemistry, 30-060 Kraków, Ingardena 3, Poland. Tel: 48 12 6632235. Fax: 48 12 6340515. E-mail: krajewsk@chemia.uj.edu.pl

ISSN 1475-6366 print/ISSN 1475-6374 online 
DOI: 10.1080/14756360701743051

effective inhibitors [9–17]. This inhibition has been habitually ascribed to the reaction of the ions with the thiol groups of cysteine residues of the enzyme, resulting in the formation of mercaptides [7–10,12–17]. This was supported by a conclusion of Shaw [9,10] that the order of effectiveness of heavy metal ions as urease inhibitors correlated with the solubility product constants of the corresponding metal sulphides. However, very importantly, heavy metal ions can also bind to functions in proteins other than thiols. These mainly include nitrogen- (histidine) and oxygen- (aspartic and glutamic acids) containing functional groups [18], and in fact, the relative frequency of sites reported as utilized by metals in metalloproteins follows the order: His > Cys > Asp > Glu. Further to the above observations, the binding sites chosen by individual heavy metal ions in proteins can be looked at in terms of the Hard and Soft Acids and Bases concept (HSAB) [18,19]. The concept holds that class B metals, soft acids, that include $Ag^{+}$, $Hg^{2+}$ and $Hg_2^{2+}$ ions, for binding prefer soft donors, such as sulphur, and accordingly exhibit the following preference binding site sequence: S > N > O. By contrast, the borderline metal ions, $Cu^{2+}$ ion being one of them, show a high affinity for nitrogen- and oxygen-containing ligands, though they are also capable of formation of stable complexes with all groups of ligands depending on availability. Finally, in addition to complex formation, in interacting with proteins heavy metal ions may be involved in redox processes [19] and may participate in redox cycling from which reactive oxygen species (ROS) are generated [20], and these are responsible for oxidations of protein functional groups, e.g thiols.

A subunit of jack bean urease, composed of 840 amino acid residues [2], contains the following numbers of the amino acid residues in question: 25 histidine residues, four of which directly involved in the architecture of the active site, 48 aspartic acid residues, one of which also in the active site, and 48 glutamic acid residues. The number of cysteine residues in the subunit is 15, of which six are accessible under non-denaturating conditions [21]. Among the accessible cysteines, Cys592 located in the mobile flap of the active site is important in that, although reckoned not to be essential for the catalysis [22], it has been shown to be critical for the enzyme activity [21], most likely through structural interactions with other active-site amino acid residues.

In view of the foregoing data in this study the effects of mono- (Ag, Hg) and di- (Cu, Hg) valent metal ions, the strongest urease inhibitors among the heavy metal ions, on the activity of jack bean urease were investigated in an effort to elucidate the modes of metal binding to the enzyme. It is of interest to note that in our previous study the inhibition of urease by $Hg^{2+}$ ions was extensively discussed [23]. Herein certain experiments with $Hg^{2+}$ were repeated for the data to be collected under the same assay conditions.

## Materials and methods

### *Materials*

Urease (from jack beans type III, nominal activity 45 units/mg solid), HEPES buffer (SigmaUltra), urea (for Molecular Biology), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and DL-dithiothreitol (DTT) were from Sigma. EDTA and the salts $AgNO_3$, $Cu(NO_3)_2.3H_2O$ and $HgCl_2$ were from POCh, Poland, and $Hg_2SO_4$ was from Aldrich. The pH of HEPES stock solution (200 mM) was adjusted with a NaOH solution to pH 7.0, and the working concentration 20 mM was prepared by dilution. Ultrapure deionized water obtained from a Simplicity 185, Millipore water purification system (resistivity 18.2 MΩ cm) was used throughout. The solutions of both the metal salts and urease were prepared in water, which was done to avoid side reactions of metal ions with the buffer that could interfere with their main reaction with urease. The measurements were performed at 22 ± 1°C.

### *Standard assay of urease activity*

The standard assay mixture for urease activity was 50 mM urea in 20 mM HEPES, pH 7.0, its volume being 20 mL. Reactions were initiated by the addition of aliquots of the enzyme-containing (0.25 mg) solution. The assay was performed for 5 min and the enzyme activity was determined by measuring the concentration of the ammonia released. For that, samples were withdrawn from the reaction mixtures and the ammonia was determined by the colorimetric phenol-hypochlorite method [24].

### *Residual activity measurements*

Equal volumes of the solution of urease (1.25 mg/mL water) and of the salts were mixed. The concentration of the metal ions in the mixtures varied from 1 to 10 μM. The mixtures were incubated with occasional stirring. During the incubations, at time intervals the aliquots of the mixture were transferred into the standard assay mixtures for enzyme residual activity determinations. All residual activities determined were expressed as the fraction of urease activity in the absence of the metal ions. It took the reactions of urease with the ions studied no longer than 10 min to reach the equilibrium, and this time was further used in all the residual activity and reactivation experiments.

### *Spectroscopic assays of thiol groups in metal ion-modified urease*

Thiol groups in pure and metal ion-modified urease were assayed by titration with a cysteine-selective

reagent DTNB. For that equal volumes of urease solution (2.5 mg/mL water) and of the metal salts were mixed, the final ion concentrations being in the range 2–20 μM. Upon incubation for 10 min, the activity of urease was assayed under standard conditions, and at the same time the other sample of the incubation mixture was DTNB-titrated. For that 1 mL of the incubation mixture was transferred to a cuvette and mixed with 1(mL 0.2 mM DTNB (prepared in 20 mM HEPES pH 7.0). The absorbance of the mixture was measured at 412 nm [25] for 15 min. Prior to these measurements, control measurements of the solutions used in the proportions corresponding to the final DTNB-urease reaction mixture were performed and subtracted when necessary.

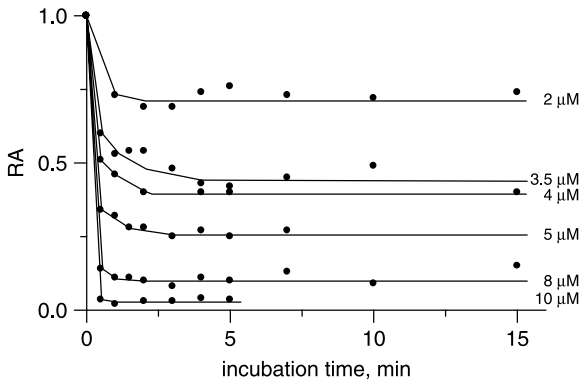


Figure 1. Time courses of the reaction of $Ag^+$ ions with urease, residual activity (RA) of urease *vs* time of incubation.

*Reactivation of metal ion-inhibited urease*

Urease was incubated with the solutions of the metal ions as in the residual activity measurements for 10 min. The concentrations of the metal ions were chosen to obtain sub-maximal inactivation of urease, i.e. with residual activity slightly higher than zero, typically amounting to a fraction of about 0.02–0.04 of the original activity. Three types of reactivation were carried out on these samples, namely with DTT, with EDTA and by dilution. For the reactivations with DTT and EDTA, to 4.5 mL volumes of the metal ion-urease incubation mixtures 0.5 mL of 20 mM DTT and of 100 mM EDTA were added to give the reactivation concentrations 2 mM DDT and 10 mM EDTA, respectively. The activity recovery of urease was observed over time against control in the absence of the metal ions, by enzyme activity determinations in the samples of the reactivation mixture withdrawn at intervals to the standard assay mixture. For the reactivation by dilution, the metal ion-urease incubation mixtures were diluted 100 times with 20 mM HEPES pH 7.0. The activity of urease was measured periodically under standard activity assay conditions in samples withdrawn from the reactivation solution.

## Results and discussion

*Residual activity measurements*

The metal ions under examination reacted with urease primarily in a concentration-dependent manner as was the case of $Hg^{2+}$ ion inhibition, extensively discussed previously [23]. Representative time courses obtained for $Ag^+$ ions are shown in Figure 1. Characteristically, during the metal ion-urease incubations the activity of the enzyme was reduced within a time not longer than 10 minutes to a non-zero constant value which varied with inhibitor concentration, with the plateaus corresponding to the fraction of the uninhibited enzyme at equilibrium. A summary of the equilibrium residual activities (RA) of urease observed for different concentrations of the ions in the incubation mixture is presented in Figures 2a–2c. As shown, for all the studied ions the plots of RA vs ion concentration are sigmoidal, located within the comparable ranges of micromolar concentrations. Consequently, the resulting $IC_{50}$ values, i.e. inhibitor concentrations bringing about a 50% inactivation of the enzyme, are of the same order for all the studied ions, whether mono- or divalent, and amount to between 2 and 3 μM. This shows that the ions react with urease with the comparable strength, this being in conformity with earlier reports in the literature [11,12,14,16]. The magnitude of $IC_{50}$ classifies the ions among strong urease inhibitors comparable for instance with 1,4-benzoquinone and tetrachloro-1,4-benzoquinone [26]. Also importantly, the plots show that mercury ions, regardless of whether $Hg^{2+}$ or $Hg_2^{2+}$, exert practically the same inhibition.

The data of Figures 2a–2c are further presented as log((1-RA)/RA) vs log [ion] in Figures 2d–2f according to the Hill equation [27]:

$$K' = \frac{1 - \mathrm{RA}}{\mathrm{RA}} \frac{1}{[\mathrm{ion}]^n} \qquad (1)$$

in which $K'$ is the equilibrium constant of the ion-urease reaction, and $n$ is the Hill constant. To construct the plots, the concentration of free ions in Equation (1) was approximated by the total concentration of the ions added, basing on that when [ion-enzyme complex] $\ll$ [free ion], [ion] in Equation (1) can be replaced by the total ion concentration. To substantiate the approximation, the molar concentration of urease in the incubation mixture was assessed. This was done by comparing the activity of urease preparation employed in this study with that reported for the pure enzyme, equal to approximately 7000 units/mg enzyme [1], and assuming a mass of urease of 550 kD [2]. The resulting concentration of the active urease in the incubation mixtures is 0.0073 μM, which is considerably lower than the concentration of the metal ions used and thus allows the above approximation.

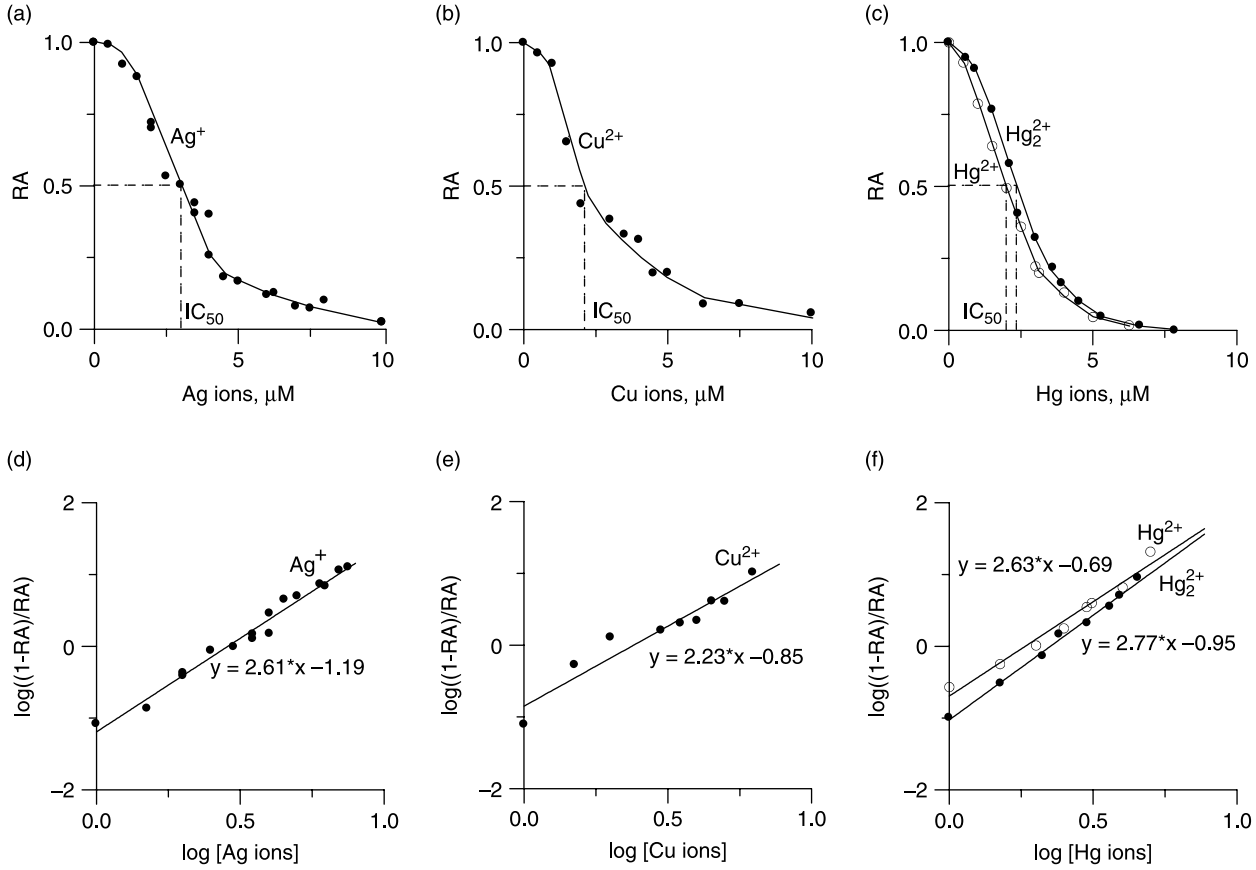


Figure 2. Equilibrium residual activity (RA) of urease as a function of total metal ion concentrations in the incubation mixtures for $Ag^{+}$ (a), $Cu^{2+}$ (b), and Hg ions (c); Logarithmic plots of ((1-RA)/RA) as a function of total metal ion concentrations in the incubation mixtures for $Ag^{+}$ (d), $Cu^{2+}$ (e), and Hg ions (f).

The constructed plots are presented in Figures 2d–2f, and contain the data for residual activity between 0.1 and 0.9. As shown, the plots are linear with slopes corresponding to *n* equal to between two and three for all the ions studied.

The manner the residual activity of urease responded to increasing concentrations of the metal ions, sigmoidal in Figures 2a–2c and linear in Figures 2d–2f, suggests that the metal ions react with urease at more than one binding site. In terms of the multisite inhibition, the Hill coefficient *n* represents the minimum number of the binding sites in a monomer of multimeric enzymes [27]. The *n* values obtained from the slopes of the lines in Figures 2d–2f indicate that in one subunit of urease three or more binding sites are involved in the reaction with the metal ions leading to the observed inhibition. These observations are important in that they support the notion that the reaction of urease with the metal ions is not restricted to cysteine residues in the active site, but involves more functional groups. If these are other cysteine residues, then how many of them involved in the reaction, or functional groups other than thiols, will be argued later.

*DTNB spectroscopic assays of thiol groups in metal ion-modified urease*

The spectroscopic titration of thiol –SH groups in metal ion-modified urease carried out with the use of DTNB was done in order to examine the involvement of urease thiol groups in the metal ion-induced inhibition. In Figure 3 the typical time courses of the

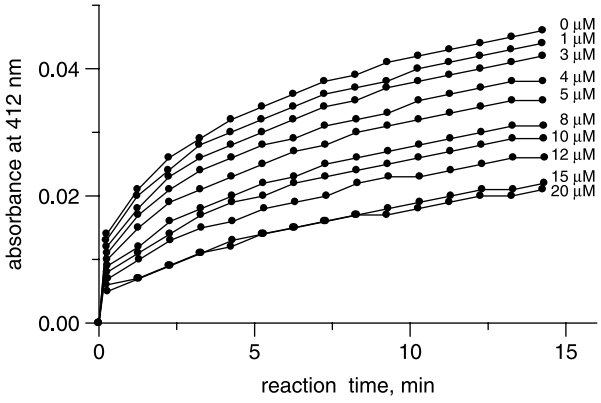


Figure 3. Spectroscopic time courses of reactions of DTNB with $Ag^{+}$-modified urease, absorbance at 412 nm *vs* time.

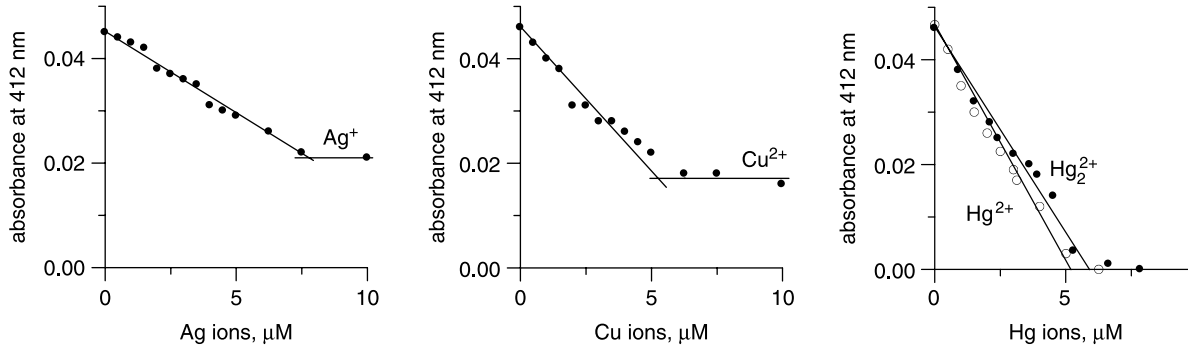


Figure 4. Final 412 nm absorbances measured during the reactions of DTNB with metal ions-modified urease, plotted against concentrations of metal ions in the incubation mixtures.

reactions of DTNB with urease modified with different concentrations of $Ag^+$ ions are presented in the form of plots of absorbance at 412 nm vs reaction time. The curve for native urease has a characteristic shape [28,29] composed of the initial phase corresponding to an abrupt reaction of 30 reactive thiols/molecule with DTNB, and the subsequent long phase corresponding to a slower reaction of DTNB with the six less reactive Cys592/molecule. The curves for $Ag^+$-modified urease are similar in shape but are located below the native enzyme, their final absorbances decreasing with an increasing $Ag^+$concentration used in the incubation mixture. This reveals that thiols of urease participate in the binding of the ions. The final absorbances measured at 15 minutes reaction time of urease with DTNB were taken as corresponding to the number of thiol groups available in a given metal ion-urease complex under non-denaturating conditions applied.

The summary of the results of the DTNB titrations of all the metal ions-modified urease is presented in Figure 4, where the final absorbances measured at 412 nm are plotted against concentrations of the metal ions in the incubation mixtures. Most interestingly, contrary to a seemingly similar pattern of behaviour of the metal ions in the reaction with urease seen in the residual activity measurements (Figure 2), the plots in Figure 4 reveal that the individual metal ions in reacting with urease engage different numbers of the enzyme thiol groups. Namely, for the complete loss of the enzyme activity, $Hg^{2+}$ ions bound to 100% of the available enzyme thiol groups, as was observed previously [23]. Likewise behaved $Hg_2^{2+}$ ions. Differently by contrast, behaved $Ag^+$ and $Cu^{2+}$ ions, which for the complete urease activity loss utilized approximately 53 and 60% of urease thiol groups, respectively, leaving the remaining 47 and 40% unbound. To account for this observation, at this point two effects may be considered. One is that $Ag^+$ and $Cu^{2+}$ ions in reacting with urease, in addition to SH groups of cysteine residues, as was revealed by DTNB titration, react with functional groups other than thiols. These may include nitrogen centres, e.g. histidine imidazole, and to a lesser but not impossible extent oxygen centres, e.g. carboxylate of glutamic and aspartic acid [18]. Also, it cannot be excluded that the ions, $Cu^{2+}$ in particular, participate in the generation of ROS giving rise to oxidations in the enzyme [19,30]. The other possible effect contributing to the observed results may be considered as originating from the size of the ions, for $Ag^+$ in particular. The diameters reported for the hydrated ions are: 2.5 Å for $Ag^+$, 6 Å for $Cu^{2+}$, 5 Å for $Hg^{2+}$ and 4 Å for $Hg_2^{2+}$ [31]. It may accordingly be viewed that the smaller size of the hydrated $Ag^+$ ion, concurrent with its higher ionic mobility, makes the accessibility of the active-site flap cysteines, the very ones whose modification results in enzyme inhibition, easier for these ions than to the other bulkier $Cu^{2+}$, $Hg^{2+}$ or $Hg_2^{2+}$ ions. This may be why the deactivation of urease by $Ag^+$ is completed before all 36 SH groups/molecule are modified.

### *Reactivation of metal ion- inhibited urease*

Reactivation of metal ion-inhibited urease with DTT and EDTA is presented in Figures 5 and 6, respectively, and that by dilution in Figure 7. As shown in Figure 5, urease alone when incubated with 2 mM DTT (control sample) underwent inactivation within 5 minutes to about 0.6 of the original activity. Accordingly, the

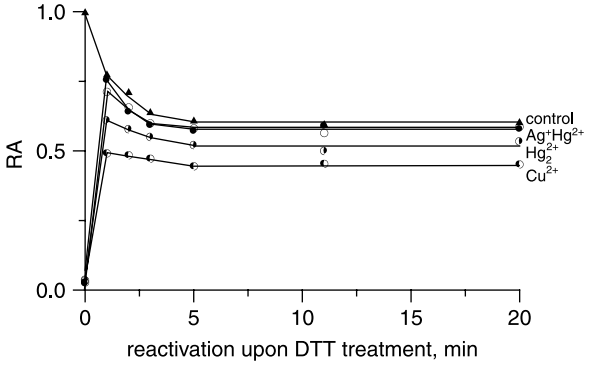


Figure 5. Reactivation of metal ions-modified urease with DTT.

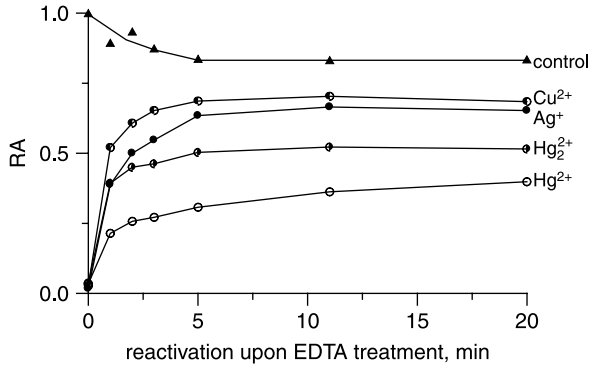


Figure 6. Reactivation of metal ions-modified urease with EDTA.

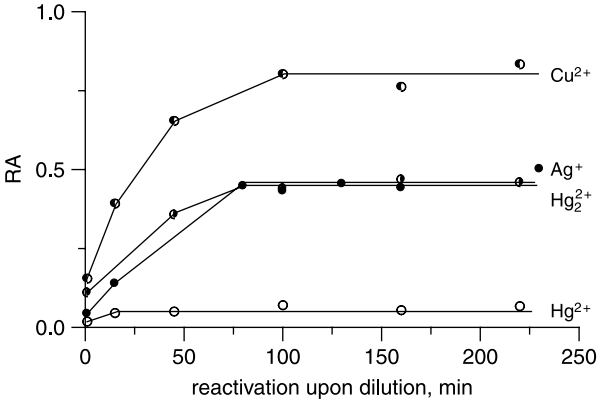


Figure 7. Reactivation of metal ions-modified urease by dilution.

reactivation curves for metal ions-inhibited urease followed the shape of the control sample. The inhibited samples recovered their activity rapidly, practically within 1 minute (or less), and within 5 minutes the reactivations achieved plateaus. $Ag^{+}$- and $Hg^{2+}$-inhibited urease restored nearly 100% of the control activity, $Hg_2^{2+}$- and $Cu^{2+}$-inhibited enzyme by contrast, about 87 and 75%, respectively. The reaction taking place in this reactivation is the disulphide interchange of the metal ions between different thiol groups, here between those of urease and DTT, resulting in the formation of DTT-metal ions complexes. Consistent with the HSAB concept, of the metals studied $Ag^{+}$, $Hg^{2+}$ and $Hg_2^{2+}$ that belong to class B metal ions, exhibit a strong preference in proteins to sulphur centres over nitrogen or oxygen centres. The ions, $Hg^{2+}$ and $Ag^{+}$ in particular, were shown to form highly stable mercaptides [32–34]. $Cu^{2+}$ by contrast, belongs to the group of borderline metal ions and consequently shows a lower affinity to sulphur. The affinity of the ions towards sulphur ligands can be measured by the magnitude of the solubility product constants $pK_{sp}$ of the respective metal sulphides. The $pK_{sp}$ values have the following approximate values [35]: 37 for CuS < 47 for $Hg_2S$ < 50 for $Ag_2S$ < 53 for HgS. This may be why $Ag^{+}$ and $Hg^{2+}$ most readily interchanged with the DTT added to the ion-urease complexes (Figure 5), whereas $Cu^{2+}$ having the lowest affinity to sulphur interchanged to the lowest degree. The reluctance of $Cu^{2+}$-modified urease to have the activity restored through the action of DTT may also be indicative of the two following $Cu^{2+}$ effects on urease. One is that $Cu^{2+}$ ions may in the aerobic conditions participate in the redox-cycling generation of ROS, an effect that was reported for instance for $Cu^{2+}$-nitrate reductase system [30]. Such generated ROS could consequently bring about inhibition of urease by oxidation of its thiol groups. These, if oxidized to sulphinic ($-SO_2H$) or sulphonic ($-SO_3H$) acid cannot be reduced back to $-SH$ groups by DTT [36], which was seen (Figure 5). Interestingly, the oxidative mechanism of $Cu^{2+}$ inhibition of urease was suggested by Hellerman et al. in an early work on urease in 1933 [6]. For reversal of the inhibition the authors used hydrogen sulphide, and like in this work, it was observed that in contrast to $Hg^{2+}$-, $Cu^{2+}$-inhibition of urease could only be partly reversed. The other possible effect of $Cu^{2+}$ on urease is that the ions in addition to thiol groups are also bound to N- or O-ligands in urease as suggested earlier by DTNB titration of $Cu^{2+}$-urease complexes (Figure 4). Although the same was demonstrated for $Ag^{+}$, its high affinity to sulphur might have driven it out of the enzyme to join DTT. Worthy of adding is that when urease was inactivated to zero activity with higher concentrations of the ions, upon addition of the same 2 mM DDT, the enzyme recovered its activity to approximately the same percent of the control (data not shown).

That both $Ag^{+}$ and $Cu^{2+}$, despite their disparate $pK_{sp}$ values, have a common pattern of behaviour in reacting with urease, and in addition to thiols in the enzyme are partly bound to N-ligands (rather than to O-ligands), is provided by the reactivation of the ion-urease complexes performed with EDTA (Figure 6). As shown, 10 mM EDTA afforded the reactivation of $Ag^{+}$- and $Cu^{2+}$-urease complexes within about 5 minutes to the highest degree, about 78 and 83% of the control, respectively, and only to about 47% for $Hg^{2+}$ and to 62% for $Hg_2^{2+}$. Interestingly, when the same reactivation was performed on urease inactivated with the metal ions to zero residual activity, EDTA restored the same activity from $Cu^{2+}$-complex, but was not capable of restoring the activity from $Ag^{+}$- and $Hg^{2+}$-complexes at all (data not shown). This is supportive of the suggestion that for the complete inactivation of urease by $Ag^{+}$ and $Hg^{2+}$ responsible is the reaction of the ions with Cys592 of the active-site flap, apparently less accessible for EDTA, while the inactivation by $Cu^{2+}$ might be a combination of some effects bringing about distortion to the architecture of the active site, the precise mechanism of which remains to be elucidated. Also, as the action of EDTA is that of a typical metal chelator, EDTA should not be capable of restoring urease activity reduced by oxidative modifications [30]. Thus, the ease of reactivation of urease by EDTA from $Cu^{2+}$-complexes

rules out the participation of oxidations by $Cu^{2+}$-mediated ROS.

Interestingly, the observations made in this work on $Cu^{2+}$ inhibition of urease are consistent with the findings reported by Follmer and Carlini [37] in their study on oligomerization of urease induced by heavy metal ions ($Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$ and $Hg^{2+}$). The authors demonstrated that unlike the other ions in the study, $Cu^{2+}$ gave rise to oligomerization of the enzyme by binding to histidine residues. Most likely through the formation of intermolecular His-$Cu^{2+}$-His bridges, the binding led to enzyme aggregation that was seen in a polyacrylamide gel electrophoresis analysis. It was suggested that this oligomerization along with the reaction of $Cu^{2+}$ with the enzyme thiols, typical of the other ions, was responsible for urease inhibition.

Importantly, the reactivation of the ion-urease complexes by dilution shown in Figure 7, supports the above presented observations. Urease recovered its activity after 100 minutes to the highest extent of 80% from the $Cu^{2+}$-complex, to 45% from the $Ag^{+}$- and $Hg_2^{2+}$-complexes, and to the lowest extent of 6% from the $Hg^{2+}$-complex. This recovery corresponds to the strength of metal binding to the functional groups of urease, the weakest for $Cu^{2+}$, the strongest for $Hg^{2+}$, with $Ag^{+}$ and $Hg_2^{2+}$ being the intermediate cases. It reflects the net effects resulting from the varying affinity of the metal ions to sulphur ligands and participation of weaker metal binding to ligands other than sulphur.

In view of the observations made in all the experiments in this study, it seems reasonable to conclude the following. In inhibiting urease the metal ions studied, whether mono- or divalent, share a common feature in that they inhibit the enzyme within the comparable micromolar range of concentrations ($IC_{50}$ values amount to between 2 and 3 $\mu$M), and also in that their inhibition is multisite involving the reaction with more than three functional groups per enzyme monomer. By contrast, the main distinguishing feature in their action on urease consists of the involvement of enzyme thiol groups in the reaction. $Hg^{2+}$ inhibition of urease is thoroughly governed by the reaction of the ions with the enzyme thiols, and the complete loss of enzyme activity involves all 36 of them available in the enzyme under non-denaturating conditions. Likewise is the inhibition by $Hg_2^{2+}$ ions, though it is easier to be reversed due to a lower affinity of these ions to sulphur. In contrast, $Ag^{+}$ and $Cu^{2+}$ ions for the complete inactivation of the enzyme require fewer enzyme thiols, 53 and 60% of them, respectively. Although $Ag^{+}$ utilizes only 53% enzyme thiols, its inhibition of urease is apparently due to the reaction with the thiols. Tentatively, responsible for this effect may be a smaller size of the ions that allows them to reach the key Cys592 in the active site more easily than Hg ions. However, a participation of $Ag^{+}$-binding to functional groups other than thiols, N-ligands in particular, cannot be excluded. $Cu^{2+}$ inhibition, by contrast, involves 60% of urease thiols and is easiest to reverse by non-sulphide means. This is clearly due to the lowest affinity of these ions, among other ions studied, to sulphur as well as to $Cu^{2+}$-binding to functional groups other than thiols, nitrogen-containing groups in particular, which is generally weaker. The combination of these effects may be responsible for distortion of the architecture of the active site, the mechanism of which remains to be elucidated.

## References


[1] Hausinger RP, Karplus PA. Urease. In: Wieghardt K, Huber R, Poulos TL, Messerschmidt A, editors. Handbook of metalloproteins. West Sussex, UK: Wiley; 2001.

[2] Takishima K, Suga T, Mamiya G. The structure of jack bean. The complete amino acid sequence, limited proteolysis and reactive cysteine residues. Eur J Biochem 1988;175:151–165.

[3] Jabri E, Carr MB, Hausinger RP, Karplus PA. The crystal structure of urease from *Klebsiella aerogenes*. Science 1995;268: 998–1004.

[4] Benini S, Rypniewski WR, Wilson KS, Miletti S, Ciurli S, Mangani S. 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. Structure 1999;7:205–216.

[5] Ha N-C, Oh S-T, Sung JY, Cha KA, Lee MH, Oh B-H. Supramolecular assembly and acid resistance of *Helicobacter pylori* urease. Nature Struct Biol 2001;8:505–509.

[6] Hellerman L, Perkins ME, Clark WM. Urease activity as influenced by oxidation and reduction. Proc Natl Acad Sci USA 1933;19:855–860.

[7] Hellerman L, Chinard FP, Deitz VR. Protein sulfhydryl groups and the reversible inactivation of the enzyme urease. J Biol Chem 1943;147:443–462.

[8] Ambrose JF, Kistiakovsky GB, Kridl AG. Inhibition of urease by silver ions. J Am Chem Soc 1951;73:1232–1236.

[9] Shaw WHR. The inhibition of urease by various metal ions. J Am Chem Soc 1954;76:2160–2163.

[10] Shaw WHR, Raval DN. The inhibition of urease by metal ions at pH 8.9. J Am Chem Soc 1961;83:3184–3187.

[11] Krawczyński vel Krawczyk T, Moszczyńska M, Trojanowicz M. Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosens Bioelectronics 2000;15:681–691.

[12] Preininger C, Wolfbeis OS. Disposable cuvette test with integrated sensor layer for enzymatic determination of heavy metals. Biosens Bioelectronics 1996;11:981–990.

[13] Toren EC, Jr, Burger FJ. Trace deteremination of metal ion inhibitors of the urea-urease system by a pH-stat kinetic method. Mikrochim Acta 1968;1049–1058.

[14] Zhylyak GA, Dzyadevich SV, Korpan YI, Soldatkin AP, El'skaya AV. Application of urease conductometric biosensor for heavy metal ion determination. Sens Actuat B 1995;24-25: 145–148.

[15] Kuswandi B. Simple optical fibre biosensor based on immobilized enzyme for monitoring of trace heavy metal ions. Anal Bioanal Chem 2003;376:1104–1110.

[16] Krajewska B. Urease immobilized on chitosan membrane. Inactivation by heavy metal ions. J Chem Tech Biotechnol 1991;52:157–162.

[17] Zaborska W, Krajewska B, Olech Z. Heavy metal ions inhibition of jack bean urease: Potential for rapid contaminant probing. J Enz Inhib Med Chem 2004;19:65–69.

[18] Rulíšek L, Vondrášek J. Coordination geometries of selected transition metal ions ($Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Hg^{2+}$) in metalloproteins. J Inorg Biochem 1998;71:115–127.

[19] Nieboer E, Richardson DHS. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. Environ Pollution B 1980;1:3–26.

[20] Halliwell B, Gutteridge MC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219:1–14.

[21] Riddles PW, Andrews RK, Blakeley RL. Zerner Jack bean urease. 6. Determination of thiol and disulfide content. Reversible inactivation of the enzyme by the blocking of the unique cysteine residue. Biochim Biophys Acta 1983;743: 115–120.

[22] Martin PR, Hausinger RP. Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. J Biol Chem 1992;267:20024–20027.

[23] Krajewska B, Zaborska W, Chudy M. Multi-step analysis of $Hg^{2+}$ ion inhibition of jack bean urease. J Inorg Biochem 2004;98:1160–1168.

[24] Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. Anal Chem 1967;39:971–974.

[25] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–77.

[26] Zaborska W, Krajewska B, Kot M, Karcz W. Quinone-induced inhibition of urease. Elucidation of its mechanisms by probing thiol groups of the enzyme. Bioorg Chem 2007;35:233–242.

[27] Segel IH. Enzyme Kinetics. London: Wiley; 1975.

[28] Todd MJ, Hausinger RP. Reactivity of the essential thiol of *Klebsiella aerogenes* urease. Effect of pH and ligands on thiol modification. J Biol Chem 1991;266:10260–10267.

[29] Srivastava PK, Kayastha AM. Significance of sulfhydryl groups in the activity of urease from pigeonpea (*Cajanus cajan* L.) seeds. Plant Sci 2000;159:149–158.

[30] Luna CM, Casano LM, Trippi VS. Nitrate reductase is inhibited in leaves of *Triticum aestivum* treated with high levels of copper. Physiol Plant 1997;101:103–108.

[31] Kielland J. Individual activity coefficients of ions in aqueous solutions. J Am Chem Soc 1937;59:1675–1678.

[32] Stricks W, Kolthoff IM. Reactions between mercuric mercury and cysteine and glutathione. Apparent dissociation constants, heats and entropies of formation of various forms of mercuric marcapto-cysteine and -glutathione. J Am Chem Soc 1953;20:5673–5681.

[33] Valladas-Dubois S. Étude électrochimique de la formation de complexes entre l'ion argent et la cystéine. Compt Rend 1950;231:53–55.

[34] Kolthoff IM, Stricks W. Argentometric amperometric titration of cysteine and cystine. J Am Chem Soc 1950;72:1952–1958.

[35] Waggoner WH. The solubility product constants of the metallic sulfides. J Chem Ed 1958;35:339–342.

[36] Krajewska B, Zaborska W. Double mode of inhibition-inducing interactions of 1,4-naphthoquinone with urease. Arylation vs oxidation of enzyme thiols. Bioorg Med Chem 2007;15:4144–4151.

[37] Follmer C, Carlini CR. Effect of chemical modification of histidines on the copper-induced oligomerization of jack bean urease (EC 3.5.1.5). Arch Biochem Biophys 2005;435:15–20.