Journal of Enzyme Inhibition and Medicinal Chemistry, February 2004 VOL. 19 (1), pp. 65–69

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Heavy Metal Ions Inhibition of Jack Bean Urease: Potential for Rapid Contaminant Probing

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(Received 23 September 2003)

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The kinetics of heavy metal ions inhibition of jack bean urease was studied by progress curve analysis in a reaction system without enzyme-inhibitor preincubation. The inhibition was found to be biphasic with an initial, small inhibitory phase changing over the time course of 5–10 min into a final linear steady state with a lower velocity. This time-dependent pattern was best described by mechanism B of slow-binding inhibition, involving the rapid formation of an EI complex that subsequently undergoes slow conversion to a more stable EI* complex. The kinetic parameters of the process, the inhibition constants K_i and K_i^* and the forward k_5 and reverse k_6 rate constants for the conversion, were evaluated from the reaction progress curves by nonlinear regression treatment. Based on the values of the overall inhibition constant K_i^*, the heavy metal ions were found to inhibit urease in the following decreasing order: $Hg^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+} > Pb^{2+} > Co^{2+} > Fe^{3+} > As^{3+}$. With the K_i^* values as low as 1.9 nM for Hg^{2+} and 7.1 nM for Cu^{2+}, 100–1000 times lower than those of the other ions, urease may be utilized as a bioindicator of the trace levels of these ions in environmental monitoring, bioprocess control or pharmaceutical analysis.

Keywords: Urease; Heavy metal ions; Time-dependent inhibition

INTRODUCTION

Enzyme inhibition-based analytical techniques have emerged as rapid, simple and cost-effective alternatives to spectrometric and chromatographic techniques routinely used in environmental monitoring, also in bioprocess and food control, and in biomedical and pharmaceutical analysis.[1–3] Requiring neither complex instrumentation nor tedious sample pretreatment, the techniques can readily be adapted for *in situ* and real-time detection of trace levels of pollutants that are inhibitors to the employed enzymes. Examples of note include:[1–3] cholinesterases used for the detection of organophosphorus and carbamate pesticides, tyrosinase used for cyanides, thiourea, benzoic acid and phenolic compounds, aldehyde dehydrogenase for fungicides, and glucose oxidase, urease, alcohol dehydrogenase, catalase and peroxidase used for heavy metal ions. Enzymes in these techniques are applied either in native or immobilized forms that are integrated with different kinds of transducers: potentiometric, amperometric, conductometric, thermometric or optical, to make up devices classified as biosensors and biosensing systems.[1–4] Offering a great potential in chemical analysis, actual application of enzyme inhibition-based systems, however, has as yet been impaired by their not quite satisfactory reliability and lack of selectivity in real samples.[5] The former can, however, be improved by utilization of stable immobilized enzyme preparations,[6,7] and the latter by developing hybrid systems of enzymes of different sensitivities to different inhibitors.[8,9]

Owing to its pronounced sensitivity, urease has been considered as a primary enzyme for application as a probe for heavy metal ions. Urease (urea amidohydrolase EC 3.5.1.5), a Ni-containing enzyme found in many plants, fungi, algae and bacteria and in soil as a soil enzyme, catalyzes the hydrolysis of urea to ammonia and carbon dioxide.[10–12] The sensitivity of urease to heavy metal ions is due to the presence of multiple cysteine residues, of which one, conserved principally in all known ureases, is located in the mobile flap of the active site of the enzyme. The participation of this cysteine residue in the catalytic process

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/14756360310001650237

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has been established by reacting urease with a number of cysteine selective reagents, including alkylating agents and disulfides, which showed that covalent modification of this active-site flap cysteine elicits urease inactivation.[13]

Numerous urease inhibition-based systems for heavy metal ions detection have been developed and studied.[8,14–24] Understandably, essential for these applications is the knowledge of the kinetics of the urease inhibition by heavy metal ions and foremost of the metal ions relative inhibitory strength. However, in the literature disparate types of this inhibition have been proposed: noncompetitive,[21,22,25–28] partial competitive,[29] and mixed,[30] resulting in different sets of inhibition constants. More recently, this inhibition has been interpreted in terms of biphasic enzyme inactivation.[28,31] Apparently, the disparities in the inhibition mechanisms proposed arise among others from an assumed experimental scheme, whether based on initial reaction rate measurements or progress curve analysis, whether performed in a system with or without enzyme preincubation, and if with the preincubation, whether in concentrated or dilute solution. The disparities may also arise from the disturbing effects of the buffers used and from the interferences of the products of the enzymatic reaction, namely NH_3 and CO_2, with the metal ions giving rise to the formation of aminocomplexes and carbonates.[17]

In our previous studies, we investigated the inhibition of jack bean urease by Ni^{2+} [32] and Hg^{2+} ions.[33] In contrast to most other studies where the observations were limited to the initial stage of the reaction carried out in an enzyme-inhibitor preincubated system, we recorded the progress curves of the reactions in two reaction mixtures, with and without enzyme-inhibitor preincubation. For both the ions the observed inhibition was time-dependent and could be best described by a slow-binding mechanism. In this work we continued the investigation into the inhibitory effect of heavy metal ions on urease by examining the inhibition by a series of ions: Cu^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+}, Fe^{3+} and As^{3+} in order to verify if they obey the same time-dependent mode of inhibition found for Ni^{2+} and Hg^{2+}, and to compare their relative inhibitory strengths. The enzymatic reactions were carried out at different inhibitor concentrations in a system without enzyme-inhibitor preincubation.

MATERIALS AND METHODS

Materials

Urease (from jack beans, type III, activity 22 units/mg protein), HEPES buffer (SigmaUltra) and urea (for Molecular Biology) were from Sigma. The salts: $CuCl_2{\cdot}2H_2O$, $CdCl_2{\cdot}2.5H_2O$, $CoCl_2{\cdot}6H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$, $Pb(NO_3)_2$, $FeCl_3{\cdot}6H_2O$ and As_2O_3 were from POCh, Gliwice, Poland. All the solutions were prepared in ultrapure, deionized water obtained from a Simplicity 185, Millipore water purification system (resistivity 18.2 MΩ cm). The pH of HEPES buffer stock solution (200 mM) was adjusted to pH 7.0 with a dilute NaOH solution using a calomel combination electrode in conjuction with a pH meter. The solution was diluted to the working concentration as required.

Enzymatic Reaction

The enzymatic reactions were performed in 20 mM HEPES pH 7.0 at 25°C. The kinetic parameters of urease in a noninhibited reaction, K_M and v_{max}, were obtained by measuring initial rates of the reactions performed at urea concentrations 2–50 mM. The metal ion-inhibited reactions were performed at one urea concentration, 50 mM, and at different inhibitor concentrations, their range depending on the metal ion inhibitory strength. The reactions were initiated by the addition of $1\,cm^3$ solution of urease ($1.25\,mg/cm^3$) to the reaction mixture (final volume $100\,cm^3$), and were monitored for 30 min by measuring ammonia concentration by the phenol-hypochlorite method[34] in samples withdrawn from the reaction mixtures at set time intervals. A curve fitting computer program (BURSTO, kindly offered by W.W. Cleland[35]) was used to fit the experimental points to the kinetic equations.

RESULTS AND DISCUSSION

Noninhibited Urease Reaction

The values of the kinetic parameters of urease in a noninhibited reaction, K_M and v_{max}, were obtained by fitting the initial reaction rates measured at a range of urea concentrations to the Michaelis–Menten equation by nonlinear regression. The values obtained are $K_M = 3.5 \pm 0.1$ mM and $v_{max} = 0.92 \pm 0.02$ mM NH_3/min.

Heavy Metal Ions-inhibited Urease Reaction

Assays of urease in the presence of heavy metal ions performed in the system without enzyme-inhibitor preincubation in which the reactions were initiated by adding enzyme to a substrate-inhibitor mixture, resulted in nonlinear, concave downward reaction progress curves for all the metal ions studied. Representative examples recorded for Hg^{2+} (data taken from[33]), Cu^{2+}, $Cd^{2+}$and Co^{2+} are presented in Figures 1a–1d. The nonlinear progress curves

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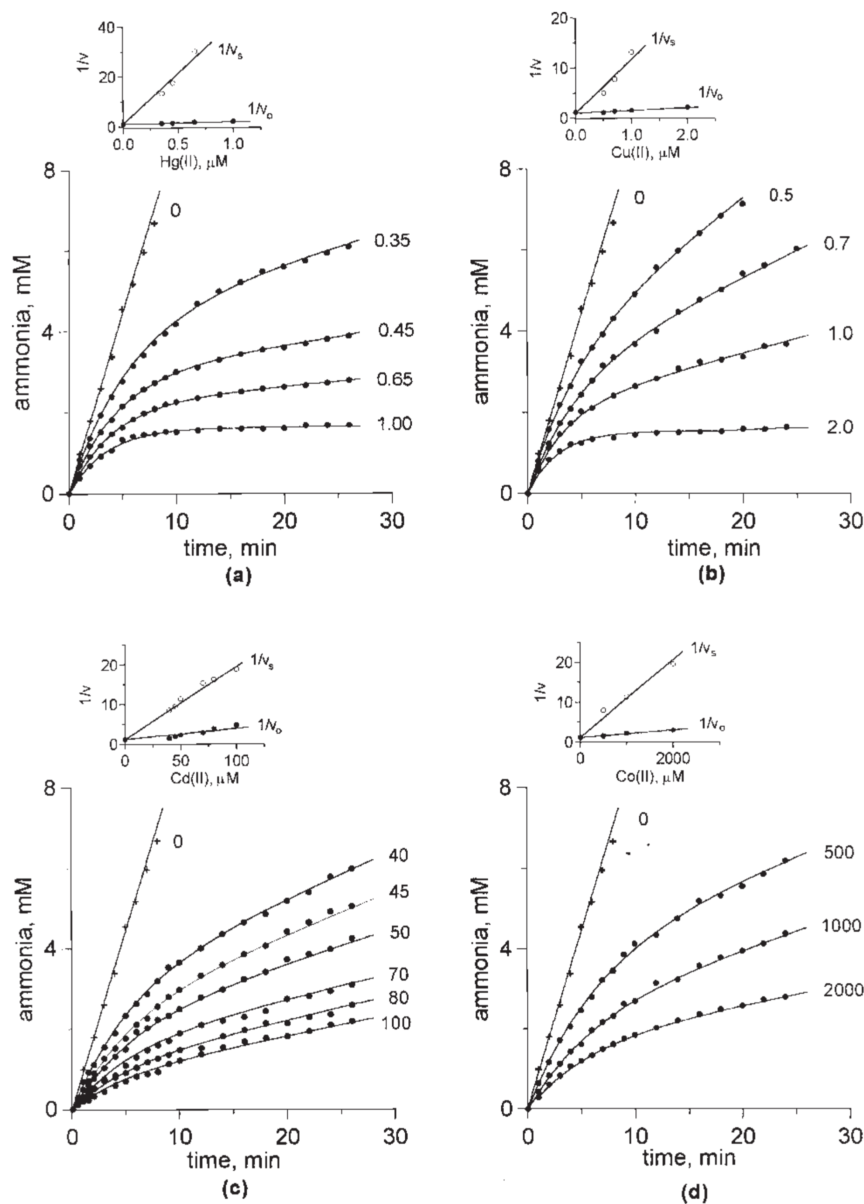
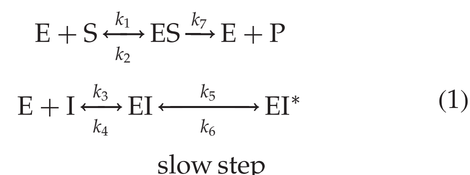


FIGURE 1 Urease reaction progress curves, of ammonia concentration produced vs time, generated by addition of urease to mixtures containing 50 mM urea and metal ions at the concentrations indicated, μM. Insets: Dixon plots of the effect of metal ions on the initial (v_o) and steady-state (v_s) rate.

clearly show that the metal ions produced a time-dependent inhibition of urease activity, in which a steady-state rate was attained slowly on the time scale of the assay with both the initial and steady-state rate decreasing with an increase in inhibitor concentration. This is supportive of mechanism B of slow-binding inhibition, found previously for the urease-Ni^{2+} and urease-Hg^{2+} systems.[32,33] To characterize the process we analyzed the experimental data according to the procedure outlined by Morrison and Walsh for competitive slow-binding inhibitors.[36]

Characteristic of slow-binding inhibitors is that they do not act on enzymes instantly but display a slow onset of the inhibition.[36] Mechanism B (Equation (1)) assumes a rapid formation of an EI complex, which next undergoes slow conversion to a more stable EI* complex.



$$\mathrm{E + S} \underset{k_2}{\overset{k_1}{\longleftrightarrow}} \mathrm{ES} \xrightarrow{k_7} \mathrm{E + P}$$

$$\mathrm{E + I} \underset{k_4}{\overset{k_3}{\longleftrightarrow}} \mathrm{EI} \underset{k_6}{\overset{k_5}{\longleftrightarrow}} \mathrm{EI^*} \qquad (1)$$

slow step

where k_1, k_2, k_3, k_4, k_5, k_6 and k_7 are rate constants.

When in system (1), without enzyme-inhibitor preincubation, the reaction is started by addition of enzyme and for inhibitor concentration > enzyme concentration reaction progress curves are described

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TABLE I Values of the kinetic parameters for the slow-binding inhibition of urease by heavy metal ions

Metal ion	K_i (μM)	K_i^* (μM)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6
Hg^{2+}	0.10 ± 0.02	0.0019 ± 0.0002	0.92 ± 0.20	0.017 ± 0.001	54 ± 12
Cu^{2+}	0.30 ± 0.12	0.0071 ± 0.0008	1.6 ± 0.7	0.040 ± 0.004	41 ± 18
Zn^{2+}	0.76 ± 0.05	0.18 ± 0.01	0.14 ± 0.01	0.044 ± 0.003	3.3 ± 0.1
Cd^{2+}	2.7 ± 0.4	0.41 ± 0.03	0.22 ± 0.04	0.040 ± 0.003	5.5 ± 0.8
Ni^{2+}*	42 ± 3	2.8 ± 0.4	0.64 ± 0.09	0.045 ± 0.004	14.2 ± 2.5
Pb^{2+}†	80	8.1	0.71	0.08	8.9
Co^{2+}	80 ± 6	8.2 ± 0.8	0.26 ± 0.02	0.030 ± 0.003	8.8 ± 0.3
Fe^{3+}	72 ± 8	27 ± 4	0.24 ± 0.07	0.145 ± 0.013	1.7 ± 0.4
As^{3+}‡	89 ± 6	34 ± 3	0.12 ± 0.01	0.076 ± 0.006	1.6 ± 0.1

*Data taken from[32]. † Pb^{2+} ions at higher concentrations produced turbidity of urease solution and temporary inactivation of the enzyme. The data are therefore only rough estimates obtained at the lowest non-turbidity Pb^{2+} concentration. ‡ As^{3+}solutions were prepared by dissolving As_2O_3 in water. The data were calculated for the total content of arsenic(III) in solution.

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by the integrated equation:

$$P(t) = v_s t + (v_o - v_s)\left(1 - e^{-k_{app}t}\right)\frac{1}{k_{app}} \quad (2)$$

where P is the concentration of product, t is time, v_o and v_s are the reaction initial and steady-state rates, respectively, and k_{app} denotes the apparent first-order rate constant for the interconversion between v_o and v_s.

Equation (2) predicts that product formation over time is a curvelinear function that displays a linear relationship with time in the initial stage of the reaction (v_o), later converting to another slower linear steady-state relationship (v_s). The equilibrium dissociation constants of the initial EI and final enzyme conformation EI* complex, K_i and K_i^* inhibition constants characterizing the inhibitory strength of inhibitor at the initial and steady-state stages of the reaction, are expressed with the corresponding rate constants by equations:

$$K_i = \frac{k_4}{k_3} \quad (3)$$

$$K_i^* = K_i \frac{k_6}{k_5 + k_6} \quad (4)$$

and k_6 is related to the reaction rates by:

$$k_6 = k_{app}\frac{v_s}{v_o} \quad (5)$$

Further, the inhibition in either stage of the reaction is described by the general equation for competitive inhibitors:

$$v_o\,(\text{or}\,v_s) = \frac{v_{max}S}{S + K_M\left(1 + \frac{I}{K_i\,(\text{or}\,K_i^*)}\right)} \quad (6)$$

where K_M and v_{max} are the Michaelis constant and maximum reaction rate of the enzyme in a noninhibited reaction.

For enzyme inhibitors conforming to mechanism B of the slow-binding mode of inhibition (Equation (1)), $K_i > K_i^*$ and $k_5 > k_6$.

The P–t data for each progress curve recorded were fitted to Equation (2) by the least squares method with use of the BURSTO program, resulting in v_o, v_s and k_{app} values. The values for the inhibition constants K_i and K_i^* were calculated according to Equation (6) from Dixon plots (insets to Figures 1a–1d). The forward (k_5) and reverse (k_6) rate constants of the EI ↔ EI* conversion were calculated by use of Equations (4) and (5). The resulting values of K_i, K_i^*, k_5 and k_6 are compiled in Table I.

The values in Table I show that the inhibitory potency of the heavy metal ions examined, if judged by the magnitude of K_i^*, forms the sequence: $Hg^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Ni^{2+} > Pb^{2+} > Co^{2+} > Fe^{3+} > As^{3+}$. Interestingly, while the k_5 and k_6 values only slowly decrease down the sequence, the values of the ratio k_5/k_6 vary as much as approximately 34-fold between Hg^{2+} and As^{3+}. As the k_5/k_6 ratio denotes the equilibrium constant for the distribution of enzyme between the two enzyme-inhibitor complexes EI and EI*, it can be treated as a measure of their relative stability, i.e. higher k_5/k_6 ratios correspond to more stable EI* complexes. This means that among the ions examined, the most stable are the Hg^{2+}- and Cu^{2+}-urease complexes. This pronounced affinity of Hg^{2+} and Cu^{2+} for urease constitutes an underlying concept for utilizing the enzyme as a probe for trace levels of these metal ions, e.g. in the environment. Obviously, essential for such an application is the ability to restore the enzyme activity inhibited by exposure to the metal ions. As we demonstrated previously for Hg^{2+} ions,[33] such a restoration can be achieved by use of dithiotreitol within 2 min.

It is interesting to note that slow-binding inhibition, most commonly involving structural analogues of intermediates of enzymatic reactions, the so called transition state analogues,[37] has rarely been reported for metal ions. A notable example is the inhibition of 6-phosphogluconate dehydrogenase by Zn^{2+} ions, with $K_i^* = 0.021\,\mu M$ and $k_6 = 0.144\,min^{-1}$.[38] In our study on the Hg^{2+} inhibition of urease,[33] it was shown that a Hg^{2+} ion reacts with cysteine residues of the enzyme to form either mercaptides, disulfide or metallodisulfide bonds in the enzyme. The latter two require sterically favoured vicinal sulfhydryl –SH

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groups. In keeping with the biphasic mechanism B of slow-binding inhibition we may therefore surmise that the initial binding of the metal ion, corresponding to the initial, little inhibitory phase of the reaction, gives rise to a slow conformational change of the enzyme which optimizes the binding . The formation of mercaptides or ion-bridging sulfides is in fact substantiated by the correlation of the metal-ion inhibitory sequence (Table I) with the solubilities of the corresponding sulfides.[39] Namely, Hg^{2+} and Cu^{2+} having the lowest sulfide solubilities exhibit the strongest inhibition of the enzyme, Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, and Pb^{2+} having higher sulfide solubilities of approximately the same order are moderate inhibitors, and As^{3+} appeared to be the weakest inhibitor among the studied ions.

Acknowledgement

This work was supported by the KBN grant no. PB 7/T09A/048/20.

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