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Inhibition of urease by Ni^{2+} ions Analysis of reaction progress curves

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Abstract

The inhibition of jack bean urease by Ni^{2+} ions was studied in 20 mM HEPES buffer pH 7.0. The inhibition was observed in two systems which differed in the order in which the components of the reaction mixture were mixed. In the first (unincubated), the reaction was initiated by adding urease to the mixture of urea and Ni^{2+} ions, and in the second (incubated), by adding urea to the mixture of urease incubated with Ni^{2+} ions prior to the reaction. It was shown that Ni^{2+} ions are a competitive slow-binding inhibitor of urease. In the first system the inhibition constants are $K_i = 0.042$ mM and $K_i^* = 0.0028$ mM, and in the second system $K_i^* = 0.0024$ mM. The inhibition was found to involve the rapid formation of a urease-Ni^{2+}complex followed by its relatively slow, reversible isomerization, with forward and reverse rate constants of 0.64 and 0.045 min^{-1}, respectively.

Keywords: Urease; Inhibition; Ni^{2+} ions; Heavy metal ions; Enzyme kinetics

1. Introduction

Studies published so far on heavy metal ions inhibition of urease of both plant and bacterial origin have aimed either at listing the metals in order of their decreasing toxicity [1–12] or at detection of their trace amounts, e.g. of Hg^{2+}, Cu^{2+} and Ag^{+} ions [9–20]. In the majority of these studies observations were limited to the initial stage of the reactions in the enzyme system which had been preincubated with the inhibitor. The Michaelis–Menten kinetics was then applied. Noncompetitive inhibition is the most frequently reported type of this inhibition [1–5,18], but partially competitive [5] and mixed [6] inhibitions were also proposed. The inhibition of urease by heavy metal ions is said to result from the reaction of these ions with a sulfhydryl group of Cys-592 in the active center of the enzyme [21] in a reaction analogous to the formation of metal sulfides. The formation of sulfides with the active center was confirmed experimentally by the correlation between the inhibitory efficiency of metal ions and the solubility products of their sulfides [1–4,7]. The following are the three selected series of inhibitory efficiency of metal ions toward urease which have been reported in the literature:

$Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+}$ [1],

$Hg^{2+} > Ag^{+} > Cu^{2+} > Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$ [3],

$Ag^{+} > Hg^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+} > Fe^{3+} > Zn^{2+} > Pb^{2+}$ [4].

Studies of the inhibition of urease by heavy metal ions are of importance. The sensitivity of urease to heavy metal ion inhibition can be employed in

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sensors for trace determinations of these ions in water, soil, wastewaters and food [8–12,14,15,17,19]. The method is rapid, simple and does not require such complex instrumentation as spectrometric techniques [9,15,16,18].

In this study, the inhibitory effect of Ni^{2+} ions on urease was reinvestigated in order to elucidate the mechanism of metal ion inhibition. The course of urease-catalyzed hydrolysis of urea was studied for longer times (30 min) than commonly used in initial reaction rate measurements. The reaction was carried out in two systems which differed in the order of addition of the components to the reaction mixture. In the first, the reaction was initiated by adding urease to the mixture of urea and Ni^{2+} ions. In the second, by first incubating urease with Ni^{2+} ions and then adding urea to the mixture. The progress curves of these reactions were recorded at different inhibitor concentrations.

The significance of Ni^{2+} ions for urease is unique, since they are a natural component of the urease active site, essential for the catalytic action of the enzyme [22], but at higher concentrations they inhibit the enzyme [1].

2. Slow-binding inhibition

2.1. Unincubated system

In slow-binding inhibition [23] the progress curves of an enzymatic reaction initiated by the addition of an enzyme to the substrate–inhibitor mixture (unincubated system) have two asymptotes corresponding to two rates: initial ν_0 ($t = 0$) and steady-state ν_s ($t \to \infty$). The initial rate ν_0 is rapid and high. After a time that varies between seconds and minutes, the rate decreases to a slower steady-state rate ν_s. If the inhibitor concentration is much higher than that of the enzyme, the equation describing the slow-binding inhibited reaction progress curve is

$$P(t) = \nu_s t + (\nu_0 - \nu_s)(1 - e^{-k_{app}t})\frac{1}{k_{app}} \quad (1)$$

where P is the concentration of the reaction product, ν_0 and ν_s are the reaction initial and steady-state rates, respectively, t stands for time, and k_{app} denotes the apparent first-order rate constant.

The dependence of ν_0, ν_s and k_{app} on the inhibitor concentration I helps to define the mechanism of this inhibition. Sculley et al. [24] proposed the following three mechanisms

1. *Mechanism A* assumes that the formation of the enzyme–inhibitor complex EI^* is a slow step. The initial reaction rate ν_0 is independent of I, whereas $1/\nu_s$ and k_{app} are linear functions of I. The following is the reaction (Scheme 1):

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

$$E + I \underset{k_4}{\overset{k_3}{\leftrightarrow}} EI^*$$

slow step

Scheme 1.

2. *Mechanism B* assumes a rapid formation of an EI complex, which next undergoes slow isomerization to EI^*. In this mechanism $1/\nu_0$ and $1/\nu_s$ are linear functions of I, and k_{app} is a hyperbolic function of I with limiting values of k_6 and $k_5 + k_6$. The reaction Scheme is as follows (Scheme 2):

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

$$E + I \underset{k_4}{\overset{k_3}{\leftrightarrow}} EI \underset{k_6}{\overset{k_5}{\leftrightarrow}} EI^*$$

slow step

Scheme 2.

3. *Mechanism B_{gen}* holds when the initial interaction of the enzyme with the inhibitor is slower than in Mechanism B, and the free enzyme and both the complexes EI and EI^* are in equilibrium. The dependencies of $1/\nu_0$, $1/\nu_s$ and k_{app} on I can either be linear or hyperbolic depending on the (k_4/k_5) ratio. Mechanisms A and B are two extreme cases of the slow-binding inhibition. Mechanism B_{gen} is a more general and more frequent form of this inhibition.

The inhibition constants K_i and K_i^* characterize the EI and EI^* complexes, respectively. The constants can be calculated from the measured reaction rates according

to the following relationships:

$$\nu_0 = \frac{\nu_{max} S}{K_M(1 + (I/K_i)) + S} \quad (2)$$

$$\nu_s = \frac{\nu_{max} S}{K_M(1 + (I/K_i^*)) + S} \quad (3)$$

The values of the forward (k_5) and reverse (k_6) rate constants of the isomerization reaction occurring in Mechanism B can be calculated from

$$k_6 = k_{app}\frac{\nu_s}{\nu_0} \quad (4)$$

$$\frac{k_5}{k_6} = \frac{K_i}{K_i^*} - 1 \quad (5)$$

and k_{app} derived by substituting ν_0 and ν_s from Eqs. (2) and (3) to Eq. (4) is a hyperbolic function of I

$$k_{app} = \frac{k_6(K_M/K_i^*)I + k_6(K_M + S)}{(K_M/K_i)I + (K_M + S)} \quad (6)$$

2.2. Incubated system

If the enzyme is incubated with the inhibitor and the reaction started later with the substrate (incubated system), reaction progress curves are observed which are different from those obtained in the unincubated system. If the final concentrations of the enzyme, of the inhibitor and of the substrate are the same as in the unincubated system, the two steady-state rates in both the systems are identical, provided that there is no significant enzyme inactivation, substrate depletion, or other enzyme-product secondary reactions.

3. Experimental

3.1. Materials

Jack bean urease, Sigma type III, with specific activity 33 μmol NH_3/min mg protein was used. HEPES (Ultra) buffer and urea (Molecular Biology Reagent) were from Sigma. $NiCl_2{\cdot}6H_2O$, p.a., was from POCh, Gliwice, Poland. Water redistilled from glass was used throughout.

3.2. Enzymatic reaction

Urease-catalyzed hydrolysis of urea: $(NH_2)_2CO + H_2O \rightarrow 2NH_3 + CO_2$ uninhibited and inhibited by Ni^{2+} ions was studied in 20 mM HEPES, pH 7.0 at 25°C. The inhibition by Ni^{2+} ions was determined in two experimental systems. In the first system (unincubated), urea was mixed with the inhibitor and the reaction was initiated by the addition of a small volume of concentrated solution of urease (1 cm^3 solution of concentration 1.25 mg urease/cm^3). In the second system (incubated), the enzyme was mixed with the inhibitor, and the mixture was incubated for 20 min at 25°C. The reaction was initiated by the addition of a small volume of concentrated solution of urea (1 cm^3 5 M solution). After mixing, at $t = 0$ the composition of the reaction mixtures in both the systems was identical: 20 mM HEPES pH 7.0, urease concentration 0.0125 mg/cm^3, urea concentration 50 mM, and the concentration of the inhibitor chosen for a given measurement. The total volume of the assay mixture was 100 cm^3.

The reaction was monitored by measuring ammonia concentration by the phenol–hypochlorite method [25] in samples removed from the reaction mixture at time intervals. Product concentration–time experimental data were fitted to Eq. (1) with use of the BURSTO computer program based on nonlinear least squares regression. The BURSTO program was kindly offered by Cleland [26].

4. Results and discussion

4.1. Uninhibited reaction

The kinetic constants of urease: the Michaelis constant K_M and the maximum reaction rate ν_{max} in 20 mM HEPES pH 7.0, in the absence of the inhibitor, were determined by measuring the initial reaction rates at urea concentrations in the range 2–50 mM. The values obtained by applying nonlinear regression to the Michaelis–Menten equation are: $K_M = 3.5 \pm 0.1$ mM and $\nu_{max} = 0.92 \pm 0.02$ mM NH_3/min (72.0 μmol NH_3/min mg).

4.2. Inhibited reaction

4.2.1. Unincubated system

The reaction progress curves recorded for a series of Ni^{2+} ion concentrations in the range 0.05–1.2 mM are presented in Fig. 1. The values of ν_0, ν_s and k_{app} were computed from the fit of the ammonia

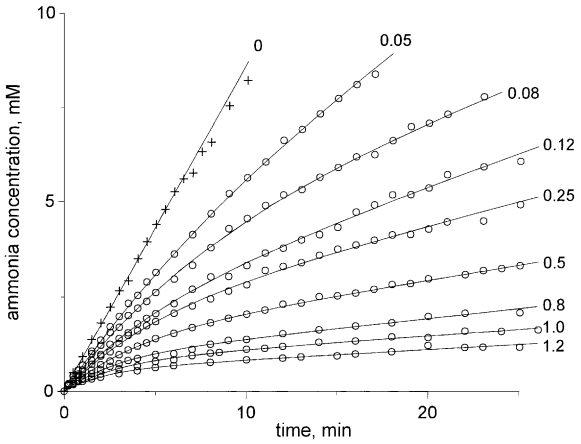

Fig. 1. Unincubated urease-urea-Ni^{2+} ion system: progress curves of urease-catalyzed hydrolysis of urea carried out in the presence of Ni^{2+} ions. Numbers denote Ni^{2+} concentration (mM).

concentration versus time data, according to Eq. (1) by using the BURSTO computer program. The linear dependencies of $1/\nu_0$ and $1/\nu_s$ on Ni^{2+} concentration (Fig. 2a and b) and the hyperbolic dependence of k_{app} on Ni^{2+} concentration (Fig. 2c) provide evidence that this inhibition obeys Mechanism B.

The values of the inhibition constants K_i and K_i^* were calculated from the ν_0 and ν_s dependence on Ni^{2+} concentration using linearised forms of Eq. (2) and (3)

$$\frac{1}{\nu_0} = \frac{K_M}{\nu_{max} S K_i} I + \frac{1}{\nu_{max}} \left(1 + \frac{K_M}{S}\right) \quad (7)$$

$$\frac{1}{\nu_s} = \frac{K_M}{\nu_{max} S K_i^*} I + \frac{1}{\nu_{max}} \left(1 + \frac{K_M}{S}\right) \quad (8)$$

The values obtained are: $K_i = 0.042 \pm 0.003$ mM and $K_i^* = 0.0028 \pm 0.0004$ mM.

The values for the forward (k_5) and reverse rate constants (k_6) of the isomerization reaction calculated from Eqs. (4) and (5) are equal to 0.64 ± 0.09 and $0.045 \pm 0.004\,min^{-1}$, respectively. Their ratio k_5/k_6, which in the state of equilibrium corresponds to $(EI^*)/(EI)$, is equal to 14 ± 2. This means that in the state of equilibrium over 90% of the enzyme–inhibitor complex is in the EI^* form, and the rest in the EI form.

4.2.2. Incubated system

Prior to the enzymatic reaction initiated by the addition of urea, urease was incubated with the

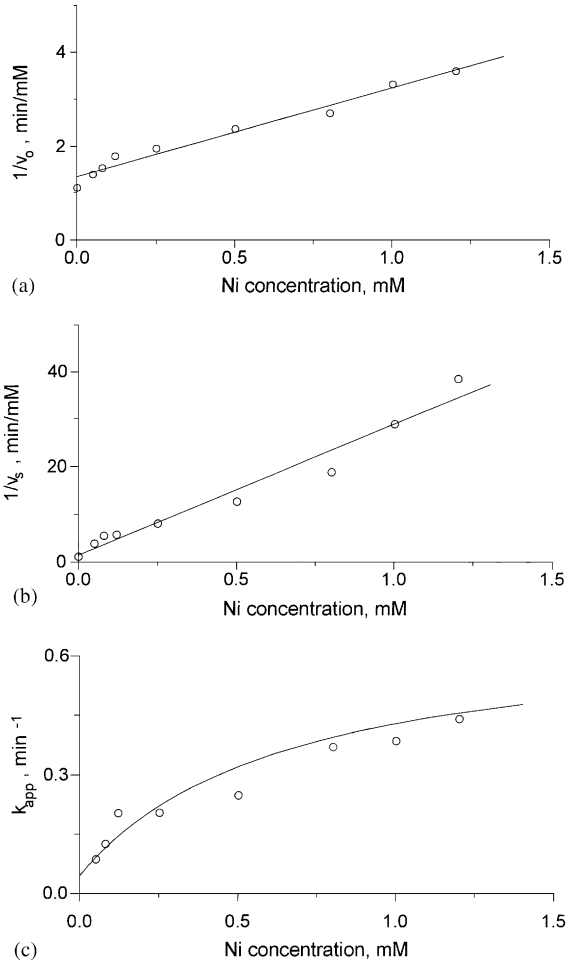

Fig. 2. Unincubated urease-urea-Ni^{2+} ion system: plots of: (a) $1/\nu_0$ vs. Ni^{2+} concentration; (b) $1/\nu_s$ vs. Ni^{2+} concentration; (c) k_{app} vs. Ni^{2+} concentration (solid line was computed from Eq. (7)).

inhibitor for 20 min (25°C). The time of incubation was chosen experimentally: 20 min appeared sufficient for inactivation of the enzyme (see the insert to Fig. 3). The reaction progress curves observed in this system are linear up to 20 min reaction, independent of Ni^{2+} ion concentration (Fig. 3). The K_i^* constant derived from the linear plot: $1/\nu_s$ versus I (Fig. 4) is $K_i^* = 0.0024 \pm 0.0003$ mM. This means that the values of K_i^* obtained in the incubated and unincubated system are equal with a confidence level of 75%. The reaction progress curves recorded in both the systems for the same Ni^{2+} concentration show agreement for

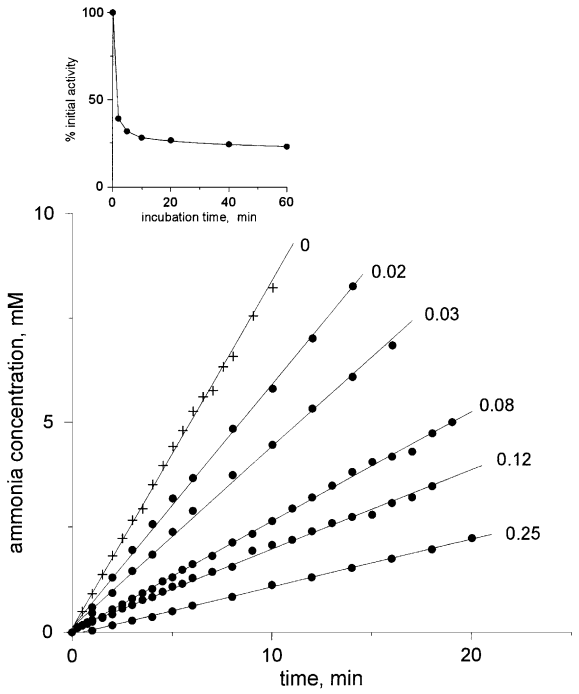

Fig. 3. Incubated urease-urea-Ni^{2+}ion system: progress curves of urease-catalyzed hydrolysis of urea carried out in the presence of Ni^{2+} ions. Numbers denote Ni^{2+} concentration (mM) (the insert shows percent inactivation of urease by incubation with Ni^{2+} ions).

the steady-state rates (see for instance the curves for 0.08, 0.12 and 0.25 mM Ni^{2+}).

In the course of this experimental work time-dependent inhibition of urease by other heavy metal ions: Hg^{2+}, Ag^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}, similar to that by Ni^{2+} ions was also observed (data not shown).

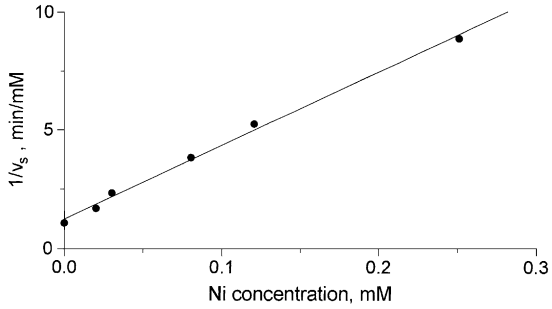

Fig. 4. Incubated urease-urea-Ni^{2+} ion system: plot of: $1/v_s$ vs. Ni^{2+} concentration.

4.3. Initial reaction rate interpretation of the experimental data

In the majority of earlier reports heavy metal ions have been classified as noncompetitive inhibitors of urease [1–5,18]. The kinetic analysis presented classified Ni^{2+} ions to be a competitive slow-binding inhibitor of the enzyme. The discrepancy between these two classifications, as will be shown below, arises from the experimental approach applied. This can be based either on the initial reaction rate measurements or on the observation of reaction progress of the system, which can either be incubated or unincubated. The majority of studies reported in the literature have investigated the incubated urease-metal ion system and measured only the initial reaction rates. If we apply the same approach to our results, and interpret the initial rates measured within the first minute of the reaction in the unincubated system (Fig. 1) in terms

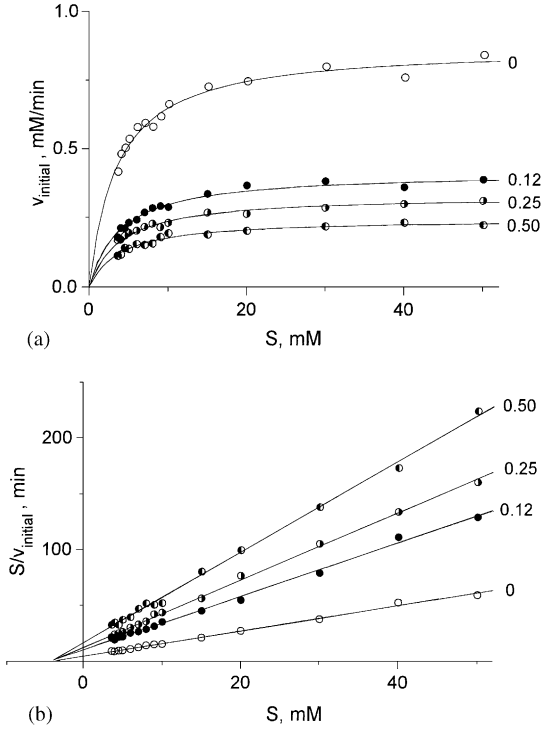

Fig. 5. Unincubated urease-urea-Ni^{2+} ion system. Initial reaction rate interpretation of the experimental data: (a) Michaelis–Menten curves: $v_{initial}$ vs. S; (b) Hanes–Woolf transformation: $S/v_{initial}$ vs. S. Numbers denote Ni^{2+} concentration (mM).

of the Michaelis–Menten kinetics, the plots in Fig. 5 are obtained. The $\nu_{initial}$–S curves in Fig. 5a and the $S/\nu_{initial}$–S straight lines in the Hanes–Woolf transformation in Fig. 5b do indeed produce representations characteristic of noncompetitive inhibition, i.e. ν_{max} decreases with an increase in I, whereas K_M is constant. The inhibition constant based on this interpretation is $K_i = 0.26$ mM, i.e. two orders of magnitude higher than the overall inhibition constant for the slow-binding inhibition K_i^*. N.B. this value depends on the choice of time for the initial rate determination: the longer the time, the lower is the value of K_i (see Fig. 1). The above analysis provides evidence that if the interpretation of the experimental data is limited only to the initial rates to which the Michaelis–Menten kinetics is applied, the classification of the inhibition will be incorrect. Also if the experimental data in the incubated system (Fig. 4) are interpreted as noncompetitive inhibition, then the calculated $K_i = 0.04$ mM. The widely disparate values of K_i prove the complexity of the experimental problem and of its interpretation.

Acknowledgements

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