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Original article

Jack bean (*Canavalia ensiformis*) urease. Probing acid–base groups of the active site by pH variation

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Abstract

A pH-variation study of jack bean (*Canavalia ensiformis*) urease steady-state kinetic parameters and of the inhibition constant of boric acid, a urease competitive inhibitor, was performed using both noninhibitory organic (MES, HEPES and CHES) and inhibitory inorganic (phosphate) buffers, in an effort to elucidate the functions exercised in the catalysis by the ionizable groups of the enzyme active site. The results obtained are consistent with the requirement for three groups utilized by urease with pK_as equal to 5.3 ± 0.2, 6.6 ± 0.2 and 9.1 ± 0.4. Based on the appearance of the ionization step with pK_a = 5.3 in v_{max}-pH, K_M-pH and K_i-pH profiles, we assigned this group as participating both in the substrate binding and catalytic reaction. As shown by its presence in v_{max}-pH and K_M-pH curves, the obvious role of the group with pK_a = 9.1 is the participation in the catalytic reaction. One function of the group featuring pK_a = 6.6, which was derived from a two-maxima v_{max}-pH profile obtained upon increasing phosphate buffer concentration, an effect the first time observed for urease–phosphate systems, is the substrate binding, another possible function being modulation of the active site structure controlled by the ionic strength. It is also possible that the pK_a = 6.6 is a merger of two pK_as close in value. The study establishes that regular bell-shaped activity–pH profiles, commonly reported for urease, entail more complex pH-dependent behavior of the urease active site ionizable groups, which could be experimentally derived using species interacting with the enzyme, in addition to changing solution pH and ionic strength.

Keywords: Boric acid; Enzyme ionization constants; pH effects; Organic buffers; Phosphate buffer; Urease

1. Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to produce ammonia and CO_2. Present in many plants, bacteria, fungi and algae and in soil as a soil enzyme, the enzyme thus plays an important role in the overall metabolism of nitrogen in nature. Primarily, urease allows plants and microorganisms to utilize urea, internally derived or external, to generate ammonia as a nitrogen source for growth [7,19,29]. Of great moment in enzymology, urease obtained from jack bean (*Canavalia ensiformis*) was the first enzyme ever crystallized (1926) [36] and the first nickel-containing enzyme identified (1975) [11].

The reaction catalyzed by urease is deceptively simple (Scheme 1). It consists of the fast hydrolysis of urea to ammonia and carbamate, followed by a spontaneous decomposition of the carbamate to ammonia and carbonic acid [7,19].

Otherwise, owing to its resonance stabilization urea is highly stable in aqueous solutions and resists decomposition. The half-time of the uncatalyzed decomposition of urea is of the order of 3.6 years, and follows a different mechanism that yields the elimination products (Scheme 1). These peculiar features render urease the most proficient enzyme identified to date [14]. For its enzymatic hydrolysis urease utilizes an active site containing a binuclear nickel center bridged by a carbamylated lysine and a hydroxide ion (Scheme 1) as was shown by the crystallographic structures resolved for three different bacterial ureases, from *Klebsiella aerogenes* [20], *Bacillus pasteurii* [4] and *Helicobacter pylori* [18]. The nearly superimposable active sites in these ureases imply that this

Abbreviations: CHES, (2-[*N*-cyclohexyloamino]ethanesulfonic acid); HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid); MES, (2-[*N*-morpholino]ethanesulfonic acid).

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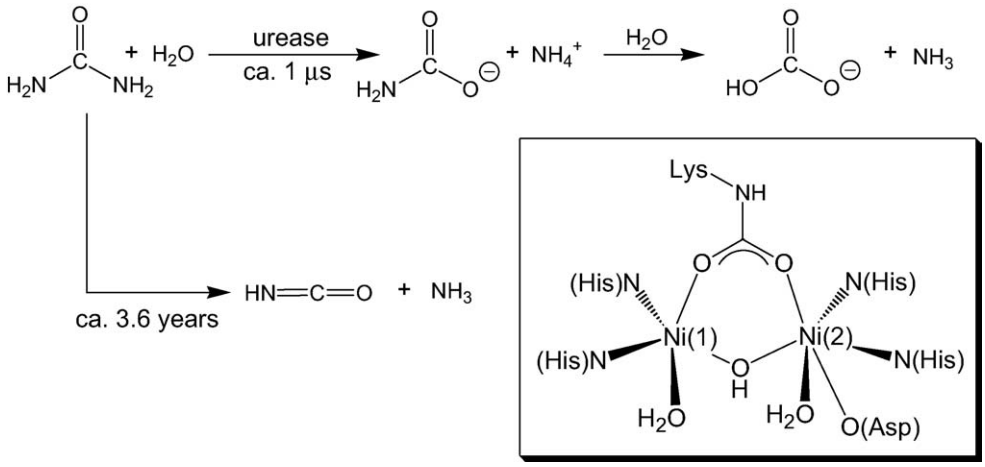

Scheme 1.

structure of an active site is common to all ureases, whether of bacterial, plant or fungal origin.

A long history and extensive research notwithstanding, urease has a catalytic mechanism that is still a matter of debate [7,19]. Final elucidation of the mechanism is of importance for strategies to combat undesirable effects brought about by the enzyme, to which belong reaction-generated ammonia and an increase in pH. These were shown to have profound medical and agricultural implications [29]. Several ureolytic bacteria have been recognized as pathogenic factors in human/animal infections of urinary and gastrointestinal tracts. In the former they are involved in the urinary stone formation, catheter encrustation and pyelonephritis, and in the latter in chronic active gastritis, peptic ulcers, both induced by *H. pylori*, and in hepatic coma. In agriculture urease is essential for converting urea fertilizers to utilizable ammonia. Too rapid a hydrolysis, however, results both in plant damage by ammonia toxicity and in the alkalization of soil, and finally in the loss of nitrogen by ammonia volatilization, thereby creating severe environmental and economic problems.

1.1. Significance of pH-variation studies

One pragmatic approach to the elucidation of enzyme mechanisms is the analysis of pH-variation studies of enzyme steady-state kinetic parameters. Such an analysis provides information on the ionization states of the components of the enzyme reaction, i.e. of the free enzyme, enzyme–substrate complex, and substrate, and thus helps to resolve the involvement of their acid–base functional groups in the catalytic mechanism [9,10].

The active site cavity of ureases features several ionizable amino acid residues that are conserved principally in all known ureases and that are thought to participate in the catalytic reaction. Accordingly, these groups along with the Ni-bound water molecules (Scheme 1), should be considered responsible for the observed pH profiles of urease kinetic parameters. Remarkably, despite numerous reports on the pH profiles of urease steady-state kinetic parameters, there is no consensus among the investigators on the number of acid–base groups required for the catalysis, their pK_as values and functions exercised in the catalytic mechanism. An array of shapes of the profiles has been obtained and interpreted by an array of ways to provide disparate results.

For most ureases the Michaelis constants K_M, falling in value in the range 1–4 mM [5,12,13,17,23,26,30,32,33,35,37,39], have been found to be only slightly dependent on pH [5,12,17,23,35,39]. Unlike K_M, the maximum reaction rate v_{max} is known to be strongly pH dependent. Most frequently, bell-shaped v_{max}-pH profiles in the pH range ca. 4.5–10.5 with the optimum pH around 7–8 have been reported for plant, bacterial and fungal ureases [1,5,13,16,17,23,25–28,31–33,35,37,39] and analyzed in terms of two macroscopic pK_a values, one on the acidic and the other on the basic side of the curve. Less frequently, three pK_as have been obtained by combining v_{max}-pH curves noted in different buffers with the data derived from K_M-pH curves [1,27]. In few instances, for urease from *H. pylori* [6,15,38], from soybean leaf [21] and from jack bean [6,22], irregular v_{max}-pH profiles exhibiting an additional optimum or a hump on the acidic side have been reported, and in some profiles they have been seemingly overlooked [23,28,38,39]. This irregularity implies that the commonly accepted bell-shaped v_{max}-pH profiles might contain some more complex features, concealed under the data. In two studies, one on jack bean [12] and the other on *K. aerogenes* urease [30] this irregularity has been interpreted as involving three and four ionizing groups, respectively, in the urease reaction mechanism.

In addition to the common pH-dependence studies of K_M and v_{max}, there are other methods of identifying enzyme ionizing groups [8,9], among them the method based on the pH-dependence of the inhibition constant K_i of enzyme competitive inhibitors. The method is valuable in that it is capable of differentiating between the enzyme groups involved in binding from those involved in the catalytic reaction [8,9]. This is because a competitive inhibitor can mimic substrate binding to enzyme but is unable to go through any catalytic steps, and accordingly, the pH-dependence of K_i can be used

to determine binding pK_as for substrate. Unlike v_{max}-pH and K_M-pH, the method has not been exploited in studies of urease.

Aiming at defining the pK_a values and the functions of ionizable groups of the urease active site in the catalysis that could help elucidate its mechanism, in this study we investigated the pH effects on jack bean urease by performing pH-dependent measurements of both the steady-state kinetic parameters and the inhibition constant of a urease competitive inhibitor, boric acid, using organic (MES, HEPES and CHES) buffers. We compare the data with those obtained previously in phosphate buffers (v_{max}-pH in 22 mM buffer and K_M^0-pH) [23] and extended here for higher phosphate concentrations and for boric acid inhibition. The buffers proposed are distinct in that while organic buffers are noninhibitory [22], phosphate buffer is a simple competitive inhibitor of jack bean urease at pH < 7.5 [23]. By combining the results we demonstrate that three ionizable groups of the urease active site are involved in the catalytic mechanism. A group with a pK_a = 5.3 is required for substrate binding and catalysis, a group with a pK_a = 9.1 has a role in the catalysis, while the third group featuring a pK_a = 6.6 participates in the binding and/or modulates the structure of the active site.

2. Results and discussion

2.1. pH profiles of v_{max} and K_M

The pH profiles of v_{max} and v_{max}/K_M obtained in the buffers examined are compiled in log–log plots in Fig. 1. Both the maximum reaction rate v_{max} (Fig. 1a) and its ratio to the Michaelis constant v_{max}/K_M (Fig. 1b) decreased at low and high pH with limiting slopes close to +1 and –1, respectively, thereby revealing that two functional groups are involved in the enzymatic process.

The data fitted to Eq. (3) gave the following pairs of values: pK_2 = 5.06 ± 0.06 and pK_1 = 9.26 ± 0.06 from the v_{max} profile, and pK_2 = 5.34 ± 0.04 and pK_1 = 8.67 ± 0.04 from the v_{max}/K_M profile. As the substrate urea has no ionizable groups at these pK_a values, it follows that the two groups are the groups of the free enzyme E (Fig. 1b), whose pK_as undergo outward displacements by about 0.28 and 0.59 pH units to the acidic and basic side, respectively, upon binding the substrate to form the enzyme–substrate complex ES (Fig. 2a). The pK_as obtained from the v_{max}/K_M profile presumably represent the values closer to the true ones, and their displacement suggests that binding of the substrate produces effects favoring the groups' deprotonation or protonation, respectively, or that v_{max} depends on more than one rate constant [8,9]. Understandably, the groups on the enzyme with the pK_2 of 5.34 and the pK_1 of 8.67 must be in opposite protonation state for optimum catalysis.

The presence of these two groups is also substantiated by the shape of the pH profile of K_M itself (Fig. 2a). Generally, the pK_M is known to be a complex function of pH that contains pK_as of both E and ES, and instead of being bell-shaped it takes up a form of a wave. Its fitting is commonly not done for the reason of being too complex. Its graphical analysis presented here shows intriguingly, that in addition to a decrease of enzyme affinity for the substrate at pH lower than ca. 5.3 and higher than ca. 9.1, a small but detectable decrease is also observed between these two values at a pH ca. 6.6–6.7. It is important to emphasize that this phenomenon occurs both in phosphate buffers as well as in MES and HEPES. In view of the complexity of this wave-like function we refrained from interpreting this profile as having a minimum at pH 7–7.5.

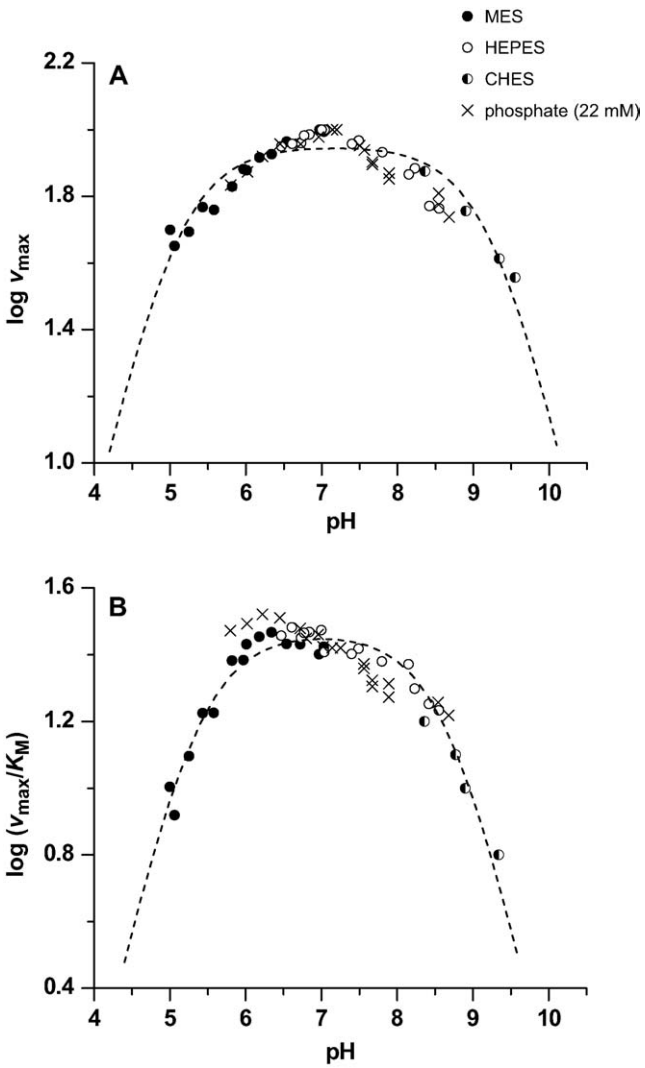

Fig. 1. Urease pH profiles of: (A) log v_{max}, and (B) log (v_{max}/K_M) (for phosphate buffers K_M^0 extrapolated to zero phosphate concentrations [23] were used).

The involvement of a ionizable group with the intermediate pK_a value of 6.77 ± 0.03 (with the corresponding higher value 8.90 ± 0.12) is also evident if the reaction rates measured in phosphate buffer at low substrate concentration (5 mM) are plotted against pH (Fig. 2b). This pK_a is not observed in the organic buffers, for which the relative curves invariably show pK_as of 5.37 ± 0.04 and 9.07 ± 0.04.

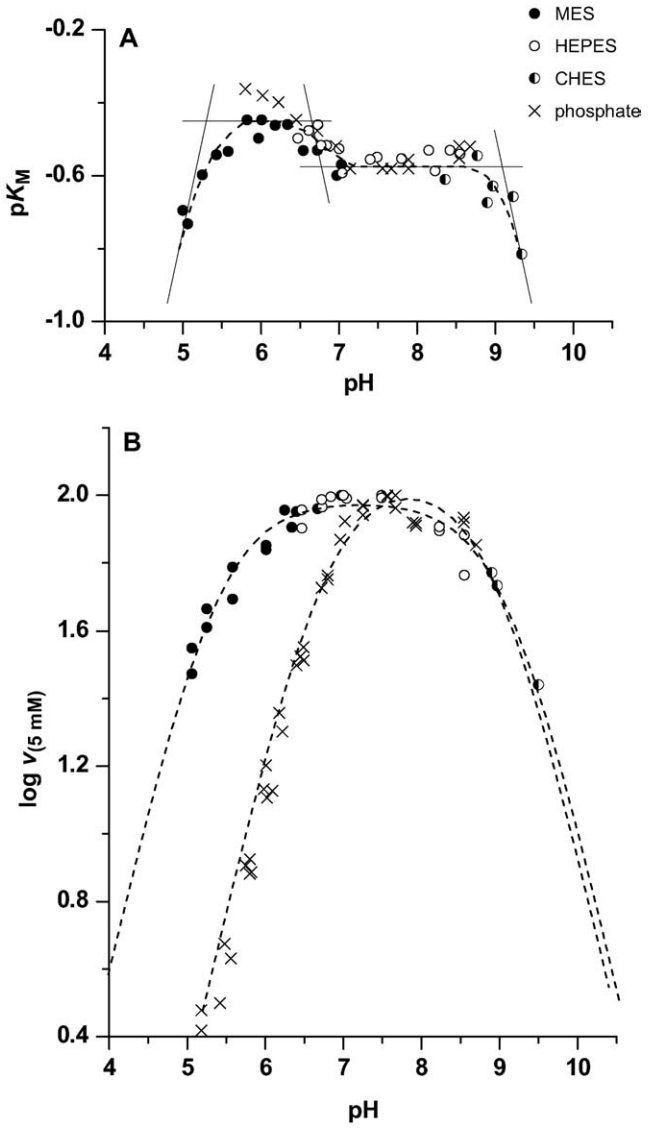

Fig. 2. Urease pH profiles of: (A) pK_M (for phosphate buffers K_M^0 extrapolated to zero phosphate concentrations [23] were used), and (B) log $v_{(5\ mM)}$.

Interestingly, the involvement of a group having an intermediate pK_a of around 6.6 was also traced in the v_{max}-pH dependence (Fig. 3). The overall curve obtained in MES, HEPES and CHES buffers, even though not overlapping accurately with that in phosphate buffers of the lowest concentration studied (22 mM), retains a bell-shape with a distinct maximum at pH 7.1–7.2. The acidic side of the curve, however, appears to be perturbed at pH of around 6.4. Hardly discernible for MES and HEPES buffers in the 'noise' of experimental points, a hump at this pH becomes ever more pronounced upon increasing the concentration of phosphate buffer to finally form another maximum located at a distance of 0.75 pH unit from the main one. The basis for this interpretation of the v_{max}-pH profiles in phosphate buffer was the fact that phosphate had unquestionably been defined to be a simple competitive inhibitor of urease. This means that K_M increases upon increasing phosphate concentration with v_{max} unchanged, in other words that the enzyme–inhibitor interaction is revealed not through v_{max} but K_M. This two-maxima v_{max}-pH profile cannot be interpreted as composed of two overlapping bell-shaped curves, as such an approach would require that each maximum be at least 2 pH units broad [9]. However, it cannot be ruled out that the pK_a of 6.6 observed here is a merger of two very close pK_as. One possible explanation of this peculiarity of the v_{max}-pH profile as revealed in phosphate buffer more distinctly than in the other buffers, is that phosphate, a urease competitive inhibitor, known to bind to the urease active site by bridging the two Ni ions using three O atoms [2], provokes a disturbance of the overall architecture of the cavity including a group with the pK_a of 6.6. Some more intricate role of phosphate that is not yet explainable with the present data is also possible, and the question remains open to further investigation and discussion in the literature.

It is important to highlight that the two-maxima activity–pH curve noted here, brought about by raising phosphate buffer concentration/ionic strength is an effect that was observed for the first time in urease–phosphate systems. Such an effect was earlier observed for *K. aerogenes* urease, however, in organic buffers containing salts to produce different ionic strengths [30]. The authors described the activity–pH curve by two curves, one with pK_as = 4.5 ± 0.5 and 6.3 ± 0.5 and the other with pK_as = 6.9 ± 0.5 and 9.1 ± 0.5, and interpreted as resulting from the existence of two distinct active states in urease governed by the ionic strength controlling the location and orientation of the essential amino acid residues.

The pH profiles of v_{max}, v_{max}/K_M and K_M obtained indicate that the three ionizable groups of the urease active site with pK_as of ca. 5.3, 6.6 and 9.0 participate both in the catalysis and in substrate binding. One possible function of the group with the pK_a = 6.6 is the binding, and the functions of the other two groups cannot be discriminated at this point.

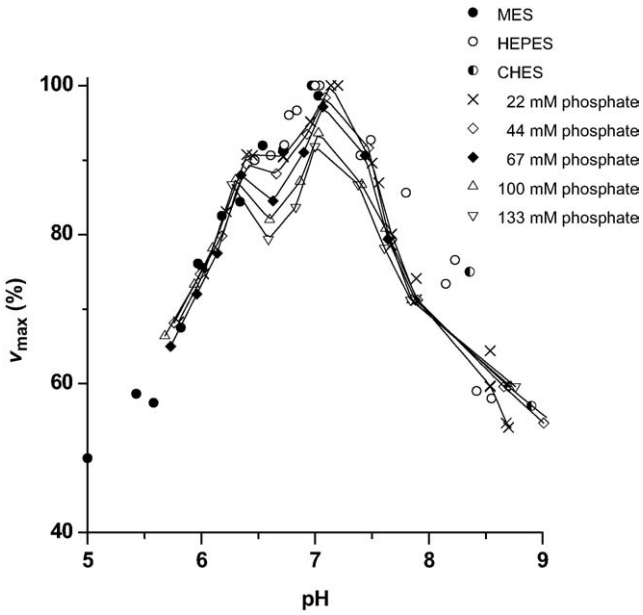

Fig. 3. Urease v_{max} versus pH curves.

2.2. pH profiles of K_i of boric acid

To further elucidate the functions fulfilled by the identified acid–base groups in the urease catalytic process, we determined the pH-dependence of the inhibition constant K_i of boric acid in both MES, HEPES, CHES and phosphate buffers (Fig. 4).

The pH–pK_i profile in MES, HEPES and CHES exhibited two ionization constants pK_a equal to 5.26 ± 0.06 and 9.39 ± 0.06 with a plateau corresponding to K_i = 0.08 mM on average. The two constants coincide with those estimated from the profiles of the kinetic parameters. Despite the possibility that the higher value might be viewed as the ionization constant of boric acid [5], a group of a pK_a close to this value must also be operative in the enzyme as shown earlier. Likewise, the group of lower pK_a is the group on the enzyme, and as revealed here in the pK_i profile, can be said to participate in the binding, meaning that the substrate binding is prevented when the group with this pK_a becomes protonated. In accord with the earlier finding, we may conclude that this group participates both in the binding and catalysis.

Unsurprisingly, the inhibition of urease by boric acid in phosphate buffer differs from that in the organic buffers. This is due to the fact that in addition to boric acid also the phosphate buffer participates in the inhibition, both compounds being urease competitive inhibitors [24], the latter only when its pH is lower than pH 7.5 corresponding to the ionization constant of $H_2PO_4^-$ ion [23], and both known to bind at the active site of the enzyme [2,3]. As shown in Fig. 4, the inhibition constant of boric acid determined at each pH in 22 mM phosphate buffer, high at low pH values (at pH 5.2, K_i = 4.7 mM) decreased with increasing pH along a linear slope +1 to approach that obtained in the organic buffers (K_i = 0.08 mM) at pH 7.2, that is when the participation of phosphate in the inhibition ceases.

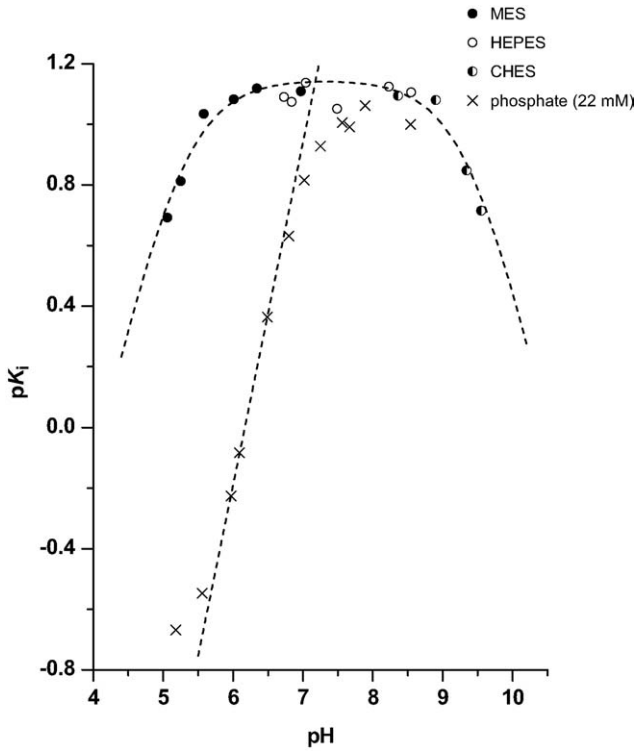

Fig. 4. Urease pH profiles of pK_i of boric acid.

2.3. pH profile of K_i of phosphate buffer [23]

Our present conclusion concerning the involvement of the two urease groups with the lower pK_as in the substrate binding, is in agreement with the conclusions that can be drawn from our previous work on phosphate buffer [23]. Namely, we showed that the pK_i–pH dependence for the buffer consists of two straight lines, one with a slope of –1 at pH 5.8–6.5 and the other with a slope of –2 at pH 6.5–7.5, intersecting at pH 6.5 (Fig. 3 in [23]). This indicates that two ionizable groups of the enzyme with pK_a values of 6.5 and < 5.8 must be protonated for phosphate binding. The slopes of the lines on the other hand, indicate that at pH < 6.5 one protonation (of a group on enzyme) takes place, whereas at pH 6.5–7.5, two protonations with pK_as that are fairly close (of a group on enzyme and of $H_2PO_4^-$) occur.

2.4. Concluding remarks

The present investigation establishes that regular bell-shaped activity–pH profiles, commonly reported for urease, indeed entail more complex pH-dependent behavior of the enzyme ionizable groups, which can be experimentally derived using species interacting with the enzyme, in addition to changing solution pH and ionic strength. The results we obtained are consistent with the requirement for three active site groups utilized by urease in its catalytic action with pK_as of 5.3 ± 0.2, 6.6 ± 0.2 and 9.1 ± 0.4, the values being the arithmetic means of all the measurements performed (Table 1).

Based on the appearance of the group with pK_a = 5.3 in v_{max}-pH, K_M-pH and K_i-pH profiles, we assigned the group as participating both in the substrate binding and catalytic reaction. As shown by its presence in v_{max}-pH and K_M-pH, one obvious role of the group of pK_a = 9.1 is the participation in the catalytic reaction. The function of the group of pK_a = 6.6 is difficult to define. Appearing mainly in the v_{max}-pH profile, the group modulates the urease activity upon increasing the phosphate concentration presumably by phosphate interacting with a group that is involved in the urease active state architecture, hence its involvement in the substrate binding cannot be ruled out. Possible is also that the pK_a = 6.6 is a resultant of two pK_as that are close in value.

Of great importance, the identification of the groups, at this point only possible by comparing the values with the protein literature data [8], provides speculative although, as known from the literature, not impossible assignments. The pK_as of 5.3 and 6.6 might be viewed as related to carboxyl and imidazole groups, or vice versa, while the pK_a of 9.1 could be related to a nickel-bridging hydroxide, recently established as the nucleophile responsible for the formation of the

Table 1
pK_a values of urease determined from pH profiles of the kinetic parameters

Profile (conditions)	pK_a	pK_a	pK_a
log v_{max} (50 mM organic and 22 mM phosphate)	5.06 ± 0.06	–	9.26 ± 0.06
log (v_{max}/K_M) (50 mM organic and 22 mM phosphate)	5.34 ± 0.04	–	8.67 ± 0.04
pK_M (50 mM organic and 0 mM phosphate)	~ 5.3	6.6–6.7	~9.1
log v (5 mM urea in 50 mM organic)	5.37 ± 0.04	–	9.07 ± 0.04
log v (5 mM urea in 22 mM phosphate)	–	6.77 ± 0.03	8.90 ± 0.12
v_{max} (22–133 mM phosphate)	–	6.4–6.6	–
p$K_{i,boric}$ (10 mM organic)	5.26 ± 0.06	–	9.39 ± 0.06
p$K_{i,phosphate}$ (data taken from Ref. [23])	< 5.8	6.5	–
Mean values	5.3 ± 0.2	6.6 ± 0.2	9.1 ± 0.4

transition state of the catalysis [4]. This group was proposed to be required in the protonated (OH^-) form in order to provide a proton to the urea NH_2 group and to form ammonia in a proton transfer step that would follow the nucleophilic attack. An alternative assignment of the high pK_a to a sulfhydryl group of a nearby cysteine is excluded by evidence that rules out its involvement in the urease catalysis [7,19]. A further reliable identification of the groups can be obtained from other studies, such as temperature variation, solvent perturbation, chemical modifications or site-directed mutagenesis [8,9].

3. Materials and methods

3.1. Materials

Jack bean urease (Sigma type III, activity 22 units mg^{-1} solid), MES, HEPES, CHES buffers (SigmaUltra) and urea (Sigma Molecular Biology) were used for all experiments. $NaH_2PO_4{\cdot}H_2O$ and $Na_2HPO_4{\cdot}12H_2O$, Na_2EDTA and boric acid were from POCh, Poland. MES, HEPES and CHES buffer stock solutions (200 mM) were prepared by dissolving the required amounts in water and had their pH values adjusted with a NaOH solution. The pH ranges were: 5.00–7.03 for MES, 6.47–8.55 for HEPES and 8.36–9.55 for CHES, as measured upon dilution to the working concentration. Overlapping pH ranges were used to ensure that any discontinuities of the parameters measured would be detected. Phosphate buffer stock solutions (222 mM) were prepared by mixing the phosphate salts in proportions to produce the pH values within the range 5.18–8.54, as measured upon dilution. The pH was measured with a calomel combination electrode (ERH-11, Hydromet, Poland) in conjunction with a pH meter (CX-731, Elmetron, Poland). Each working buffer solution contained 1 mM Na_2EDTA. Redistilled water was used throughout.

3.2. Urease assay

The activity of urease was assayed in reaction mixtures (25 ml) containing 0.625 mg enzyme and urea-buffer or urea-inhibitor-buffer of designed concentrations. The concentrations of urea (2–450 mM) and of boric acid (0–2 mM) were selected accordingly to the urease activity at a given pH. Enzymatic reactions were initiated by the addition of 0.25 ml of a urease concentrated solution (2.5 mg enzyme ml^{-1} freshly prepared in 10 mM buffer) to the reaction mixture. The amount of ammonia liberated was determined by the phenol–hypochlorite method [40] in samples removed from the reaction mixtures, against the calibration curves determined individually for each buffer, its pH and concentration, with special attention paid to CHES that was found to inhibit the 625 nm color development. The initial reaction rates v_o at each substrate concentration were calculated from the readouts corresponding to 5 min reaction. The measurements were performed at 22 ± 1 °C.

3.3. Determination of K_M, v_{max} and K_i

The Michaelis constants K_M and the maximum reaction rates v_{max} were determined from the initial reaction rates v_o measured at a range of urea concentrations S in 50 mM MES, HEPES and CHES buffers and in phosphate buffers of concentrations between 22 and 133 mM, at each pH indicated. The K_M and v_{max} values were obtained using Eq. (1). The values of v_{max} and $v_{5\ mM\ urea}$ presented in all figures are expressed as percent of their maximal values corresponding to the optimum pH.

The inhibition constants of boric acid, K_i, were determined in 10 mM MES, HEPES and CHES and 22 mM phosphate buffers. At each pH, the initial reaction rates v_o were measured for two series of solutions, one containing a range of urea concentrations and the other containing a range of boric acid concentrations I at one urea concentration selected at a non-saturating level. The K_i values were obtained from the Dixon plots according to Eq. (2b).

3.4. Data analysis

Data were fitted using the following equations. The kinetic parameters K_M and v_{max} were obtained by fitting substrate saturation curves v_o–S to Eq. (1). Data for competitive inhibition described by Eq. (2a) were fitted to the Dixon plot, Eq. (2b), and K_i values were obtained with use of the v_{max} values obtained from Eq. (1) [34]. Data of bell-shaped pH curves

were fitted using Eq. (3), and the MacCurveFit v.1.5 program was used to obtain the ionization constants pK_1 and pK_2 [9,10].

$$v_o = \frac{v_{max} S}{K_M + S} \quad (1)$$

$$v_o = \frac{v_{max} S}{K_M\left(1 + \frac{I}{K_i}\right) + S} \quad (2a)$$

$$\frac{1}{v_o} = \frac{K_M}{v_{max} S K_i} I + \frac{1}{v_{max}}\left(1 + \frac{K_M}{S}\right) \quad (2b)$$

$$X = \frac{X_{(opt)}}{1 + \frac{[H^+]}{K_2} + \frac{K_1}{[H^+]}} \quad (3)$$

In the above equations v_o and v_{max} are the initial and maximum rates, respectively, K_M is the Michaelis constant, S and I are the substrate and inhibitor concentrations, respectively, and K_i is the inhibition constant. In Eq. (3) X stands for v_{max}, v_{max}/K_M or $1/K_i$, and $X_{(opt)}$ for their theoretical maximal values corresponding to all enzyme groups in their preferred ionization state, while K_1 and K_2 denote the ionization constants of the enzyme active site basic and acidic groups involved in the enzymatic reaction.

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References

[1] A. Barth, H.J. Michel, A contribution on the mechanism of the enzymatic cleavage of urea, Biochem. Physiol. Pflanzen 163 (1972) 103–109.

[2] S. Benini, W.R. Rypniewski, K.S. Wilson, S. Ciurli, S. Mangani, Structure-based rationalization of urease inhibition by phosphate: novel insights into the enzyme mechanism, J. Biol. Inorg. Chem. 6 (2001) 778–790.

[3] S. Benini, W.R. Rypniewski, K.S. Wilson, S. Mangani, S. Ciurli, Molecular details of urease inhibition by boric acid: insights into the catalytic mechanism, J. Am. Chem. Soc. 126 (2004) 3714–3715.

[4] S. Benini, W.R. Rypniewski, K.S. Wilson, S. Miletti, S. Ciurli, S. Mangani, 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 7 (1999) 205–216.

[5] J.M. Breitenbach, R.P. Hausinger, *Proteus mirabilis* urease. Partial purification and inhibition by boric acid and boronic acids, Biochem. J. 250 (1988) 917–920.

[6] S.D. Cesareo, S.R. Langton, Kinetic properties of *Helicobacter pylori* urease compared with jack bean urease, FEMS Microbiol. Lett. 9 (1992) 15–22.

[7] S. Ciurli, S. Mangani, Nickel-containing enzymes, in: I. Bertini, A. Sigel, H. Sigel (Eds.), Handbook on Metalloproteins, Marcel Dekker, New York, 2001, pp. 669–707.

[8] W.W. Cleland, Determining the chemical mechanisms of enzyme-catalyzed reactions by kinetic studies, Adv. Enzymol. 45 (1977) 273–386.

[9] W.W. Cleland, The use of pH studies to determine chemical mechanisms of enzyme-catalyzed reactions, Meth. Enzymol. 87 (1982) 390–405.

[10] M. Dixon, E.C. Webb, in: Enzymes second ed, Longmans, London, 1964, pp. 116–145.

[11] N.E. Dixon, C. Gazzola, R.L. Blakeley, B. Zerner, Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J. Am. Chem. Soc. 97 (1975) 4131–4133.

[12] N.E. Dixon, P.W. Riddles, C. Gazzola, R.L. Blakeley, B. Zerner, Jack bean urease (EC 3.5.1.5). V. On the mechanism of action of urease on urea, formamide, acetamide, *N*-methylurea, and related compounds, Can. J. Biochem. 58 (1980) 1335–1344.

[13] H. Eng, J.A. Robertson, G.W. Stemke, Properties of urease from *Ureaplasma urealyticum*: kinetics, molecular weight, and demonstration of multiple enzyme isoelectric point forms, Can. J. Microbiol. 32 (1986) 487–493.

[14] G. Estiu, K.M. Merz Jr., The hydrolysis of urea and the proficiency of urease, J. Am. Chem. Soc. 126 (2004) 6932–6944.

[15] D.J. Evans Jr., D.G. Evans, S.S. Kirkpatrick, D. Graham, Characterization of the *Helicobacter pylori* urease and purification of its subunits, Microb. Pathogen. 10 (1991) 15–26.

[16] A.S. Fahmy, N.Y. Abadir, B.M. Abd-Alla, Purification and characterization of urease from ruminal fluid of camel (*Camelus dromedarius*), J. Camel. Pract. Res. 5 (1998) 143–155.

[17] M. Fidaleo, R. Lavecchia, Kinetic study of enzymatic urea hydrolysis in the pH range 4–9, Chem. Biochem. Eng. Q. 17 (2003) 311–318.

[18] N.-C. Ha, S.-T. Oh, J.Y. Sung, K.A. Cha, M.H. Lee, B.-H. Oh, Supramolecular assembly and acid resistance of *Helicobacter pylori* urease, Nat. Struct. Biol. 8 (2001) 505–509.

[19] R.P. Hausinger, P.A. Karplus, Urease, in: K. Wieghardt, R. Huber, T.L. Poulos, A. Messerschmidt (Eds.), Handbook of Metalloproteins, Wiley, West Sussex UK, 2001, pp. 867–879.

[20] E. Jabri, M. Carr, R.P. Hausinger, P.A. Karplus, The crystal structure of urease from *Klebsiella aerogenes*, Science 268 (1995) 998–1004.

[21] P.S. Kerr, D.G. Blevins, B.J. Rapp, D.D. Randall, Soybean leaf urease: comparison with seed urease, Physiol. Plant. 57 (1983) 339–345.

[22] B. Krajewska, M. Chudy, M. Drozdek, Z. Brzózka, Potentiometric study of urease kinetics over pH 5.36–8.21, Electroanal. 15 (2003) 460–466.

[23] B. Krajewska, W. Zaborska, The effect of phosphate buffer in the range of pH 5.80–8.07 on jack bean urease activity, J. Mol. Catal., B Enzym. 6 (1999) 75–81.

[24] B. Krajewska, W. Zaborska, M. Leszko, Z. Brzózka, Inhibition of jack bean urease by a mixture of boric acid and phosphate buffer, Pol. J. Chem. 73 (1999) 359–366.

[25] K.J. Laidler, The influence of pH on the rates of enzyme reactions, Trans. Faraday Soc. 51 (1955) 550–561.

[26] M.W. Lubbers, S.B. Rodriguez, N.K. Honey, R.J. Thornton, Purification and characterization of urease from *Schizosaccharomyces pombe*, Can. J. Microbiol. 12 (1996) 132–140.

[27] K. Lynn, Some properties and purifications of urease, Biochim. Biophys. Acta 146 (1967) 205–218.

[28] P.R. Martin, R.P. Hausinger, Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease, J. Biol. Chem. 267 (1992) 20024–20027.

[29] H.L.T. Mobley, R.P. Hausinger, Microbial ureases: significance, regulation, and molecular characterization, Microbiol. Rev. 53 (1989) 85–108.

[30] S. Mulrooney, T. Zakharin, R.A. Schaller, R.P. Hausinger, Dual effects of ionic strength on *Klebsiella aerogenes* urease pH-dependent activation and inhibition, Arch. Biochem. Biophys. 394 (2001) 280–282.

[31] K. Myrbäck, Zur Wirkungsweise der Hydrolasen, Acta Chem. Scand. A 1 (1947) 142–148.

[32] M.A. Pearson, I.S. Park, R.A. Schaller, L.O. Michel, P.A. Karplus, R.P. Hausinger, Kinetic and structural characterization of urease active site variants, Biochemistry 39 (2000) 8575–8584.

[33] Y. Qin, J.M.S. Cabral, Kinetic studies of the urease-catalyzed hydrolysis of urea in a buffer-free system, Appl. Biochem. Biotechnol. 49 (1994) 217–240.

[34] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1975.

[35] P.K. Srivastava, A.M. Kayastha, Studies on the histidine residues in pigoenpea (*Cajanus cajan* L.) urease, J. Mol. Catal., B Enzym. 16 (2001) 81–89.

[36] J.B. Sumner, The isolation and crystallization of the enzyme urease, J. Biol. Chem. 69 (1926) 435–441.

[37] P.V. Sundaram, K.J. Laidler, The urease-catalyzed hydrolysis of some substituted ureas and esters of carbamic acid, Can. J. Biochem. 48 (1970) 1132–1140.

[38] M. Taylor, C.S. Goodwinn, Q.N. Karim, Two urease activities with different pH optima in *Campylobacter pylori* and similar organisms, FEMS Microbiol. Lett. 55 (1988) 259–262.

[39] M.J. Todd, R.P. Hausinger, Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*, J. Biol. Chem. 262 (1987) 5963–5967.

[40] M.W. Weatherburn, Phenol–hypochlorite reaction for determination of ammonia, Anal. Chem. 39 (1967) 971–974.