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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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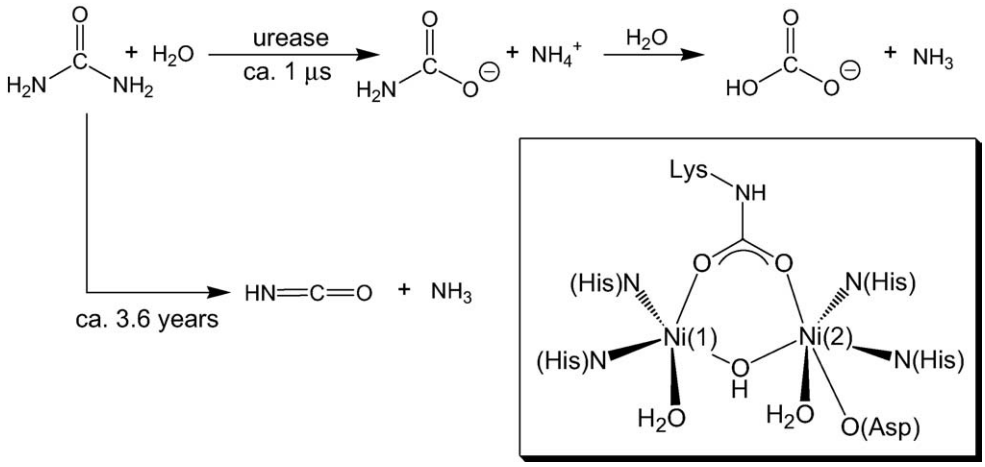

Scheme 1.

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