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Review

Ureases I. Functional, catalytic and kinetic properties: A review

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ABSTRACT

Ureases (urea amidohydrolases, EC 3.5.1.5) are a group of highly proficient enzymes, widely distributed in nature, whose catalytic function is to catalyze the hydrolysis of urea, its final products being carbonic acid and ammonia. The products and the resulting increase in pH of the reaction environment are consequential characteristics of the action of ureases. Apart from its natural significance, ureases-catalyzed hydrolysis of urea is important in that it has great potential for practical applications. In view of this importance, this article offers a review of the properties of the enzymes, where in addition to the established knowledge, the recent findings are presented. Special emphasis is put on the functional and practical properties of ureases that can be customized and exploited in a diversity of important applications, notably medical, analytical, environmental and engineering.

Contents

1. Introduction

Ureases (urea amidohydrolases, EC 3.5.1.5) are a group of enzymes widespread in nature among plants, bacteria, fungi, algae and invertebrates that, although with different protein structures, exercise a single catalytic function, that is the hydrolysis of urea, its final products being ammonia and carbonic acid. Deceptively simple, this function is frequently looked at as a response of nature to the ubiquitous presence of urea. Functionally, ureases belong to the superfamily of amidohydrolases and phosphotriestreases [1]. The primary common feature of the enzymes is the presence of metal centres in their active sites, whose task is to activate the substrate and water for the reaction. Among other dinuclear metallohydrolases in the superfamily, ureases are unique in that they possess Ni(II) ions in the active site.

In urease-catalyzed hydrolysis of urea, the two protagonists of the reaction, urea and urease are special in the development of natural sciences. Urea, first discovered in human urine by

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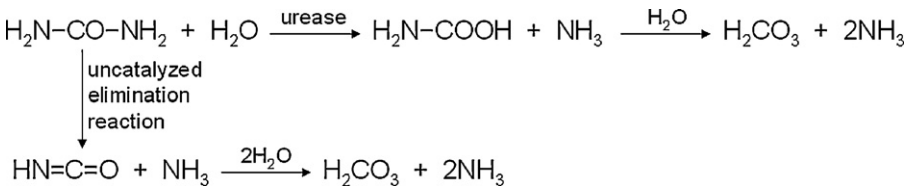

Scheme 1.

Hillaire M. Rouelle in 1773, later became the first organic compound synthesized from inorganic materials (Wöhler, 1828). That ammonia in urine derives from the fermentation of urea was first recognized in 1798 by Fourcroy and Vauquelin, but the first ureolytic microorganism, *Micrococcus ureae* was isolated from urine much later in 1864 by van Tieghem. By contrast, the first ureolytic enzyme was obtained in 1874 by Musculus from putrid urine, and as proposed by Miquel in 1890, it was named urease. The discovery of urease in soybean (*Glycine max*) by Takeuchi in 1909 on the other hand assured a plentiful source of the enzyme for increasingly intensified investigations (data taken from Ref. [2]). Of landmark significance in biochemistry, in 1926 James B. Sumner crystallized urease from jack bean (*Canavalia ensiformis*) to show the first time ever that enzymes are proteins and can be crystallized [3]. Equally importantly, ascertained by Dixon et al. in 1975, jack bean urease was the first enzyme shown to possess nickel ions in the active site, essential for activity [4].

Since its discovery by Takeuchi, urease has been a subject of extensive research that included its occurrence and roles in nature, the mechanisms by which it functions, the specificity of action and reaction to foreign compounds. More recently, the research included amino acid sequencing, crystal structures of native ureases, their mutants and complexes with inhibitors, and the genetic organization. Necessary for the understanding of the molecular basis of the catalytic mechanism of urease, this knowledge is also crucial for controlling the processes occurring with the participation of the enzyme. These include both natural processes and those man-devised, in which the enzyme is utilized. The latter ones have emerged, as concomitantly with the theoretical research, the practical potential of ureases has been increasingly studied for various laboratory, technical and biotechnological applications.

In view of both the significance of the processes in which ureases take part and their possible exploitation in practical applications, this article offers a review of the properties of the enzymes, where in addition to the established knowledge, the recent findings are presented. Special emphasis is put on the functional and practical properties of ureases with the intention that this be an introduction to a follow-up article dealing with how they can be customized by immobilizations. A large number of references are offered to provide the guidance through the source literature.

2. Enzymatic cleavage of urea and proficiency of ureases

The reaction catalyzed by urease is the hydrolysis of urea, its products being carbamate and ammonia [5–7], the former further hydrolyzing spontaneously to another molecule of ammonia and carbonic acid (Scheme 1).

Otherwise, owing to its resonance energy estimated at 30–40 kcal/mol [8,9] urea is highly stable in aqueous solutions and resists decomposition. The uncatalyzed decomposition of urea observed in aqueous solutions [10,11], also proven theoretically [12], is an elimination reaction that results in isocyanate and ammonia (Scheme 1). The reaction was found slow, proceeding at a rate independent of pH between 2 and 12 [13]. By contrast, the uncatalyzed hydrolysis of urea has never been observed [14]. These peculiar features render it difficult to assess the catalytic proficiency of urease. This is because the catalytic proficiency, defined as the ratio of the effective second-order rate constant for free enzyme, k_{cat}/K_M, to the rate constant of the uncatalyzed reaction, k_{uncat}, [15,16] requires that the rate constants k_{cat} and k_{uncat} be for the same reaction mechanism occurring in the presence and absence of enzyme. With the constant k_{uncat} for the uncatalyzed urea hydrolysis experimentally unavailable, urease proficiency has been roughly estimated to be higher than 10^{14} by referring to the uncatalyzed decomposition via elimination reaction [17–19]. Recently, to estimate the true urease proficiency, the uncatalyzed hydrolysis constants k_{uncat} were derived theoretically, one from the data on the hydrolysis of 1,1,3,3-tetramethylurea (Me_4U) [20] and another from mechanistic simulations [21]. The values are compiled in Table 1 along with the experimental ones for the uncatalyzed decomposition and for the urease (plant and bacterial)-catalyzed hydrolysis. The values show that with the Me_4U data, urease is an enzyme with efficiency, rate enhancement and proficiency comparable to other relatively highly proficient C—N hydrolases [15,20], however, with those from the mechanistic simulations, the 10^{30}-fold rate enhancement and proficiency of the order of 10^{32}, though viewed by some as overestimated [12], classify urease as the most proficient enzyme known to date [15,16,21].

Intriguingly, sound documentation notwithstanding, the hydrolytic action of urease [5–7,22] has recently been challenged by a postulate derived from quantum chemical [23,24] and molecular dynamics [25] simulations that the elimination mechanism can compete with the hydrolytic one for the urease-catalyzed urea cleavage, both mechanisms resulting in the same final products H_2CO_3 and NH_3 (Scheme 1). Effectively, this postulate is in keeping with an early discussion on the cyanate mechanism of urease [2,5,26] and its recent experimental reassessments based on model dinickel centres [27,28].

3. Occurrence of ureases and their functions

Ureases are enzymes widely occurring in nature. They are synthesized by numerous organisms, including plants, bacteria, algae, fungi and invertebrates, and also occur in soils as a soil enzyme. The substrate urea for the reaction is readily available. Its pervasive presence arises chiefly from urine excretion by animals and from the decomposition of N-compounds from dead organisms [29], and also from its application as a fertilizer. Thus, owing to their occurrence, ureases play a prominent role in the overall nitrogen metabolism in nature. Their key function is to provide organisms with nitrogen in the form of ammonia for growth.

3.1. Ureases in plants

Urease, beside urea amidolase, is an essential urea-degrading enzyme in plants that catalyzes urea assimilation after uptake into plant cells [29,246]. Not entirely yet elucidated, higher plants were shown to possess various urea transport systems, passive and active, which allow them to optimize N-nutrition depending on the nitrogen form available from external environment or internally synthesized. From external environment, plants assimilate urea through roots as urea, but essentially as ammonia generated from urea hydrolysis, and this is possible due to the presence of ureases in soils, a fact exploited in urea fertilization practices.

Table 1a
Kinetic constants of uncatalyzed and urease-catalyzed decomposition of urea at neutral pH and 38 °C.

Decomposition of urea	k (s^{-1})	$\tau_{1/2}$	E_a (kcal/mol)	K_M (mM)	Efficiency k_{cat}/K_M (s^{-1} M^{-1})	Refs.
Uncatalyzed elimination reaction	6.3×10^{-9}	3.5 y	32.5	–	–	[10]
Uncatalyzed hydrolysis[a]	6.0×10^{-11}	365 y	22.9	–	–	[20]
Uncatalyzed hydrolysis[b] (37 °C)	2.5×10^{-27}	8.8×10^{18} y	–	–	–	[21]
Jack bean urease-catalyzed hydrolysis	5913	118 μs	6.64	2.9	2.0×10^{6}	[123]
K. aerogenes urease-catalyzed hydrolysis (37 °C)	3500	198 μs	–	2.8	1.25×10^{6}	[131]

Table 1b
Rate enhancement and proficiency of urease acting on urea relative to uncatalyzed decomposition of urea (data taken from Table 1a).

Decomposition of urea (referral to urease-catalyzed hydrolysis)	Urease rate enhancement k_{cat}/k_{uncat}		Urease proficiency $(k_{cat}/K_M)/k_{uncat}$ (M^{-1})	
	Jack bean	*K. aerogenes*	Jack bean	*K. aerogenes*
Uncatalyzed elimination reaction	9.3×10^{11}	5.6×10^{11}	3.2×10^{14}	2.0×10^{14}
Uncatalyzed hydrolysis[a]	9.8×10^{13}	5.8×10^{13}	3.4×10^{16}	2.1×10^{16}
Uncatalyzed hydrolysis[b]	2.4×10^{30}	1.4×10^{30}	8.2×10^{32}	5.0×10^{32}

[a] Theoretical data derived from the hydrolysis of 1,1,3,3-tetramethylurea (Me_4U) [20].
[b] Theoretical data derived from mechanistic simulations [21].

Importantly, high inputs of urea fertilizers applied may constitute a serious hazard both to plants and the environment (see Section 3.3). To enhance fertilization practices, urea is also applied through the foliage. Absorbed rapidly, foliar-applied urea, however, can be toxic in high concentrations [29]. Clearly, further knowledge on the mechanisms of urea-related plant nutrition is needed for the development of balanced strategies of urea-fertilization for best and sustainable agricultural crop production.

In plant cells on the other hand, urease participates in the metabolism of N-containing compounds [29,30,246]. Therein, apart from being acquired from external environment, urea is an important intermediate resulting from two metabolic processes: arginase-catalyzed breakdown of arginine [31] and degradation of purines and ureides [32,247]. Metabolized rapidly, urea practically does not accumulate, however, constantly generated may serve as an N-source. It has also been hypothesized that due to the generation of ammonia, urease fulfils a defense function against plant pathogens [33]. In the same context, recently, evidence has been provided that independent of their ureolytic activity, ureases also exhibit insecticidal [34,35] and antifungal properties [36,37], suggestive of their function in plant defense system. Present virtually in all plants, urease is especially abundant in leguminous seeds, those of soybean (*Glycine max*) containing 0.012% urease/dry mass and those of jack bean (*Canavalia ensiformis*) 0.07–0.14%, the latter thus being one of the commonest sources of the enzyme [3,38,39].

3.2. Ureolytic bacteria pathogenic to humans and animals

Among numerous ureolytic bacteria, of special interest are those that are pathogenic to humans and animals. The pathogenesis is due to the effects arising from urea hydrolysis, which are an increase in pH (up to ca. 9.2) and the toxicity of the released ammonia and of its derivatives [40]. Urea, the major metabolic nitrogenous waste product of most terrestrial animals, is produced in the liver, carried in the bloodstream to the kidneys, and excreted in urine, its serum concentration in healthy humans amounting to 1–10 mM, and urine concentration to around 0.5 M [40,41]. Additionally, some 20–25% of all urea produced is estimated to remain in the intestinal tract, its concentration in the stomach being 1.7–3.4 mM. This renders urea readily available to ureolytic bacteria making the urinary and intestinal tracts the most common sites of ureolytic bacteria infections in humans [40,41].

3.2.1. Ureolytic bacteria in urine

The infection of the urinary tract increases the pH of urine, typically neutral or slightly acidic, and may cause a number of complications. One is the necrosis of kidney tissue responsible for acute pyelonephritis. Another, more frequent one is the precipitation of normally soluble ions in urine, leading to the formation of urinary stones (also implicated in catheter encrustation). Chemically, the major urinary stones are composed of struvite $MgNH_4PO_4{\cdot}6H_2O$ and carbonate apatite $Ca_{10}(PO_4)_6CO_3$ [40–43], and the most common bacterium responsible for their formation is *Proteus mirabilis*, also *Ureaplasma urealyticum*, other implicated bacteria being of *Pseudomonas*, *Klebsiella* and *Staphylococcus* spp. [40,43]. Ureolytic-infection-induced stones are estimated to constitute 15–20% of all urinary stones.

Incidentally, by the same token the precipitation takes place in urine outside the human body, especially when source separated from household wastewater. The precipitation is undesirable in that it causes clogging in pipes, and in consequence increases maintenance costs [44]. Conversely, when performed in a controlled manner, the precipitation is utilized for the phosphorus- and nitrogen recovery in wastewater and urine treatment processes [44]. This is done because human urine contributes ca. 80% of the total N and ca. 45% of the total P load to municipal wastewater [45]. This proposed biological recovery of the two dominant nutrients together as struvite, particularly from the urine separately collected in a no-mix toilet system, presents an interesting alternative to their chemical removal in urine recycling.

3.2.2. Helicobacter pylori

Helicobacter pylori is the primary ureolytic bacterium infecting the intestinal tract [40,41,46,47]. The bacterium typically colonizes the mucosal lining of the stomach, where the increase in pH of the strongly acidic environment allows the bacterium that requires pH 6–8 to grow, to persist in the hostile conditions. Concomitantly it incurs damage to the host tissue, thereby giving rise to gastritis and gastroduodenal ulcers. The damaging factors are ammonia and monochloramine, the latter resulting from the oxidative burst created by immune cells. Ammonia has been shown to have a direct cytotoxic effect on gastric epithelial cells, while monochloramine can induce mutagenic DNA damage, which in the case of chronic infection with *Helicobacter pylori*, is believed to contribute to the development of stomach cancer. In addition to gastric complications, the infection of the intestinal tract is a causative factor of hepatic coma.

Interestingly, the action of urease in the upper intestinal tract has been exploited in a non-invasive urease breath test for the diagnosis of bacterial infections of *Helicobacter pylori*. In the test, ^{13}C- or ^{14}C-labelled urea is ingested, and if the bacterium is present in the stomach, the urea is converted into isotope-labelled carbon

dioxide. This is absorbed into the blood and exhaled in the breath, where it is detected by mass spectrometer or scintillation counter [48].

3.3. Soil urease and ammonia volatilization problems

Of great importance in agriculture is the ureolytic activity of soils [49–52]. This activity derives from microorganisms [52,53], but foremost from soil urease [50]. A remnant of dead plant and microbial cells, the enzyme is extracellular, but owes its stability to the immobilization on clays and humic substances [54,55]. The presence of this stable form of urease in soils allows urea to be used as an efficient N-fertilizer. Due to its high N content, chemical stability, and low production costs urea now makes up over 50% of the total N-fertilizers applied worldwide. The role of soil urease is in making urea available to plants through converting it into ammonia. Significant though it is, the hydrolysis may also have adverse effects. Namely, if too rapid, it may result in unproductive loss of nitrogen by ammonia volatilization, while ammonia toxicity and alkalinity along with accumulated nitrite may induce plant damage by affecting seed germination, seedling growth and early plant growth in soil, thereby causing severe environmental and economic problems [49–51].

Ammonia volatilization is also a problem faced in management of livestock wastes, these being presently produced in increasing amounts due to considerably intensified farming practices [56]. This volatilization entails a number of undesirable consequences. One is that livestock slurry, a valuable fertilizer for crop production, has its value considerably reduced by loss of nitrogen. Another one is that ammonia is a source of pollution, and besides, it contributes to odour that may have an adverse impact on people and animals.

In a similar context, attempts have been made to recycle urine to use as flush water. The idea is to suppress urease activity in urine to avoid ammonia emission [57]. Given that an adult passes approximately 1.5 L of urine per day and 10 times this amount water is used for flushing, the process is expected to help save water and relieve the water shortage problem.

In all the instances that require the control of urease activity (medical, agricultural, environmental), to counteract its deleterious effects, the use of enzyme inhibitors is proposed (see Section 8).

3.4. Acid ureases

Acid ureases are a distinct subgroup among ureases. Their salient characteristic is that unlike typical (neutral) ureases with the optimal activity at pH close to neutral (Table 2) they have the optimal pHs in the range 2–4.5. They are found to be produced mainly in intestinal (*Lactobacillus*, *Streptococcus*, *Escherichia*, *Morganella* and *Bifidobacterium*) [58–63] and soil bacteria (*Arthrobacter mobilis*) [64], and remarkably, it has not been explained to date why bacteria growing in the alimentary tract, whose pH is neutral, produce acid ureases. Like neutral ureases, acid ureases are nickel-containing enzymes with related K_M values, but their activities are mostly lower (Table 2). Also, like neutral ureases they are inhibited by acetohydroxamic acid, Hg^{2+}, Cu^{2+}, Ag^{+} ions and by p-chloromercuribenzoic acid [59–61,64].

Table 2
Kinetic parameters of selected ureases.

Ureases	K_M[a] (mM)	Activity[a] (μmol urea/min mg)	pH_{opt}[a]	p*I*	Refs.
Plants					
Canavalia ensiformis (jack bean)	2.9–3.6	2700–3500	7.0–7.5	5.0–5.1	[120,123,128,130,136–140]
Glycine max (soybean)	0.2–0.6	650–800	7	–	[34,36,82,141]
Cajanus cajan (pigeon pea)	3.0	3120	7.3	–	[83]
Gossypium hirsutum (cotton seeds)	0.12–0.15	14.5	8.0	–	[36]
Fungi					
Aspergillus nidulans	1.33	670	8.5	–	[142]
Aspergillus niger	3.0	1341	8.0	–	[143]
Coccidioides immitis	4.1	1750	8.0	5.5	[88,102]
Schizosaccharomyces pombe	1.03	700–800	8.2	–	[87]
Bacteria					
Aerobacter aerogenes	2.8	690	7.5	–	[144]
Arthobacter oxydans	12.5	219	7.6	4.3–4.7	[145]
Bacillus pasteurii	17.3	2500	8.0	4.6	[53,77,105,146,147]
Brevibacterium ammoniagenes	32	3570	7	4.1	[148]
Brucella suis	5.60	540	7.0	5	[149]
Helicobacter pylori	0.2–0.8	1700	8.0–8.2	5.9; 5.93; 5.99	[94–97]
Klebsiella aerogenes	2.8	2500	7.75	–	[131]
Proteus mirabilis	13	2000	7.5	5.2–5.9	[132,140]
Providencia rettgeri	10.5	30.6	7.5	5.1–5.2	[140,150]
Providencia stuartii	9.3	7100	–	5.4	[109,140]
Selenomonas ruminantium	2.2	1100	8.0	–	[151]
Staphylococcus leei	1.66	730	–	–	[92]
Staphylococcus saprophyticus	9.5	1979	6.0; 7.0	4.7	[91]
Staphylococcus xylosus	–	1573	7.2	4–5	[152]
Ureaplasma urealyticum	2.5	180,000	6.9–7.5	5.0–5.2; 4.60	[153,154]
Acid ureases					
Streptococcus mitior	2.0	230	4.5	4.6	[61]
Lactobacillus fermentum	2.7	220	2	4.8	[59]
Lactobacillus reuteri	2.8	290	2	4.7	[60]
Arthrobacter mobilis	3.0	2370	4.2	6.8	[64]
Algae					
Nitellopsis obtusa	4.5	–	–	–	[155]
Invertebrates					
Land snail *Otala lactea*	0.11	0.79	8.4–9.0	–	[156]

[a] In comparing these values it should be remembered that they are dependent on the purity of the enzyme, and on conditions in which they were determined, i.e. temperature, buffer, its pH and concentration.

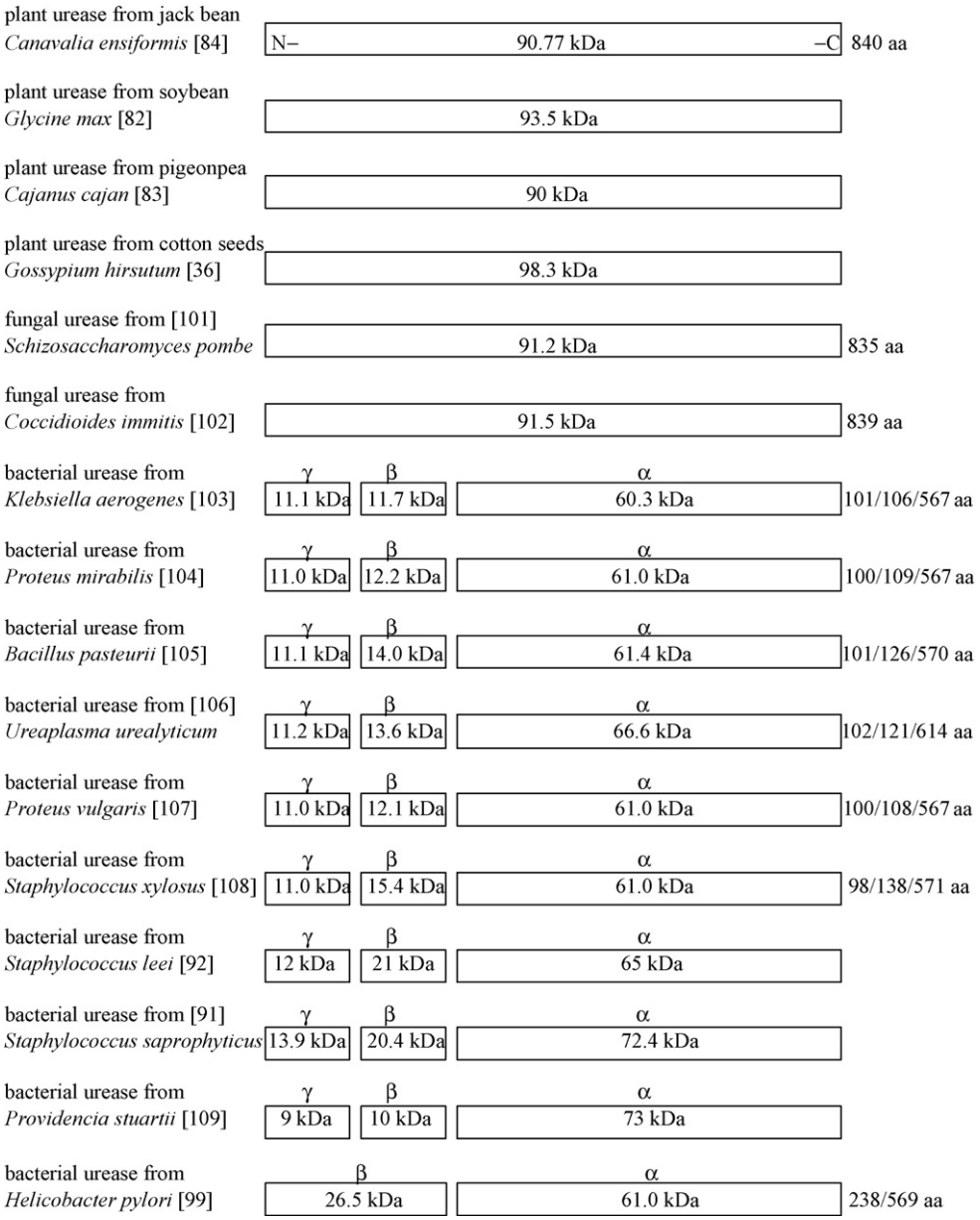

Fig. 1. Structural subunits of ureases.

Acid ureases, those from *Lactobacillus* sp. in particular, are now commercially available in soluble and insoluble form, and are used for the elimination of urea in alcoholic beverages [59–65]. This is done to prevent the formation of ethyl carbamate, known to be carcinogenic, from the reaction of urea and ethanol taking place during alcohol manufacturing and preservation. With the acidic pH of alcoholic beverages (e.g. pH of sake, 4.4; pH of wine, 3.2 [63]), unlike neutral ureases, acid ureases thus meet the requirements of the process.

3.5. Ureases in calcium carbonate biomineralization

Promoting calcium carbonate formation is another important function of ureases in nature [66]. Urea hydrolysis is counted beside photosynthesis and sulphate reduction, as one of the pathways of microbial-induced $CaCO_3$ precipitation occurring commonly in various natural environments, such as soils, geological sediments and natural waters [66–68]. Though not entirely resolved, the role of bacteria in the process is thought to be 3-fold, namely (i) to increase the alkalinity of the environment that favours the precipitation of $CaCO_3$, (ii) to increase the concentration of dissolved inorganic carbon, and (iii) to serve as crystal nucleation sites. By both increasing the pH and providing carbonate ions (Scheme 1), the hydrolysis of urea in the presence of Ca^{2+} ions gives rise to the precipitation of carbonates, interestingly, in different crystal polymorphs, along the following overall equation [67,68]:

$$H_2N{-}CO{-}NH_2 + 2H_2O + Ca^{2+} \xrightarrow{\text{urease}} 2NH_4^+ + CaCO_3 \downarrow$$

Further to gaining a better understanding of calcification processes in nature, if performed in a biomimetic manner, this biocatalytic calcification offers potential in innovative biotechnological applications [69]. These include preparation of advanced carbonate materials [70–72], cleaning waste- and ground-waters of excess soluble Ca^{2+} [73] and of radionuclide $^{90}Sr^{2+}$ [74], and its use as a microbial sealant for cementing surface cracks and fissures in building [75–77] and historic monument restoration [78] sectors. The bio-precipitation of $CaCO_3$ is also used as a plugging

agent in oil reservoirs. The plugging is done for prevention of sand transportation during oil production from unconsolidated reservoir formations and, by decreasing the permeability of porous areas of the reservoirs, for enhancement of secondary oil recovery [248,249].

A similar mechanism has been hypothesized to be involved in the biomineralization of calcium carbonate by invertebrates for the formation of shells [79,80]. The hypothesis holds that inorganic carbon in the form of HCO_3^- is derived from the reaction of carbonic anhydrase, whereas ammonia produced from urease-catalyzed hydrolysis of urea serves as a proton acceptor, thereby allowing $CaCO_3$ to precipitate. In this connection, an intriguing urease-hypothesis on big mass extinctions on the Earth was outlined [80]. The hypothesis suggests that the partial pressure of atmospheric CO_2 equal to $\sim$ 560 ppmv, by setting seawater pH at $\sim$ 7.9 concurrent with the dead zone of urease activity typical of urease-reliant marine organisms, results in the cessation of $CaCO_3$ biomineralization. This, according to the hypothesis, has led in the past and may lead again, if the present level of CO_2 equal to $\sim$383 ppmv (seawater pH $\sim$8.1) continues to grow, to the disruption of the marine ecosystem, thereby triggering "the kill mechanism" for mass extinctions of the Earth's species.

4. Protein structures of ureases

Plant and fungal ureases are made up of identical subunits, typically of ca. 90 kDa (Fig. 1), most commonly assembled as trimers α_3 and hexamers α_6 [36,81–83]. The α subunit of jack bean (*Canavalia ensiformis*) urease is composed of 840 amino acids [84,85], its molecular mass without Ni(II) ions amounting to 90.77 kDa, hence a mass of the hexamer, the 12 nickel ions included, being 545.34 kDa (590 kDa by a sedimentation method [81]). Other examples of homohexameric structures of plant ureases are those of soybean [82], pigeon pea [83] and cotton seeds [36] enzymes, but compositions such as α_2 are also known for urease from the leaf of mulberry (*Morus alba*) [86] and that from fungi *Schizosaccharomyces pombe* [87], or α_4 for fungal *Coccidioides immitis* urease [88].

Unlike plant and fungal, bacterial ureases are composed of three distinct subunits, one large (α, 60–76 kDa) and two small (β, 8–21 kDa and γ, 6–14 kDa) (Fig. 1), commonly forming $(\alpha\beta\gamma)_3$ trimers, resulting in the enzyme molar masses between 190 and 300 kDa. The typical examples are *Klebsiella aerogenes* [89] and *Bacillus pasteurii* [90] ureases. Other stoichiometries, however, were also reported, examples being $(\alpha\beta\gamma)_4$ for *Staphylococcus saprophyticus* [91] and $(\alpha\beta\gamma)_5$ for *Staphylococcus leei* [92] ureases. Coincidentally, acid ureases are also reported to be composed of three subunits, similar to those of neutral bacterial ureases, α (66–68 kDa), β (15–17 kDa) and γ (8–14 kDa), their structures being either $(\alpha_1\beta_2\gamma_1)_2$ [59–61] or $(\alpha\beta\gamma)_3$ [64]. By contrast, the ureases of *Helicobacter* species are composed of two subunits, α (61–66 kDa) and β (26–31 kDa) [93–100], which for *Helicobacter pylori* urease were shown to form a dodecameric complex $((\alpha\beta)_3)_4$ [100].

Remarkably, though composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi exhibit high homology of amino acid sequences. When compared, the $(\alpha\beta)$ unit of *Helicobacter* urease and the $(\alpha\beta\gamma)$ unit of the other bacterial ureases align with the jack bean urease monomer in a manner where the α subunits align with the carboxy-terminal two thirds of this monomer, and the other subunits, with its amino-terminal one third (Fig. 1). This suggests that all ureases are evolutionary variants of one ancestral enzyme. Of importance, the active sites in all known ureases are always located in the α subunits.

All plant and bacterial ureases, except that from *Helicobacter pylori* are cytoplasmic [41,110]. *Helicobacter pylori* urease differs in that upon lysis of some of the bacteria, the released enzyme associates with the surface of intact cells and accounts for ca. 30% of the total activity [111]. It has been hypothesized that this external location of urease, along with the special dodecameric structure of the enzyme and its low K_M value (Table 2) that allows it to be effective in low substrate concentrations, provides protection to the bacterium against the hostile acidic environment and enables it to inhabit the gastric lumen. However, mainly because the purified enzyme is irreversibly inactivated below pH 5, the role of the enzyme at the extracellular location has been a subject of controversy [112].

Even though jack bean urease was the first enzyme obtained in the crystalline form [3], its crystal structure has not as yet been determined. The best resolution obtained at 3.5 Å only allowed to assign the octahedral crystals of this urease to the cubic space group $F4_132$ [113], and to see its molecule as built of two trimers α_3, each being structurally related to the $(\alpha\beta\gamma)_3$ trimer of bacterial ureases [114]. Also another urease of plant origin from pigeon pea had its structure preliminarily resolved at 2.5 Å, with crystals of the rhombohedral space group $R32$ [115]. Successfully determined X-ray structures by contrast have bacterial ureases from *Klebsiella aerogenes* and *Bacillus pasteurii*, the former resolved at 2.2 Å with crystals of the cubic space group $I2_13$ [89,113], and the latter at 2.0 Å resolution with crystals of the hexagonal space group $P6_322$ [90,116]. Consistent with the high homology of amino acid sequences (63% (α), 46% (β) and 61% (γ)) [90], the overall structures of these ureases were shown to be equivalent. The molecules $(\alpha\beta\gamma)_3$ are tightly associated, 3-fold symmetric trimers of $\alpha\beta\gamma$ units, each molecule containing three dinuclear Ni-centres located in the α subunits at a distance of ca. 50 Å from one another, entirely independent. The amino acid residues ligating the nickel ions in the active centres proved to be conserved in both ureases. More recently, also the crystal structure of *Helicobacter pylori* urease was resolved [100], this being of importance for the understanding of the survival mechanism of the bacterium.

5. Active centre of urease and reactive cysteine residues in the enzyme

The knowledge on the urease active site was provided by the crystal structures resolved for bacterial ureases from *Klebsiella aerogenes* [89,117] and *Bacillus pasteurii* [90]. The active site was shown to contain a binuclear nickel centre (Fig. 2a), in which the Ni—Ni distances were found close in value in both ureases, 3.7 and 3.5 Å in *Bacillus pasteurii* and *Klebsiella aerogenes* enzyme, respectively. In the centre the nickel(II) ions are bridged by a carbamylated lysine through its O-atoms, with Ni(1) further coordinated by two histidines through their N-atoms, and Ni(2) by two histidines also through N-atoms and additionally by aspartic acid through its O-atom. Besides, the Ni ions are bridged by a hydroxide ion (WB), which along with two terminal water molecules, W1 on Ni(1), W2 on Ni(2), and W3 located towards the opening of the active site, forms an H-bonded water tetrahedral cluster filling the active site cavity. It is this cluster that urea replaces when binding to the active site for the reaction. As a result of the above ligations, Ni(1) is pentacoordinated and Ni(2) hexacoordinated, and their coordination geometry is pseudo square pyramidal and pseudo octahedral, respectively. Crucially, the fact that the two ureases have a nearly superimposable active site implies that it is common to all ureases.

In addition to the amino acid residues directly involved in the architecture of the active site, in the urease catalysis functional are also the residues composing the mobile flap of the site. Mainly through H-bonding, the residues participate in the substrate binding, stabilize the catalytic transition state and accelerate the reaction. The flap is thought to act as a gate for the substrate. In the structure of *Bacillus pasteurii* urease the flap was found in the open

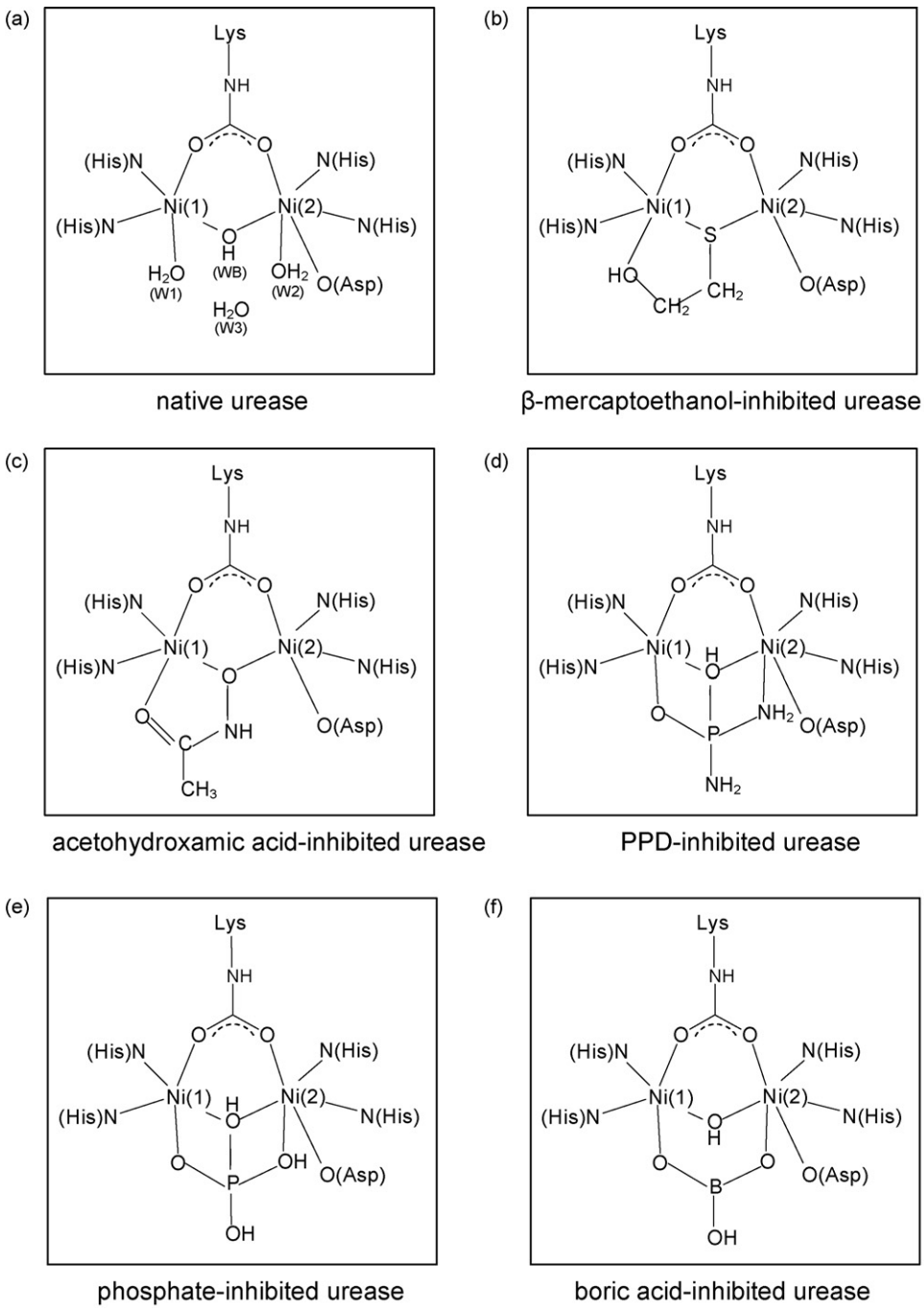

Fig. 2. Schematic structures of the active site of urease: (a) native [90], and inhibited by (b) β-mercaptoethanol [166], (c) acetohydroxamic acid [169], (d) PPD [90], (e) phosphate [195], and (f) boric acid [196].

conformation, while its closed conformation is apparently needed for the reaction [90]. Note that the coordination of urea to the active site of urease has never been observed in a resting state of the enzyme.

Among the amino acid residues in the flap there is one cysteine, Cys319 by *Klebsiella aerogenes* numbering, Cys322 by *Bacillus pasteurii* numbering and Cys592 in jack bean urease. Although determined not to be essential in the catalysis, as was evidenced by site-directed mutagenesis studies [118], this cysteine is judged to be involved in the catalysis, as was demonstrated with cysteine-reactive agents [119–122]. In view of the structural studies, one role suggested for this cysteine residue is in positioning other key residues in the active site appropriately for the catalysis. This flap cysteine in particular, but also other cysteine residues in ureases effectively enable enzyme cysteine-targeted inhibition (see Section 8).

Ureases are cysteine-rich enzymes. Apart from the flap Cys592, jack bean urease was proven by disulfide titration in non-denaturating conditions to contain five other cysteine residues per subunit that are more reactive [119,120]. With additional nine cysteine residues disclosed only in denaturating conditions, the overall number of cysteines per jack bean urease subunit amounts to 15, hence 90 cysteines per molecule. By contrast, *Klebsiella aerogenes* urease revealed nine cysteines per ($\alpha\beta\gamma$) unit, eight in the α and one in the β subunit, hence the overall number of cysteines in this urease is 27 per molecule [117,121].

6. Proposed reaction mechanisms for urease-catalyzed urea hydrolysis

The mechanisms of urease-catalyzed hydrolysis of urea presently contemplated are those by Benini et al. [90] and Karplus et al. [18]. Admittedly, taking their origins from the mechanism put forward by Zerner's group [123], they assume that in the active site of urease (Fig. 2a), urea binds to the more electrophilic Ni(1) ion with the oxygen atom of its carbonyl group, owing to which the carbonyl carbon becomes more electrophilic, hence more susceptible to nucleophilic attack. Upon replacing W1–W3 waters, urea is further bound to Ni(2) through the nitrogen of one of its amino groups (nonleaving-N), making its binding overall bidentate [90]. This binding is believed to facilitate the nucleophilic attack of water on the carbonyl carbon, resulting in the formation of a tetrahedral intermediate from which NH_3 and carbamate are released. While Benini et al. [90] propose that this nucleophilic attack is performed by the bridging hydroxide, simultaneously acting as a general acid that provides protons to the leaving NH_3 molecules, Karplus et al. [18,124] argue that it is His320 located in the mobile flap of the active site that is poised to act as the general acid in this protonation, consistent with the assumed reverse protonation mechanism. Furthermore, Karplus et al. do not entirely rule out a monodentate binding of urea only to Ni(1), with Ni(2) delivering a water molecule as the nucleophile to the carbonyl carbon atom of urea [18,110], this mechanism being also supported by molecular dynamics calculations [126], and heavy-atom isotope effect and kinetic investigations of the hydrolysis of formamide [127] and semicarbazide [250] by urease. All things considered, the proposed mechanisms of urea hydrolysis catalyzed by urease contain a number of controversies that remain to be clarified.

7. Kinetic properties of ureases and enzyme substrates

Ureases typically exhibit simple Michaelis–Menten behaviour, though at high concentrations substrate and product inhibitions are seen (see Section 8). Kinetic characteristics of ureases from different sources, including their Michaelis constants K_M, activities, optimum pHs and isoelectric points p*I*, are compiled in Table 2.

Typically, the K_M values amount to 1–4 mM and, as was shown for jack bean [123,128–130] and bacterial ureases [131,132], are practically invariant with pH. Unlike K_M, the activity of ureases is strongly dependent on pH, the enzymes being active in a pH range of ca. 4.5–10.5 with the optimum activity at pH 7–8. Frequently interpreted as bell shaped, thereby indicating the involvement of two active-site functional groups in the catalysis of pK_as ~ 6.5 and ~ 9 [128,131], the activity–pH profiles of ureases were shown to possess shapes with two activity maxima, one dominant at pH 7–8 and the other one at an acidic pH [96,133–135]. These shapes imply the involvement of either three (pK_as: 3.0, 6.25 and 9.0 [123] or 5.3, 6.6 and 9.1 [133] for jack bean urease) or four functional groups (pK_as: 4.5, 6.3, 6.9 and 9.1 for *Klebsiella aerogenes* urease [134]). Regrettably, the identity of the functional groups detected by the pH-variation studies has not been elucidated.

Although originally believed to be absolutely urea-specific, today a number of urease substrates are known which, however, are hydrolyzed at a much lower rate than urea (Table 3). Among the substrates two distinct groups are seen, namely urea analogues and phosphoric acid amides and esters. The reaction with compounds other than urea is complex, which is because most of them are both enzyme substrates and inhibitors (see Section 8).

Table 3
Substrates of ureases.

Substrates	K_M (mM)	Refs.
Urea: $H_2N—CO—NH_2$	1–4	Table 2
Hydroxyurea: $H_2N—CO—NHOH$	1.25–1.6; 125	[83,157]
Dihydroxyurea: HOHN—CO—NHOH	12.5	[158]
Semicarbazide: $H_2N—CO—NHNH_2$	60	[159]
Formamide: $H_2N—CO—H$	1060; 516	[123,160]
Acetamide: $H_2N—CO—CH_3$	750; 240	[123,130]
Thioacetamide: $H_2N—CS—CH_3$	83	[130]
Thiourea: $H_2N—CS—NH_2$	70; 210; 40	[83,130,161]
Methylurea: $H_2N—CO—NHCH_3$[a]	220; 120; 1000	[83,123,162]
Ethylurea: $H_2N—CO—NHC_2H_5$	340	[162]
Methyl carbamate: $H_2N—CO—OCH_3$	490	[162]
Ethyl carbamate: $H_2N—CO—OC_2H_5$[a]	420	[162]
Amides and esters of phosphoric acid		
Phosphoroamidate	–	} [163,164]
Diamidophosphate (DAP)	–	
Phenylphosphorodiamidate (PPD)	–	
Phosphoric triamide (PTA)	–	

[a] Ref. [159] reports that these compounds are not urease substrates.

8. Urease inhibitors

Ureases are inhibited by a number of compounds. Best investigated are presented in Table 4. Studies of the inhibitions are done to provide insights into the molecular mechanism of urease action as well as to assure compounds that could effectively control enzyme activity.

Substrate urea, product ammonium ions, and substrate analogues are weak inhibitors of urease. The kinetic analysis of the inhibition by substrate analogues is difficult; typically in the initial phase of their action urease loses activity to further undergo reactivation [7,158].

As revealed by pH-dependent kinetic study, thiols inhibit urease competitively in their thiolate anion form R-S^- [165]. The crystal structure of urease with β-mercaptoethanol (Fig. 2b) [166], a model compound of this group [165,167,168], demonstrated that β-mercaptoethanol binds to urease by displacing all four water/hydroxide molecules in the active site. The inhibitor S-atom bridges the Ni ions in place of WB, reducing the Ni—Ni distance to 3.1 Å, while its OH group coordinates to Ni(1) in place of W1. This results in that both Ni ions in the active site are penta-coordinated.

A similar penta-coordination was also observed for the binding of acetohydroxamic acid $CH_3—CO—NHOH$ (Fig. 2c) [169]. Here the bridging that shortens the Ni—Ni distance to 3.5 Å, is provided by the acidic hydroxamate oxygen, with the carbonyl oxygen ligating the Ni(1) ion. Acetohydroxamic acid, a representative of numerous acylhydroxamic acids R-NHOH studied as inhibitors of plant [7,81,170–172], bacterial [95,173–176], fungal [142] and soil [177] ureases, was shown to be a slow-binding inhibitor with moderate strength. Owing to its low toxicity, it is one of the most intensively studied inhibitors for medical therapies to be used in ureolytic bacteria-induced pathological conditions (Section 3.2) [40,42,43,178,179].

Amides and esters of phosphoric acid (Fig. 3) are also slow-binding inhibitors of urease, classified as the strongest inhibitors. The kinetic analysis of their inhibition implied that irrespective of the compound, the inhibition is always brought about by the same diamidophosphate (DAP), a product of their hydrolysis [147,164,180]. This contention was verified by the crystal structure of phenylphosphorodiamidate (PPD)-inhibited urease [90], in which the presence of DAP in the active site was demonstrated (Fig. 2d). In the structure, the tetrahedral DAP molecule nearly perfectly replaces the cluster of four water molecules seen in the native enzyme. One oxygen of DAP replaces WB and as an OH group bridges the Ni ions, retaining them at a distance of 3.8 Å. The other oxygen and one nitrogen replace W1 and W2 and bind to Ni(1) and Ni(2), respectively, while the other nitrogen of DAP is directed towards the opening of the active site. In this structure, DAP is viewed as a transition state analogue, and it was its particular binding that provided the basis for the mechanism of urease

Table 4
Inhibitors of ureases.

Inhibitors	Type of inhibition	K_i[a] (mM)			
		Plant[b]	Refs.	Bacterial	Refs.
Urea (substrate)	Uncompetitive	$(3–6.4) \times 10^3$	[219,220]	–	–
Ammonium ion (product)	Noncompetitive	2–118	[221,222]	–	–
Urea analogues					
Hydroxyurea	Competitive	1.45–3.6; 100[c]	[223,83[c]]	0.13 (*Ss*)[d]; 0.23 (*Ba*)[e]; 1.04 (*Bs*)[f]	[91,148,149]
Formamide		404	[160]	–	–
Thiourea		70; 23[c]	[130,83[c]]	26.12 (*Bs*)	[149]
Ethylurea		26	[224]	–	–
Methylurea		980[c]	[83[c]]	–	–
Thiols					
β-Mercaptoethanol	Competitive	0.72	[168]	0.55 (*Ka*)[g]; 4.1 (*Hp*)[h]	[165,225]
Acylhydroxamic acids					
Acetohydroxamic acid	Competitive slow-binding	0.004; 0.016	[81,172]	0.0026 (*Ka*); 0.002 (*Hp*)	[165,176]
Amides and esters of phosphoric acid					
Phosphoric triamide (PTA)	Competitive slow-binding	2.44×10^{-6}	[147]	3.22×10^{-5} (*Bp*)[i]	[147]
Phenylphosphorodiamidate (PPD)		1.6×10^{-7}	[147]	6×10^{-7} (*Bp*); 9.4×10^{-8} (*Ka*)	[147,165]
4-Chlorophenylphosphorodiamidate		4.1×10^{-8}	[147]	3.5×10^{-7} (*Bp*)	[147]
N-(diaminophosphinyl)benzamide		1.62×10^{-6}	[147]	6.9×10^{-6} (*Bp*)	[147]
N-(diaminophoshinyl)-4-fluoro-benzamide		–	–	4.0×10^{-6} (*Hp*)	[176]
Phosphate buffer (pH <7.5)	Competitive	19 (pH 7)	[128]	40 (pH 7) (*Ka*)	[165]
Boron compounds					
Boric acid	Competitive	0.12; 0.23; 0.08; 0.35[c]	[226,227,133,228[c]]	0.099 (*Pm*)[j]; 0.34 (*Ka*)	[132,165,121]
Butylboronic acid		1.8[c]	[228[c]]	0.547 (*Pm*)	[132]
Phenylboronic acid		2.5[c]	[228[c]]	1.26 (*Pm*)	[132]
4-Bromophenylboronic acid		0.3[c]	[228[c]]	0.124 (*Pm*); 0.37 (*Ka*)	[132,121]
F^-	Uncompetitive slow-binding	0.83; 0.02	[168,172]	0.17 (*Ka*)	[125]
Heavy metal ions					
$Hg^{2+} \approx Ag^+$	Competitive slow-binding	1.9×10^{-6}	[203]	–	–
Cu^{2+}		7.1×10^{-6}		–	–
Zn^{2+}		1.8×10^{-4}		–	–
Cd^{2+}		4.1×10^{-4}		–	–
Ni^{2+}		2.8×10^{-3}		–	–
Pb^{2+}		8.1×10^{-3}		–	–
Co^{2+}		8.1×10^{-3}		–	–
Bismuth compounds					
Bi(EDTA)	Competitive	1.74	[210]	2.46 (*Ka*)	[210]
$Bi(Cys)_3$	Competitive	1.84	[210]	–	–
Ranitidine bi citrate	Noncompetitive	1.17	[210]	–	–
Quinones					
1,4-Benzoquinone	Competitive slow-binding	4.5×10^{-5}	[215]	–	–
2,5-Dimethyl-1,4-benzoquinone		1.2×10^{-3}	[215]	–	–
Tetrachloro-1,4-benzoquinone		4.5×10^{-7}	[216]	–	–

[a] For slow-binding inhibitors the overall inhibition constants K_i^* are given. In comparing the K_i values it should be remembered that they are dependent on the purity of the enzyme, and on conditions in which they were determined, i.e. temperature, buffer, its concentration and pH.
[b] These are data for jack bean urease except for [c]data for pigeonpea urease; [d]*Staphylococcus saprophyticus*; [e]*Brevibacterium ammoniagenes*; [f]*Brucella suis*; [g]*Klebsiella aerogenes*; [h]*Helicobacter pylori*; [i]*Bacillus pasteurii*; [j]*Proteus mirabilis*.

catalysis formulated by Benini et al. [90] (Section 6). An interesting group of compounds within this class of inhibitors are derivatives of thiophosphoric acid, chiefly amides (Fig. 3), which were shown to effectively be only precursors that become inhibitors upon their conversion into oxygen analogues [181,182]. Due to their efficacy, a variety of derivatives of both phosphoric and thiophosphoric acids have been intensively studied for retarding urease hydrolysis in soils [177,181–191,251,252] and against ureolytic bacteria infections [95,174–176,180,192].

Phosphate buffer, very common in the kinetic studies of urease, and long known to be inhibitory at neutral pHs [194], had its inhibitory strength shown to be pH-dependent. This strength decreases with an increase in pH to cease at pH 7.0–7.5 [128,165]. The inhibitory action of the buffer was ascribed to $H_2PO_4^-$ ion [128,165,168], a point verified by the crystal structure of urease–phosphate complex at pH 6.3 [195]. The mode of phosphate binding (Fig. 2e) was shown to be similar to DAP (Fig. 2d), with the Ni—Ni distance reduced to 3.5 Å.

Boric and boronic acids are rapidly binding urease inhibitors, comparatively weak. For boric acid, the maximum inhibitory activity was observed at pH between 6 and 9 [132,133], suggestive of its action in the molecular form $B(OH)_3$. The crystal structure of boric acid-inhibited urease (Fig. 2f) [196] revealed that $B(OH)_3$ replaces in the active site W1–W3 water molecules, leaving in place the bridging WB. The inhibitor binds to the Ni ions with its two oxygen atoms, whereas its third oxygen points towards the cavity opening, the Ni—Ni distance being 3.6 Å. Interestingly, a recent DFT study of boric acid-urease complex did not exclude a possibility of a strong covalent bond formation between the bridging oxygen and boron [197].

In contrast to the above inhibitors, the data on fluoride inhibition are less consistent. Namely, in a comprehensive study of *Klebsiella aerogenes* urease [125], fluoride was found to be an uncompetitive slow-binding inhibitor, however, for jack bean urease, by virtue of F^- binding to an active-site nickel ion, this inhibition was defined as competitive, while its time-dependent character also suggested

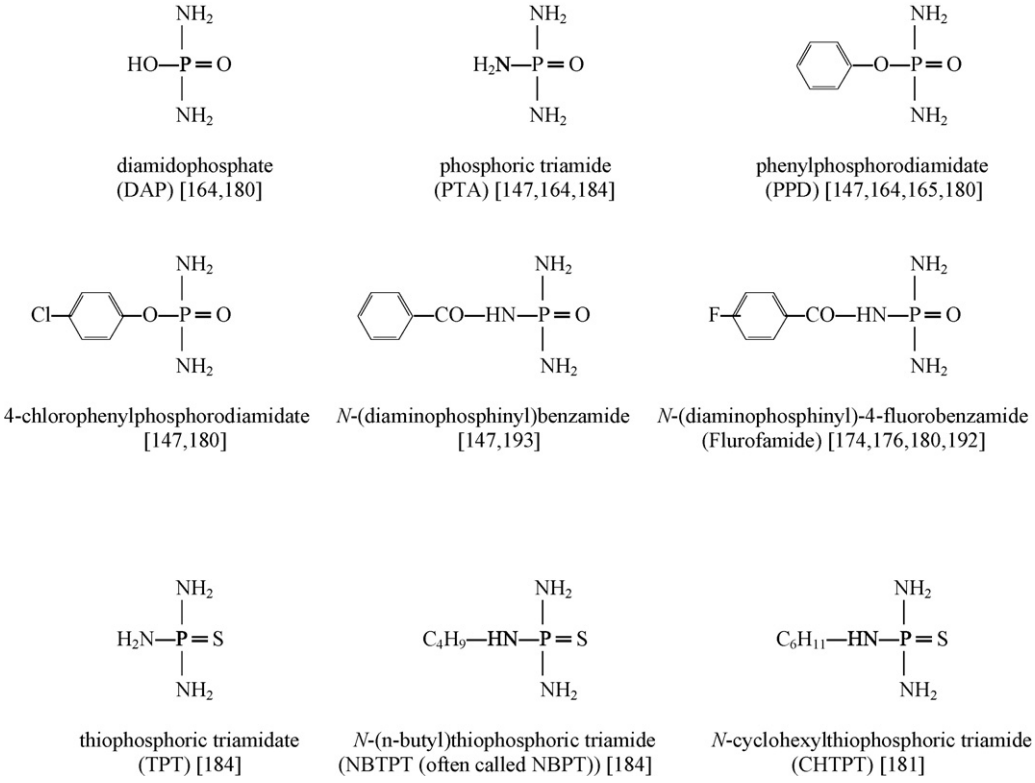

Fig. 3. Amides and esters of phosphoric and thiophosphoric acids applied as urease inhibitors.

that it be uncompetitive [168], and in [172] it was interpreted as competitive slow-binding.

Heavy metal ions inhibit both plant [198–203] and bacterial ureases [148,174] at the following approximate order of effectiveness: $Hg^{2+} \approx Ag^{+} > Cu^{2+} \gg Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$ [200,203], with Hg^{2+}, Ag^{+} and Cu^{2+} ions nearly always listed as the strongest inhibitors [198–201,203–206]. Classified on the basis of the initial reaction rates measurements as noncompetitive [199–201,205], in the reaction progress curve studies this inhibition was best described as slow binding [202,203]. This inhibition has been habitually ascribed to the reaction of the ions with the thiol groups of the enzyme, resulting in the formation of mercaptides [198–203,205–207]. However, evidence was also provided that Cu^{2+} and quite likely Ag^{+} ions, in addition to SH groups, coordinate to nitrogen- (histidine) and possibly oxygen- (aspartic and glutamic acids) containing functional groups in urease [208,209]. In practice, this inhibition is important for two reasons. One is that in view of heavy metal ion pollution, appropriate levels of urease activity in agricultural soils may be endangered. The other one is that this inhibition may be exploited in constructing urease inhibition-based sensing systems [201,204,206] for in situ and real time determination of trace levels of the ions, e.g. in environmental monitoring, food control and biomedical analysis.

Likewise, the involvement of urease thiol groups was found in the inhibition of the enzyme by bismuth compounds [210]. The data on this inhibition [210–212] are of medical importance, because bismuth compounds are widely used as bactericidal agents in the treatment of peptic ulcers and *Helicobacter pylori* infections.

The inhibition of ureases by quinones on the other hand has been mainly tested for their potential application with urea fertilizers [50,213]. The inhibition was reported non-competitive [214], but in other reports also slow-binding [215,216]. The inhibitory action of quinones proved to be either through covalent modification of enzyme thiols (Michael-type addition) [217], or through redox cycling resulting in the oxidation of the thiols, which was the case of naphthoquinone [218]. In the latter inhibition, H_2O_2 was shown to participate, its inhibition constant being K_i = 3.24 mM [218].

Most recently, a group of novel inhibitors, *P*-methyl phosphinic and thiophosphinic acids, were designed, synthesized and studied [229]. They proved to be competitive inhibitors, simple and slow binding, respectively, with K_i constants varying between $1.7 \cdot 10^{-4}$ and 0.34 mM for *Bacillus pasteurii* urease. *P*-methyl thiophosphinic acids appeared to be stronger inhibitors than their oxygen analogues.

A variety of other compounds were tested for their inhibitory potential towards ureases. Among those are ketones (α,β-unsaturated [230], α-hydroxyketones [231] and cyclic β-triketones [232]), Schiff base metal (Cu, Ni, Co, Cd, Mn) complexes [233,234], and notable for medicinal usage, compounds of natural origin, garlic- [235] and herbs-derived [236,237]. Furthermore, there are reports on impact of pesticides on ureases [238–245], which is unquestionably of prime significance, given the importance of adequate control of soil urease activity in the economic use of urea-based fertilizers.

9. Concluding remarks

The foregoing discussion has attempted to summarize properties of ureases, both well established and newly found. The emphasis was placed on the processes in which the enzymes participate and their relevant practical properties. Enzymes with a long history, implicated in numerous natural processes and of great potential for practical applications, ureases are still an enigma in that their catalytic mechanism has not as yet been resolved, too few inhibitors are known for effective and dependable control of urease activity in a safe manner, and additionally, there is a demand for robust reliable urease preparations with properties customized for chosen applications.

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