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Jack bean urease: The effect of active-site binding inhibitors on the reactivity of enzyme thiol groups

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Abstract

In view of the complexity of the role of the active site flap cysteine in the urease catalysis, in this work we studied how the presence of typical active-site binding inhibitors of urease, phenylphosphorodiamidate (PPD), acetohydroxamic acid (AHA), boric acid and fluoride, affects the reactivity of enzyme thiol groups, the active site flap thiol in particular. For that the inhibitor–urease complexes were prepared with excess inhibitors and had their thiol groups titrated with DTNB. The effects observed were analyzed in terms of the structures of the inhibitor–urease complexes reported in the literature. We found that the effectiveness in preventing the active site cysteine from the modification by disulfides, varied among the inhibitors studied, even though they all bind to the active site. The variations were accounted for by different extents of geometrical distortion in the active site that the inhibitors introduced upon binding, leaving the flap either open in AHA-, boric acid- and fluoride-inhibited urease, like in the native enzyme or closed in PPD-inhibited urease. Among the inhibitors, only PPD was found to be able to thoroughly protect the flap cysteines from the further reaction with disulfides, this apparently resulting from the closed conformation of the flap. Accordingly, in practical terms PPD may be regarded as the most suitable inhibitor for active-site protection experiments in inhibition studies of urease.

Keywords: Urease; Inhibition; Phenylphosphorodiamidate; Acetohydroxamic acid; Boric acid; Fluoride; Enzyme thiols; DTNB

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1. Introduction

James B. Sumner crystallized the enzyme urease from jack bean, *Canavalia ensiformis*, a bushy annual tropical American legume grown mainly for forage, in 1926, to show the first time ever that enzymes can be crystallized [1]. For his discovery Sumner was awarded the Nobel Prize in 1946. Nearly 50 years later in 1978 jack bean urease was shown by Dixon et al. [2] to possess nickel ions, this discovery opening a new field of bioinorganic chemistry of nickel. Even today, more than 80 years after Sumner's discovery, a catalytic mechanism of urease has not been fully resolved, and the enzyme, due to its multiple implications is still extensively studied.

Urease (urea amidohydrolase EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and CO_2. A variety of ureases are found in bacteria, fungi, higher plants, and in soil as a soil enzyme [3]. Medically, bacterial ureases are important virulence factors implicated in the pathogenesis of many clinical conditions such as pyelonephritis, hepatic coma, peptic ulceration, and the formation of infection-induced urinary stones. In agriculture, by contrast, a hydrolysis of fertilizer urea by soil urease, if too rapid, results in unproductive volatilization of nitrogen and may cause ammonia toxicity or alkaline-induced plant damage. Understanding the catalytic mechanism of urease is therefore of utmost importance for the development of strategies to be used to counteract the undesirable effects brought about by urease activity.

1.1. Structural characteristics of native ureases

While plant and fungal ureases are known to mostly be homohexamers α_6, bacterial ureases typically are heterotrimers $(\alpha\beta\gamma)_3$, whose $(\alpha\beta\gamma)$ units exhibit high homology of amino-acid sequences with the α subunit of jack bean urease [3,4]. Composed of only two subunits that form a dodecameric complex $((\alpha\beta)_3)_4$ [5], *Helicobacter pylori* urease is notably exceptional. Importantly, in all known ureases the active sites are always located in the α subunits.

The knowledge on the urease active site has been provided by the crystal structures resolved for ureases from two bacteria, *Klebsiella aerogenes* [6,7] and *Bacillus pasteurii* [8]. The active site was shown to contain a binuclear nickel centre (Fig. 4a), in which the Ni–Ni distances was found close in value in both ureases, 3.7 and 3.5 Å in *B. pasteurii* and *K. aerogenes* enzyme, respectively. In the centre the nickel(II) ions are bridged by a carbamylated lysine through its O atoms, with Ni1 further coordinated by two histidines through their N atoms, and Ni2 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 Ni1, W2 on Ni2, and with 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, Ni1 is pentacoordinated and Ni2 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, including jack bean urease whose crystal structure has not been resolved to date.

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 *B. pasteurii* urease (later used in the discussion of our results) the flap was found in the open conformation, while its closed conformation is apparently needed for the reaction. 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 acids in the flap there is one cysteine, Cys319 by *K. aerogenes* numbering, Cys322 by *B. 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 [9], this cysteine is judged to be involved in the catalysis, as was demonstrated by reacting the enzyme with cysteine-reactive agents, alkylating and disulfides [10–13]. In view of the structural studies, one role suggested for this cysteine is in positioning other key residues in the active site appropriately for the catalysis, which is why when this residue is chemically modified the flap loses its mobility and the reaction is perturbed.

Apart from Cys592, jack bean urease was proven by disulfide titration with 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) [10] and 2,2′-dithiodipyridine (DTDP) [11] in non-denaturing conditions, to contain five other cysteine residues per subunit that are more reactive. With additional nine cysteine residues disclosed only in denaturing conditions, the overall number of cysteines per jack bean urease subunit amounts to 15, hence 90 cysteines per molecule. By contrast, *K. aerogenes* urease, when titrated with 2,2′-dithiodipyridine (DTDP) in denaturing conditions 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 [7,12].

Taking into account the peculiarities of the active site flap cysteine in the urease catalysis, it might be inferred that equilibrating the enzyme with active-site binding inhibitors might have an effect on the reactivity of this enzyme residue. In view of this complexity in this work we studied how the presence of typical active-site binding inhibitors of urease, phenylphosphorodiamidate (PPD), acetohydroxamic acid (AHA), boric acid and fluoride, affects the reactivity of enzyme thiol groups, the active site flap thiol in particular, and analyze the effects observed in terms of the structures of the inhibitor–urease complexes.

2. Experimental

2.1. Materials

Urease (from jack beans, type III, activity 16 U/mg solid), urea (for Molecular Biology), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) and acetohydroxamic acid (AHA) were from Sigma. Phenylphosphorodiamidate (PPD) was from Avocado-Research Chemicals, boric acid and sodium fluoride from POCh, Poland. Phosphate buffer (50 mM) was prepared by adjusting pH of phosphoric acid to pH 7.8 with NaOH, and 2 mM EDTA was added to all enzyme-containing solutions. pH 7.8 of phosphate buffer was chosen for the study to avoid interference of the inhibition of urease by phosphate known to be operative at pHs below 7.5 [14], with the inhibition by the inhibitors under study, and also because DTNB titration of thiols in proteins requires alkaline pH [15].

2.2. Standard urease activity assay

The standard urease assay mixture consisted of 100 mM urea in 50 mM phosphate buffer, pH 7.8, with 2 mM EDTA, its volume being 25 mL. Reactions were initiated by the

addition of small aliquots of the enzyme-containing (0.5 mg) solution, and the activity was determined by measuring ammonia concentration by the colorimetric phenol–hypochlorite method [16] in samples withdrawn from the reaction mixture at 5 min reaction. The measurements were performed at ambient temperature.

2.3. Urease–inhibitors reactions

Solutions of urease (2.0 mg solid/mL in 50 mM phosphate buffer, pH 7.8) and of the inhibitors, PPD, AHA, boric acid and fluoride, were mixed 1:1. The concentrations of the inhibitors in the reaction mixtures were: 0.04 μM for PPD, 0.075 mM for AHA, 50 mM for boric acid and 1 mM for fluoride. The mixtures were incubated with occasional stirring. During the incubations, periodically 0.5 mL aliquots were transferred into the standard assay mixtures for enzyme residual activity determinations. All residual activities of inhibitor–urease complexes were normalized to the activity measured for native urease, accounted as a control activity of 100%. For further experiments 10 min equilibration time was chosen as sufficient for the reactions of the inhibitors with urease. For IC_{50} determinations, solutions of urease (2.0 mg solid/mL in 50 mM phosphate buffer, pH 7.8) and of different concentrations of the inhibitors, were incubated for 10 min. Upon incubation urease had its residual activity determined under standard conditions.

2.4. Spectroscopic progress curves of the reactions of DTNB with urease–inhibitor complexes

The samples of urease (2.0 mg/mL in 50 mM phosphate buffer, pH 7.8) were equilibrated for 10 min with the inhibitors, their concentrations in the mixtures being 0.5 μM PPD, 0.5 mM AHA, 50 mM boric acid and 20 mM fluoride. The equilibrated samples were subjected to the reaction with DTNB. For that 2.5 mL of the mixture was transferred to a cuvette (light path 5 cm) and mixed with 2.5 mL 0.15 mM DTNB (prepared in 50 mM phosphate buffer, pH 7.8). The spectroscopic progress curves of the reaction of DTNB with urease–inhibitor complexes, absorbance at 412 nm vs time [15], were registered for 15 min. Prior to these measurements, control measurements of the solutions used in the proportions corresponding to the final DTNB–urease reaction mixture were performed and subtracted when necessary. To convert the 412 nm-absorbance progress curves into DTNB-modified enzyme thiols vs time progress curves, the number of thiols in urease was calculated according to the protocol proposed previously [17]. In brief, assuming that the 15-min absorbance measured for the free urease corresponds to 36 thiols per enzyme molecule (the number revealed in non-denaturing conditions), the 15-min absorbances of the inhibitor-modified enzyme samples were transformed by direct absorbance/thiols proportion into the number of inhibitor-modified thiols per urease molecule.

3. Results and discussion

The inhibitors of urease used in this study, phenylphosphorodiamidate (PPD), acetohydroxamic acid (AHA) boric acid and fluoride, are presented in Table 1 along with their kinetic characteristics. PPD (through DAP, the product of its hydrolysis), AHA and boric acid have been invariably reported as competitive inhibitors for both plant and bacterial ureases, PPD and AHA slow-binding [18–21], and boric acid simple [19,24–27]. The reports on fluoride inhibition by contrast, are less consistent. Namely, in a comprehensive

Table 1
Kinetic characteristics of the inhibitors under study for different ureases

Inhibitor	Type	Urease (buffer pH)	K_i[a] (mM)	Ref.
PPD[b]	Competitive (slow-binding)	J. bean (100 mM THAM, pH 7.0)	1.6×10^{-7}	[18]
		B. pasteurii (100 mM THAM, pH 7.0)	6×10^{-7}	[18]
		K. aerogenes (100 mM Hepes, pH 7.75)	9.4×10^{-8}	[19]
AHA	Competitive (slow-binding)	J. bean (22 mM phosphate, pH 7.0)	0.016	[20]
		J. bean (20 mM phosphate, pH 7.0)	0.004	[21]
		K. aerogenes (100 mM Hepes, pH 7.75)	0.0026	[19]
F^-	Uncompetitive (slow-binding)[c]	J. bean (22 mM phosphate, pH 7.0)	0.02	[20]
		J. bean (140 mM phosphate, pH 7.0)	0.83	[22]
		K. aerogenes (100 mM Hepes, pH 7.0)	0.17	[23]
Boric acid	Competitive (simple)	J. bean (buffer-free system, pH 7.0)	0.12	[24]
		J. bean (50 mM Hepes, pH 7.0)	0.08	[25]
		Pigeonpea (50 mM Tris-acetate, pH 7.0)	0.35	[26]
		P. mirabilis (25 mM Hepes, pH 7.5)	0.1	[27]
		K. aerogenes (100 mM Hepes, pH 7.75)	0.33	[19]

[a] For slow-binding inhibitors the overall inhibition constants K_i^* are given.
[b] In the inhibition of urease by PPD, the actual inhibitor is diamidophosphate, DAP, the product of PPD hydrolysis (see text).
[c] F^- was found to be an uncompetitive slow-binding inhibitor for *K. aerogenes* urease [23], 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 that it be uncompetitive [22], and in [20] it was interpreted as competitive slow-binding.

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In order to establish how much time the inhibitors required for their reactions with urease to attain the equilibrium, the kinetic curves of these reactions, enzyme residual activity (RA) vs time, were recorded. Fig. 1 shows that the equilibria were attained within comparatively short times not longer than 5 min. Based on these results, in further studies the enzyme was equilibrated with the inhibitors systematically for one time of 10 min. Correspondingly, by equilibrating urease with different concentrations of the inhibitors and determining the enzyme residual activity (Fig. 2) we obtained IC_{50} values, i.e. inhibitor concentrations bringing about a 50% inactivation of the enzyme, presented in Table 2. Thus in terms of the inhibitory strength towards urease the inhibitors studied form the order: boric acid $< F^- <$ AHA $<$ PPD, consistent with the order of the inhibition constants (Table 1).

Having established the time required for the inhibitor–urease complex formation, we further tested if the inhibitors have an effect on the reactivity of the enzyme thiol groups. For that we performed experiments in which the number of urease thiol groups and their accessibility were assessed by spectroscopic titration with DTNB, a typical disulfide thiol-selective agent. The results are presented in Fig. 3 in the form of spectroscopic progress curves for the reaction of DTNB with urease and inhibitor–urease complexes, DTNB-modified thiols in urease vs reaction time. The curves for native urease have a

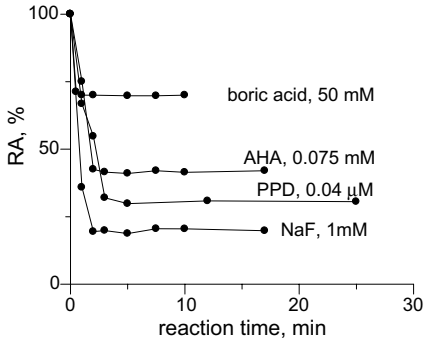

Fig. 1. Kinetic curves of the reaction between urease and the inhibitors under study: PPD, AHA, boric acid and fluoride. Urease was mixed with the chosen concentrations of inhibitors and had its residual activity assayed periodically over time in standard conditions.

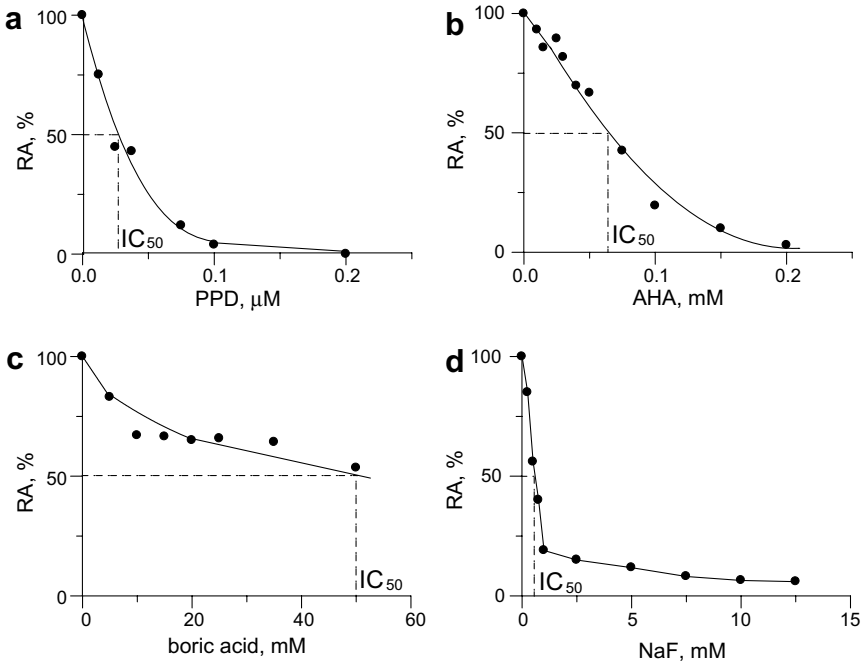

Fig. 2. Plots of urease residual activity (RA) as a function of inhibitors concentration in the mixtures equilibrated for 10 min with: (a) PPD, (b) AHA, (c) boric acid and (d) fluoride.

characteristic shape [12,28], composed of the initial phase corresponding to an abrupt reaction of 30 reactive thiols/molecule with DTNB, and the subsequent long phase corresponding to a slower reaction of DTNB with the six less-reactive Cys592/molecule. The curves for the complexes of urease obtained at excess inhibitors are similar in shape but are always located below the native enzyme, thereby revealing that some thiols, following

Table 2
Numbers of thiols in native urease and in inhibitor–urease complexes inaccessible for disulfide reagents, DTNB (this work) and DTDP [12]

Inhibitor	IC_{50} (mM)	Mol thiol/mol jack bean urease (composition α_6) inaccessible for DTNB (this work)	Mol thiol/mol *K. aerogenes* urease (composition $\alpha_2\beta_4\gamma_4$) inaccessible for DTDP [12]
None	—	0 (0)[a]	0 (0)[a]
PPD	2.5×10^{-5}	6.8 (1.1)	2.2 (1.1)
AHA	0.06	0.9 (0.15)	0.4 (0.2)
Boric acid	50	1.6 (0.3)	—
F^-	0.6	3.6 (0.6)	—

In brackets are given the numbers of inaccessible thiols per active site urease calculated according to the urease composition assumed.

[a] These values are zero on assumption used that under non-denaturing conditions all thiols of urease are accessible for DTNB.

the enzyme equilibration with the inhibitors, became inaccessible for DTNB. Unlike for thiol-reactive inhibitors, Hg(II) ions [17] and quinones [29], the curves practically did not change their location upon increasing the inhibitor concentrations (data not shown), indicating that further inhibitor binding did not occur, i.e. no more enzyme thiols could further become inaccessible for DTNB.

The numbers of the enzyme thiols in the inhibitor–urease complexes inaccessible for DTNB, were calculated as a difference between the curves recorded in the absence and presence of the inhibitors. These numbers, mol thiol/mol urease (±17 %), are listed in Table 2, where they are compared with the analogous numbers obtained for *K. aerogenes* urease through titration with DTDP [12]. Importantly, the numbers found for jack bean and *K. aerogenes* urease are, within the experimental error never higher than six and two, respectively (note that the composition assumed for *K. aerogenes* urease was $\alpha_2\beta_4\gamma_4$, only later corrected to be $(\alpha\beta\gamma)_3$ [7]), suggesting that the maximum number of thiols protected from disulfide modification is that of the active site flap cysteines, Cys592 and Cys319, respectively. Thus, the inhibitors studied, though not being thiol-reactive do have an effect on the reactivity of these particular thiols. Remarkably, the numbers of disulfide-inaccessible thiols per urease active site (given in Table 2 in brackets) for PPD- and AHA-equilibrated enzymes are in agreement between jack bean and *K. aerogenes* urease. This confirms that the inhibitors interact with the active site of urease in a similar way and play the same role in preventing the active site cysteine from disulfide modification regardless of the origin and composition of the enzyme. The effects produced by individual inhibitors, however, are not identical even though not only by assumption but also as evidenced by their crystal structures (Fig. 4), the inhibitors occupy the active site. Specifically, PPD protects 1.1 mol thiol/mol active site from the DTNB modification, while AHA only 0.15 mol thiol/mol active site, with fluoride and boric acid being the intermediate cases. This shows that the action of PPD is stoichiometric in respect to Cys592 and that of AHA, fluoride and boric acid sub-stoichiometric. Visibly, the numbers do not correlate with the strength with which the inhibitors bind to urease as expressed by their IC_{50} values (Table 2). In an attempt to account for these differences we will discuss the results in terms of the structures of the inhibitor–urease complexes.

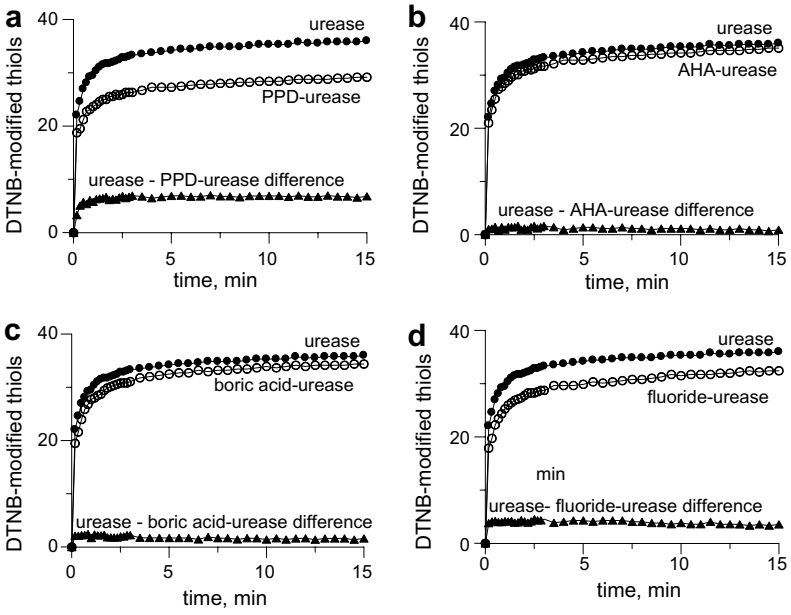

Fig. 3. Spectroscopic progress curves for the reaction of urease with DTNB in the absence and presence of excess inhibitors: (a) PPD, (b) AHA, (c) boric acid and (d) fluoride. Absorbance at 412 nm was converted into number of DTNB-modified urease thiols and is presented as a function of time. Presented are also thiol number differences between the curves recorded for urease and inhibitor–urease complexes.

Schematic structures of the active site of urease, native and inhibited with the inhibitors under study are presented in Fig. 4. Among these structures only the one with fluoride is hypothetical [23], while the other structures were solved for urease from *Bacillus pasteurii* and reported, native in [8], PPD-inhibited in [8], AHA-inhibited in [30] and boric acid-inhibited in [31]. The inhibitor–urease structures are reviewed in short below.

PPD, phenylphosphorodiamidate was found to inhibit urease in an indirect way, as was confirmed by the PPD-inhibited urease crystal structure [8]. The structure revealed that the actual inhibitor that binds to the enzyme active site is DAP, diamidophosphate, the product of the enzymatic hydrolysis of PPD. 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 the bridging hydroxide WB and as an OH group bridges the two Ni ions. The other oxygen and one nitrogen replace W1 and W2 and bind to Ni1 and Ni2, respectively, while the other nitrogen of DAP is directed towards the opening of the active site. The Ni–Ni distance is 3.8 Å. Thus the DAP binding does not alter the coordination and overall geometry of the active site. Very importantly, the DAP-bound structure of urease shows the mobile flap of the active site closed, opposite to the native enzyme. In this structure, DAP can be viewed as a transition state analogue, and it was its particular binding that has provided the basis for one of the mechanisms of urea binding and catalysis [8].

AHA, acetohydroxamic acid also inhibits urease [30] by displacing all four water/hydroxide molecules in the active site. In the structure, the acidic hydroxamate oxygen replaces the bridging hydroxide WB and forms a symmetrical bridge between the Ni ions

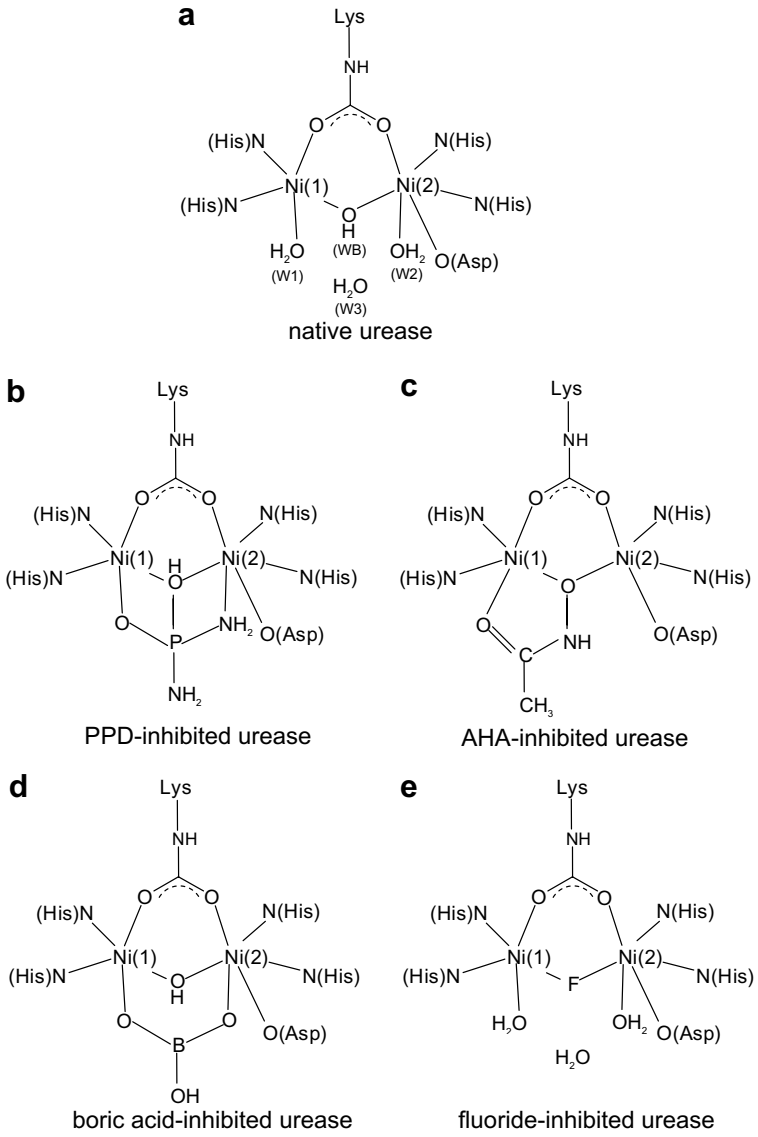

Fig. 4. Schematic structures of the active site of urease: (a) native [8], and inhibited by (b) PPD [8], (c) AHA [30], (d) boric acid [31] and (e) fluoride [23].

shortening the Ni–Ni distance to 3.5 Å. The carbonyl oxygen of the inhibitor on the other hand, being placed in the position of W1, ligates the Ni1 ion. As a result of the inhibitor binding, both Ni ions are pentacoordinated. The flap of the site was found in the open conformation.

Boric acid $B(OH)_3$, a neutral, planar, trigonal molecule, unlike DAP and AHA, replaces in the active site only three water molecules, W1, W2 and W3, leaving in place the bridging hydroxide, WB [31]. The inhibitor binds to the Ni ions with its two oxygen

atoms, whereas its third oxygen points towards the active site opening. The molecule occupies the space symmetrically between the Ni ions, slightly shortening the distance between them to 3.6 Å. The flap of the active site was found in the open conformation.

Fluoride speculatively binds to the urease active site by displacing one water/hydroxide molecule, either W1, W2 or WB, but most likely the bridging hydroxide WB [23], the argument for this particular location being that the substrate urea binds to the fluoride-inhibited enzyme. It can consequently be surmised that the fluoride-occupied active site mimics that of the native enzyme and that its flap, in order to allow urea in, is open.

The structures presented above have a number of common features, namely all of the inhibitors investigated, to inhabit the urease active site, replace water/hydroxide molecules therein and bind to the Ni ions. Another common feature is that this binding does not change the overall architecture of protein ligands of the Ni ions. By contrast, the main distinguishing feature of the structures is the conformation of the flap, open or closed. The open conformation was found in the structures of native urease and of AHA-, boric acid- and tentatively of fluoride-inhibited urease, with the closed one only in PPD-inhibited enzyme.

Interestingly, the data suggest that the conformation of the flap be correlated with the number of the DTNB-inaccessible thiols in urease (Table 2), namely, the open conformation allows DTNB to react with the thiol groups, whereas the closed one prevents this reaction. Accordingly, those of the inhibitor–urease complexes that like native urease have the flap open in their structures, i.e. AHA, boric acid and fluoride, are not capable of protecting the thiols from DTNB. However, the numbers of the DTNB-inaccessible thiols in these complexes were not found to be exactly zero like in the native enzyme but higher. When compared with the corresponding structures they support the suggestion that they are dependent on the degree of distortion introduced to the active site by the inhibitor upon binding, namely, the greater is the distortion the lower is this number. Of the complexes showing the flap open, the one with AHA can be reckoned as having the most distorted structure, and this may be why the flap does not close and consequently its thiols are exposed to the reaction with DTNB. The number of thiols in this complex prevented from this reaction is indeed the lowest among the complexes investigated. Likewise, boric acid and fluoride, leave the flap open and are not capable of protecting all of the six thiols from DTNB, though the numbers of thiols protected are higher than that in the AHA-complex. This may be due to the fact that boric acid is viewed in the structure with urease as a substrate analogue and the fluoride-complex is in fact the best resembling the active site of native urease. Also, in interpreting the above data concerning AHA, boric acid and fluoride, it cannot be excluded that these are not the active site flap cysteines that are protected from the reaction with DTNB, but some of the five other urease cysteines, this effect arising from the structural distortions caused by inhibitor binding. However, to assign which residues these might be is impossible with the results obtained. In sharp contrast to AHA, boric acid and fluoride, PPD inhibits urease by binding DAP, a tetrahedral molecule that out of the inhibitors studied is best fitting the active site cavity. This is certainly why the binding allows the flap to close the active site, as a result of which the flap is fixed in the closed conformation and its six thiols are soundly secured from a further reaction with DTNB.

From the foregoing analysis it is possible to conclude that the effectiveness in preventing the active site cysteine in urease from the modification by cysteine-reactive reagents, such as disulfides, varies among active-site binding inhibitors. The variations observed can be

accounted for by different extents of geometrical distortion in the active site that the inhibitors introduce upon binding. Among the inhibitors studied DAP, resulting from PPD, not only best fits the active site but also mimics the substrate, thereby preserving the geometry of the active site practically intact and allowing the flap to close, which effectively makes Cys592s unavailable for further reactions. Accordingly, in practical terms for active-site protection experiments in inhibition studies of urease, among competitive inhibitors, which by definition are more suitable than uncompetitive ones, PPD should be regarded as the most suitable and effective inhibitor to be used.

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