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Hydrogen peroxide-induced inactivation of urease: Mechanism, kinetics and inhibitory potency

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ABSTRACT

Evidence has been collected that H_2O_2 resulting from redox reactions taking place in some inhibitor systems, is responsible for the inactivation of urease. Accordingly, in this study the inactivation by H_2O_2 exogenously added to urease (jack bean) was investigated. The reaction accountable for the inactivation was the oxidation of the enzyme thiol groups. The reaction was studied at pHs over 6.2–8.2. At each pH, the first-order kinetics was obeyed, the resulting dependences of k_{obs} on H_2O_2 concentration being hyperbolic. Analyzed with Kitz-Wilson method, the k_{inact} appeared to be invariant with pH, while the inhibitor binding constant K_I decreased with increasing pH. Consequently, the process grew faster with an increase in pH, the second-order rate constants increasing from 0.0032 M^{-1} s^{-1} at pH 6.2 to 0.0615 M^{-1} s^{-1} at pH 8.2, consistent with higher susceptibility of thiolate anions to oxidation by H_2O_2 compared to reduced thiols. The reactions always resulted in irreversible inactivation of urease, indicative of the oxidation of thiol groups to either sulfinic or sulfonic acid, independent of the extent of inactivation. Analysis of the numbers of urease thiol groups modified with H_2O_2 revealed that for the complete inactivation, out of 36 thiols /molecule available under non-denaturating conditions, the rapid oxidation of 30 highly reactive –SH groups was responsible for a 50% loss in enzyme activity, and the slower oxidation of the remaining six –SH groups, for the other 50%. The enzyme was protected by active-site binding inhibitors, boric acid and fluoride, from the inactivation, suggesting that the six thiols are the active-site flap Cys-592s. For comparison with other urease inhibitors, the IC_{50} was determined for 20 min incubation with H_2O_2, its value changing from 242 mM at pH 6.2 to 11 mM at pH 8.2.

1. Introduction

Irrespective of the origin, whether bacterial, fungal, algal, plant or soil, ureases (urea amidohydrolases, EC 3.5.1.5) exert one catalytic function that is the hydrolysis of urea, its final products being ammonia and carbonic acid [1]. The product ammonia and an increase in pH consequent to the reaction, may trigger an array of deleterious complications, notably in medicine and agriculture. In the former, bacterial ureases may serve as a virulence factor, giving rise to pathological conditions, such as peptic ulcer disease, gastric cancer and hepatic coma resulting from the infection of the gastrointestinal tracts (primarily with *Helicobacter pylori*), and to kidney stone formation and pyelonephritis, resulting from the infection of the urinary tracts (chiefly with *Proteus mirabilis* and *Ureaplasma urealyticum*). In agriculture by contrast, a hydrolysis of fertilizer urea by soil urease, if too rapid may lead to unproductive volatilization of nitrogen and may cause ammonia toxicity and alkaline-induced plant damage. Various strategies have been utilized to counteract these complications, one of them being to incapacitate urease with use of inhibitors [2,3].

Though composed of different types of subunits, ureases from different sources extending from bacteria to plants and fungi, exhibit similar amino acid sequences and share common catalytic characteristics [1,4]. A pivotal catalytic characteristic common to all ureases is the presence of nickel(II) ions in the active site, essential for activity. As revealed by the crystal structures of bacterial ureases from *Klebsiella aerogens* [5], *Bacillus pasteurii* [6] and *Helicobacter pylori* [7] and recently, by the structure of plant urease from *Canavalia ensiformis* [8], the active sites of the enzymes contain a binuclear nickel centre where nickel(II) ions are bridged by a carbamylated lysine and a hydroxide, Ni(1) being further coordinated by two histidine residues, and Ni(2) by two histidine residues and an aspartic acid residue. Crucially, the contention that ureases have a conserved active site and consequently the same mechanism of catalysis, allows the generalization of the results over all ureases independent of their origin.

Another important characteristic of ureases is the presence of multiple cysteinyl residues in the enzymes bearing thiol groups. For instance, urease from jack beans (*Canavalia ensiformis*), a protein composed of six identical subunits, each of 90.77 kDa, was

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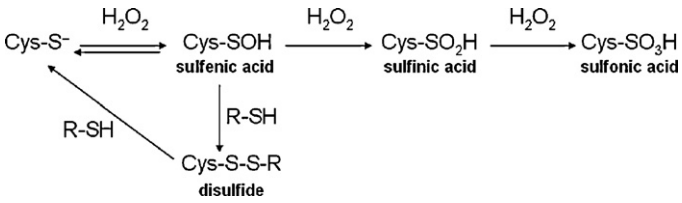
proven to contain 90 cysteinyl residues per molecule (15 per subunit) [9]. Fifty four residues are buried in the molecule and are disclosed only in denaturating conditions. Of the 36 residues disclosed in non-denaturating conditions by contrast, 30 are highly reactive, but without major impact on the enzyme activity, the remaining six (Cys-592) being less reactive, but importantly, of critical importance for the catalysis. The Cys-592s are located in the mobile flap of the active site, one per each of the six sites in the hexameric molecule. Although not essential, the residue is assessed to have a role in positioning other key residues in the active site appropriately for the catalysis, which is why when chemically modified, it restricts the mobility of the flap, hence, the reaction is perturbed and the enzyme activity reduced [9,10]. The presence of this particular cysteine residue in the active site is another important common characteristic of ureases from various sources.

Ureases are inhibited by a number of compounds [1]. Those interacting with the active site of the enzyme include hydroxamic acids [11], amides and esters of phosphoric acid [6], boric acid [12,13], fluoride [14], phosphate [15], β-mercaptoethanol [16], and phosphinic acids [17], whereas to the group of the inhibitors reacting with the thiol groups of the enzyme belong heavy metal ions [18], bismuth compounds [19], quinones [20], and L-ascorbic and dehydroascorbic acid in the presence of Fe^{3+} ions [21]. Interestingly, it was shown that the inhibitions by compounds from the two latter groups, namely by 1,4-naphtoquinone and by dehydroascorbic acid-Fe^{3+}, were mediated by H_2O_2 endogenously generated from redox reactions taking place in the systems.

1.1. Interactions of H_2O_2 with proteins

Consequential for the activity and functions, the interactions of H_2O_2 with proteins are viewed as mainly consisting of oxidation, thiol groups of cysteinyl residues (Cys-SH) being the most likely targets [22–24]. The lowest state of Cys-SH oxidation is sulfenic acid (Cys-SOH) (Scheme 1), which is usually unstable and either reacts with any accessible thiol to form a disulfide (–S–S–) or undergoes further oxidation to sulfinic (Cys-SO_2H) or sulfonic acid (Cys-SO_3H). The disulfide is stable and resistant to further oxidation. Of the four oxidation states, sulfenic acid and disulfide are reducible back to Cys-SH (reversible oxidation), whereas sulfinic and sulfonic acids are not (irreversible oxidation). Importantly, being itself a mild oxidant, H_2O_2 reacts more readily with thiolate anion Cys-S^- rather than with the reduced Cys-SH, which is on account of stronger nucleophilic properties of thiolates *versus* thiols [25,26].

Given the involvement of H_2O_2 in urease inhibition systems, in this study the inhibition by H_2O_2 exogenously added to urease was investigated. A series of experiments was performed in an effort to assess the mechanism, kinetics and the inhibitory potency of H_2O_2 towards urease. The inactivation was found irreversible with a pH-dependent kinetics. When correlated with the numbers and reactivities of the enzyme thiol groups, the kinetic data revealed that to entirely abolish the enzyme activity, the oxidation of the active-site flap cysteine residue is required.



Scheme 1. Oxidation path of thiol groups in proteins.

2. Materials and methods

2.1. Materials

Urease (from jack beans, type III, nominal activity 40.1 units/mg solid), urea (for Molecular Biology), D,L-dithiothreitol (DTT), glutathione (GSH), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), and catalase (from bovine liver, activity 1340 units/mg solid) were from Sigma. Hydrogen peroxide, 30% p.a. (8.44 M by manganometric titration) was from Lach-Ner. The solution was diluted daily to the working concentrations. Phosphate buffers 200 mM with pHs 6.2, 6.7, 7.2, 7.7 and 8.2 were prepared by adjusting pH of phosphoric acid with a NaOH solution, and 20 mM solutions were prepared by diluting the concentrated stocks. Ultrapure, deionized water obtained from a Simplicity 185 Millipore water purification system (resistivity 18.2 MΩ cm) was used throughout. All the enzyme mixtures contained 1 mM EDTA. The measurements were performed at ambient temperature (22 ± 1 °C) under aerobic conditions in a lab protected from the sunlight.

2.2. Standard urease activity assay

The standard urease assay mixture contained 100 mM urea in 20 mM phosphate buffer, pH 7.2, 1 mM EDTA, its volume being 20 mL. The reactions were initiated by the addition of small aliquots of the enzyme-containing (0.3 mg) solution. The assay was run for 5 min and the enzyme activity was determined by measuring the concentration of the ammonia released. For that samples were withdrawn from the reaction mixtures and the ammonia was determined by the colorimetric phenol-hypochlorite method [27]. Of note, all the measurements were done at H_2O_2 concentrations lower than 5 mM in the final solutions subjected to the ammonia analysis, as above this concentration, H_2O_2 interfered with the phenol-hypochlorite method.

2.3. Inactivation of urease by H_2O_2

The inactivation of urease by H_2O_2 was assayed as the decrease in urease activity over time of urease-H_2O_2 reaction. Urease (1.5 mg/mL) and H_2O_2 solutions were prepared in 20 mM phosphate buffer of a chosen pH. The solutions were mixed 1:1, which was time zero of the reaction. The mixtures were incubated with occasional stirring, and at time points, samples (0.4 mL) were taken for residual activity determinations in the standard assay mixture. All inactivation measurements were monitored relative to control samples, which had the same composition except that H_2O_2 was omitted. They remained stable over the course of experiments and their activity was set to 100% at each time point. For the most part the measurements were performed in triplicate. The inactivation was studied at pHs 6.2, 6.7, 7.2, 7.7 and 8.2 in the presence of H_2O_2 inactivating concentrations ranging from around 4–30 mM at pH 8.2 to 40–350 mM at pH 6.2.

2.4. Urease protection against H_2O_2 inactivation

Urease protection studies were carried out at pH 8.2, the urease concentration in the final incubation mixture always being 0.75 mg/mL. For the protection with thiols, urease was pre-incubated with different concentrations of DTT and GSH from the range 5 to 100 mM. For the protection with boric acid and fluoride, the enzyme was pre-incubated with 50 mM boric acid and 20 mM fluoride. The pre-incubation lasted 10 min in all cases. Following the pre-incubation, each sample of the protected urease was incubated with 21.1 mM H_2O_2 for 60 min, and the inactivated urease had its activity assayed always against the urease control samples

that contained the protectors at the same concentrations as in the samples with H_2O_2. Further, urease was protected with catalase. For that the enzyme was inactivated with 21.1 mM H_2O_2 for 20 min, after which the mixture was incubated with catalase (268 units/mL) for 10 min. The activity of urease was next followed up to 40 min.

2.5. Reactivation of H_2O_2-inactivated urease

Urease reactivation was performed at pH 8.2 and the urease concentration in the final incubation mixture was 0.75 mg/mL. Two types of reactivation were performed, namely with 10 and 50 mM thiols (DTT, GSH) and by dilution. For the reactivation with the thiols, urease was first reacted with H_2O_2 concentrations from the range 4.22 to 42.2 mM for 20 min. Next H_2O_2 was depleted by incubating the mixtures with catalase (268 units/mL) for 10 min. Reduced DTT and GSH were then added to final concentrations of 10 and 50 mM and the mixtures were incubated for 10 min. The recovery of the enzyme activity was measured in the standard assay mixture and the residual activity was expressed as the activity of the control samples containing the same 10 and 50 mM concentrations of the thiols. For the reactivation by dilution, urease was equilibrated with 21.1 mM H_2O_2. Next, at different times of incubation between 10 and 40 min, the mixture was diluted 100 times with 20 mM phosphate buffer pH 8.2. The activity of urease in the diluted solutions was measured periodically in the standard conditions.

2.6. Spectroscopic determination of thiol groups in H_2O_2-inactivated urease

5,5′-Dithiobis(2-nitrobenzoic) acid (DTNB) was used to examine the numbers and reactivity of thiol groups in native and H_2O_2-inactivated urease. Both the urease-H_2O_2 inactivations and the reactions of the resulting inactivated enzyme with DTNB were carried out in 20 mM phosphate buffer pH 8.2. Two types of thiol group determinations in H_2O_2-inactivated urease were performed. In one, thiol groups were assayed over the time of enzyme incubation with 21.1 mM H_2O_2, and in the other, urease was incubated for the same time of 20 min but with different H_2O_2 concentrations ranging from 1.05 to 84.4 mM. For the former, at time intervals of incubation and for the latter, at 20 min incubation with a given H_2O_2 concentration, two samples of the incubation mixtures were withdrawn. One sample was used for the activity determination in the standard assay mixture and the other for DTNB spectroscopic titration [28]. Prior to the titration, catalase (0.2 mL containing 268 units in 20 mM phosphate buffer pH 8.2) was added to the urease-H_2O_2 samples (1 mL) to remove H_2O_2. The treatment lasted 10 min. To perform the titration, 0.8 mL of 0.15 mM DTNB (prepared in 20 mM phosphate buffer pH 8.2) was added to 1.2 mL of the incubation mixture in a cuvette. The absorbance of the mixture was measured at 412 nm for 15 min. Additionally, the background titration of the solution containing catalase at the same concentration as that used for H_2O_2 removal from urease solutions, was measured and subtracted from the titrations of H_2O_2-modified urease to derive the true absorbances corresponding to the free thiols in urease. The number of thiols modified in urease by H_2O_2 was calculated according to the protocol proposed previously [18,20]. In brief, assuming that the 15 min absorbance measured for the free urease corresponds to 36 thiols per enzyme molecule that is the number revealed in non-denaturating conditions [9], the 15 min absorbances of H_2O_2-modified urease samples were converted by direct absorbance/thiols proportion into the number of free thiols, and these were subtracted from 36.

3. Results and discussion

3.1. Kinetics of urease inactivation by H_2O_2

Urease was reacted with different concentrations of H_2O_2 in 20 mM phosphate buffer at five pHs, 6.2, 6.7, 7.2, 7.7 and 8.2. The residual activities (RA) were plotted as ln RA *versus* time, giving linear dependences in each case. The representative semi-log plots obtained for pH 6.2 and 8.2 are shown in Fig. 1a and c, respectively.

The plots demonstrate that in the pH range 6.2–8.2 urease was inactivated by H_2O_2 in a time- and concentration-dependent process, following first-order kinetics:

$$\mathrm{RA} = \mathrm{e}^{-k_{\mathrm{obs}}t} \tag{1}$$

where k_{obs} is the first-order rate constant observed at a given H_2O_2 concentration, and t is time. The plots also show that with increasing pH the process became faster and accordingly required lower H_2O_2 concentrations, those at pH 6.2 being in the range of 42–337 mM and at pH 8.2, in the range of 4–32 mM. The replots of the observed inactivation rate constants k_{obs} as a function of H_2O_2 concentration resulted in hyperbolic dependences (Fig. 1b and d). This indicates that the inactivation is not a simple bimolecular process, as in this case a straight line would be expected. This saturation kinetics also indicates that the inactivation is irreversible. Under these conditions, the inactivation data were analyzed with Kitz-Wilson method [29] to generate the maximal rate constant for inactivation, k_{inact}, and the inhibitor binding constant, K_I, according to the following equation:

$$k_{\mathrm{obs}} = \frac{k_{\mathrm{inact}} I}{K_{\mathrm{I}} + I} \tag{2}$$

where I is the inhibitor concentration. k_{inact} is the first-order rate constant for inactivation at high (saturating) inhibitor concentration, whereas K_I represents the concentration of inhibitor producing half-maximal inactivation rate. Like K_M for the substrate in the Michaelis–Menten equation, it is a measure of inhibitor binding affinity, i.e. the lower is K_I, the higher is the inhibitor affinity to the enzyme. At low (non-saturating) inhibitor concentration ($I \ll K_I$) on the other hand, the kinetics corresponds to a simple bimolecular mechanism and k_{obs} becomes a second-order rate constant expressed as:

$$k_2 = \frac{k_{\mathrm{inact}}}{K_{\mathrm{I}}} \tag{3}$$

To obtain the parameters k_{inact} (min^{-1}) and K_I (mM) for the studied process, double-reciprocal plots $1/k_{obs}$ *versus* $1/H_2O_2$ were drawn for each pH (Fig. 2). They gave straight lines in the studied ranges of H_2O_2 concentrations. The values of the calculated parameters are presented in Table 1. The k_2 values calculated from Eq. (3) and expressed in $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ are included in Table 1.

The data in Table 1 demonstrate that the inactivation process grew faster with pH, its k_2 increasing from 0.0032 $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ at pH 6.2 to 0.0615 $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ at pH 8.2. Most interestingly, as shown in Fig. 2, the lines $1/k_{obs}$ *versus* $1/H_2O_2$ intersect on the *Y*-axis. This provides evidence that the inactivation of urease by H_2O_2 is a process that proceeds over the pH range 6.2–8.2 with the same maximal rate constant k_{inact}, its value being $0.135 \pm 0.047\ \mathrm{min}^{-1}$ on average, and

Table 1
Kinetic parameters for the inactivation of urease by H_2O_2 at different pHs.

pH	k_{inact} (min^{-1})	K_I (mM)	k_2 ($\mathrm{M}^{-1}\,\mathrm{s}^{-1}$)
6.2	0.120 ± 0.010	630 ± 90	0.0032 ± 0.0001
6.7	0.141 ± 0.047	227 ± 90	0.0103 ± 0.0007
7.2	0.148 ± 0.045	103 ± 41	0.0239 ± 0.0015
7.7	0.139 ± 0.016	53 ± 8	0.0442 ± 0.0014
8.2	0.125 ± 0.028	34 ± 10	0.0615 ± 0.0031

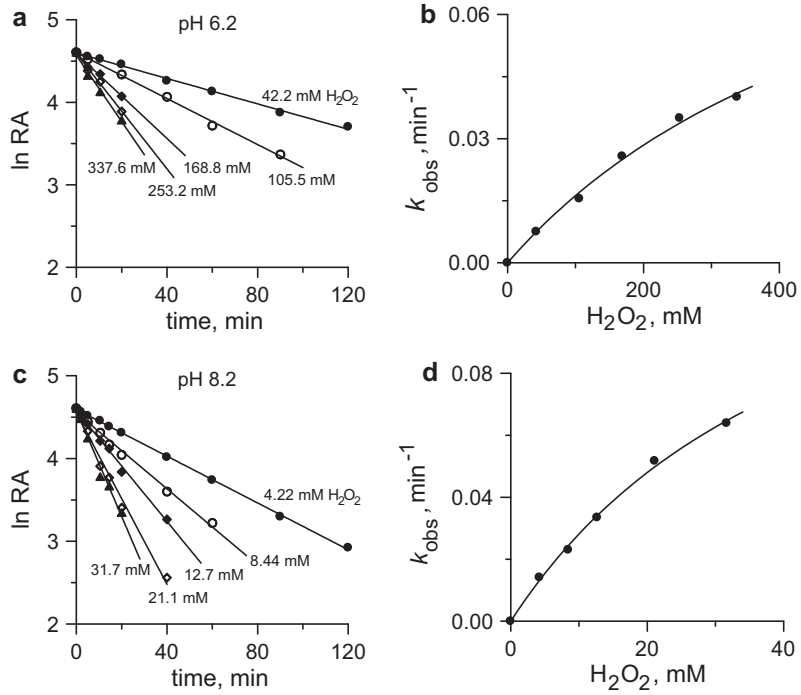


Fig. 1. Inactivation of urease by H_2O_2: semi-log plots of residual activity (RA) *versus* time at pH (a) 6.2, and (c) 8.2. Replots of k_{obs} *versus* H_2O_2 concentration at pH (b) 6.2, and (d) 8.2. The solutions of urease (1.5 mg solid/mL) and of H_2O_2 were mixed 1:1. During the incubations at time intervals aliquots were transferred into the standard assay mixture for RA determinations. The RA values are reported as percent of the control activity.

that the increase in the inactivation rate observed with increasing pH originates from a decrease in K_I, corresponding to a growth of the H_2O_2-urease affinity. The K_I had its value reduced ca. 20-fold from 630 mM at pH 6.2 to 34 mM at pH 8.2 (Table 1). As was the case with other enzymes [23,24,30–40], this observation points to the thiol groups of urease as the target of the oxidative inactivation by H_2O_2, and consequently, the pH-dependent inactivation observed can be accounted for by the fact reported for both low-molecular thiol compounds [25,26] and enzymes [23,24,30–33], that thiol groups react with H_2O_2 as deprotonated thiolate anions $–S^-$ more readily than as protonated –SH groups. Given the fact that the pK_a of Cys-SH in proteins normally falls in the range 8.0–8.5 [41], the k_2 values growing with pH (Table 1) confirm this feature of the reaction. To address this point more specifically, the pH profile of the inactivation rate, log k_2 *versus* pH, was drawn (Fig. 3). The profile shows that the log k_2 values initially grow with pH at a positive unit slope, to level off at higher pHs, which is due to approaching the pK_a value of the enzyme thiols. Though not fully developed, the profile roughly yields a pK_a of ca. 7.9–8.0, close in value to the pK_a of 8.0 revealed by the pH dependence of iodoacetamide inactivation of *Klebsiella aerogenes* urease [10].

Interestingly, the above observations account for the results of our previous work on dehydroascorbic acid inhibition of urease in the presence of Fe^{3+} ions [21]. Therein it was found that this inhibition was primarily mediated by H_2O_2 generated endogenously in the system, and that it was favoured by increasing pH.

When set beside other enzymes, the inhibition of urease by H_2O_2 appeared slower. The values of k_2 reported for instance for protein tyrosine phosphatases (PTPs) are of the order 10–20 M^{-1} s^{-1} [30], but a value of 0.73 M^{-1} s^{-1} was also demonstrated in the inhibi-

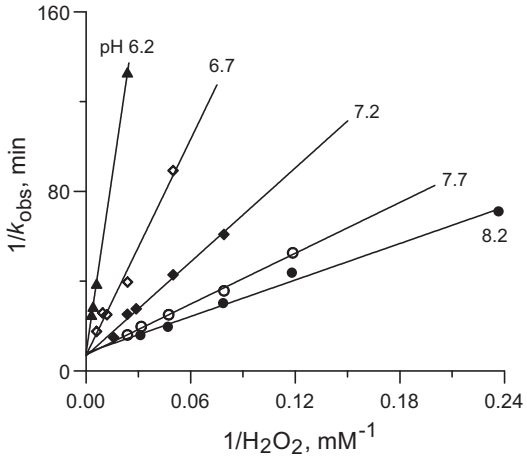


Fig. 2. Kitz-Wilson analysis of urease inactivation by H_2O_2 at pHs between 6.2 and 8.2 ($1/k_{obs}$ *versus* $1/H_2O_2$ concentration).

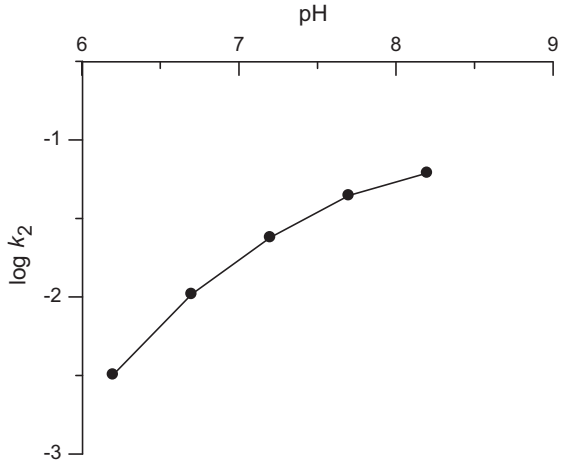


Fig. 3. pH profile of the rate of urease inactivation by H_2O_2 (log k_2 *versus* pH).

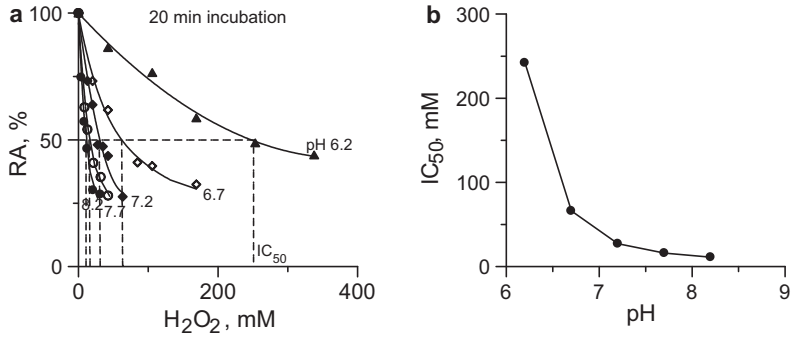


Fig. 4. (a) Dose–response curves for 20 min incubation of urease with H_2O_2 at the pHs studied and (b) change of the resulting IC_{50} values with pH.

tion of PTPα by *o*-quinones [38]. The markedly higher k_2 values apparently derive from a special character of PTPs. The activity of the enzymes was evidenced to be regulated among others by reversible redox processes involving oxidation of the active-site cysteine by H_2O_2 produced endogenously [42]. To fullfil this function the enzymes are furbished with special features, foremost among them being remarkably low pK_a values of the essential cysteine (4.7–5.4), which allows the enzymes to have the residue in a thiolate form at physiological pHs. Also, unlike urease that will be shown later to be oxidized irreversibly (Section 3.3), PTPs are oxidized by H_2O_2 reversibly. In the process their active-site cysteine is oxidized either to sulfenic acid –SOH [30] or to a disulfide bond –S–S– formed with the nearby cysteine [32,34,39]. The disulfide bond protects cysteine-sulfenic acid from further oxidation, thereby allowing it to be reduced back to the thiol by cellular reductants, hence ensuring efficiency to the redox regulation of the enzymes.

To compare the inhibitory strength of H_2O_2 with other urease inhibitors, the dose–response curves for 20 min incubation at each pH studied were constructed (Fig. 4a) and the resulting IC_{50} values (mM) are presented as a function of pH in Fig. 4b. The IC_{50} values change from 242 mM at pH 6.2 to 11 mM at pH 8.2.

To clarify the mechanism of H_2O_2 inactivation of urease, further experiments were performed in the quickest inactivation system at pH 8.2.

3.2. Urease protection against H_2O_2 inactivation

Thiol protection experiments of urease against H_2O_2 consisted of the pre-incubation of the enzyme with DTT and GSH at different concentrations prior to the addition of H_2O_2. The results for DTT are presented in Fig. 5. They show that DTT protected urease in a concentration-dependent manner and that only the concentrations 25 mM or higher were capable of preventing the inactivation with 21.1 mM H_2O_2. The action of DTT in the pre-incubation mixture apparently consisted of its covalent binding to urease thiol groups thereby protecting them from the interactions with the inhibitor. This is an important observation in that it points to the thiol groups as those exclusively involved in the inactivation of the enzyme. The action of DTT could also be that of scavenging H_2O_2 in the incubation mixture. In this context, the data suggest that under the experimental conditions (pH 8.2) H_2O_2 reacted more readily with the enzyme than with DTT. Apparently, this is consistent with the higher H_2O_2 reactivity of thiolates compared to protonated thiols. With the pK_a = 9.1 [26], in the experiment DTT was in the protonated form, hence less reactive than the enzyme thiolates (Section 3.1). Likewise in the protection experiments behaved GSH, though its protective potency was found ca. 1.2 times weaker than that of DTT (data not shown).

To determine if the active site Cys-592 is involved in the inactivation by H_2O_2, two active-site binding inhibitors, boric acid [12,13] and fluoride ion [14], were included in the protection of urease. When equilibrated with the enzyme, the inhibitors by occupying the active site restrict the accessibility of inactivators to the active-site functional groups [43]. Fig. 6 demonstrates that 50 mM boric acid and 20 mM fluoride afforded to protect 24% and 12% of urease activity from the loss as compared to the respective control samples. These observations are soundly supportive of the involvement of the active-site flap Cys-592 in the process and will be later confirmed by the DTNB-spectroscopic quantification of urease thiol groups (Section 3.4).

Further, to ascertain that the inactivation under study was provoked exclusively by H_2O_2 and not by other reactive oxygen species (ROS) to which H_2O_2 readily converts in the presence of metal ions via the Fenton reaction $Me^{(n-1)+} + H_2O_2 \rightarrow Me^{n+} + OH^- + {}^{\bullet}OH$, a protection of urease with catalase, a H_2O_2-decomposing enzyme, was performed. The metal ions in the reaction could be some trace transition metal ions present in the system, although non-metallic conditions were assured by using ultra-pure deionized water and EDTA, or importantly, Ni(II) ions from the active site of urease. The result presented in Fig. 6 shows that the addition of catalase to the H_2O_2-urease incubation mixture, where the enzyme had 30% of its initial activity, did not cause any further activity loss over 40 min of further incubation. This proves that under the experimental conditions, H_2O_2 alone was responsible for the observed inactivation with no other ROS participating in the process, and that Ni(II) ions were not a target of H_2O_2 oxidation.

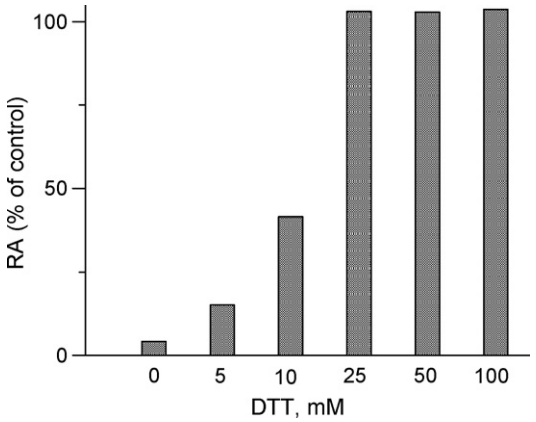


Fig. 5. DTT protection of urease against H_2O_2 inactivation at different DTT concentrations at pH 8.2. Urease (0.75 mg solid/mL) was pre-incubated with DTT for 10 min. Next, the mixture was incubated with 21.1 mM H_2O_2 for 60 min, and the activity of urease was measured in the standard assay mixture. The RA values are reported as percent of the control activity.

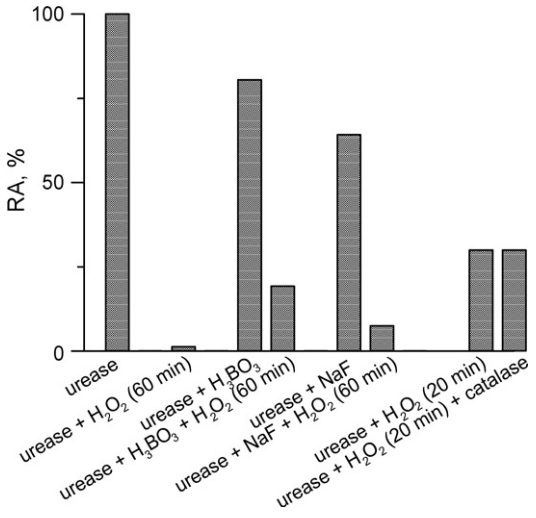


Fig. 6. Protection of urease against H_2O_2-inactivation by boric acid, fluoride and catalase at pH 8.2. Urease (0.75 mg solid/mL) was pre-incubated with 50 mM boric acid and 20 mM NaF, for 10 min. Next, the mixtures were incubated with 21.1 mM H_2O_2 for 60 min, and the activity of urease was measured in the standard assay mixture. For the protection with catalase, urease was incubated with 21.1 mM H_2O_2 for 20 min, followed by the incubation with catalase (268 units/mL, 10 min), after which urease activity was assayed at 40 min. The RA values are reported as percent of the control activity.

3.3. Reactivation of H_2O_2-inactivated urease

To investigate if the inactivation of urease by H_2O_2 is reversible by reductants, the enzyme was first inactivated by H_2O_2 at different concentrations. Upon removing the excess H_2O_2 with catalase, DTT and GSH were added at two concentrations, 10 and 50 mM. The inactivations with different H_2O_2 concentrations were done to examine whether, by being brought to different levels of activity, urease thiol groups were oxidized to different oxidation states. Remarkably, it was found that none of the experiments was capable of reversing the inactivation. The representative data for 10 mM DTT are shown in Fig. 7. These observations are consequential in that they prove that irrespective of the extent of inactivation, urease was always inactivated irreversibly, having its thiol groups oxidized to sulfinic $-SO_2H$ or sulfonic $-SO_3H$ acid. This finding is unlike that in our previous study of urease inhibition by dehydroascorbic acid-Fe^{3+}-generated H_2O_2 [21]. It was suggested in the study that as a result of the inhibition half of the thiol groups of urease were oxidized to sulfenic acid and the remaining half, to further thiol oxidation states. A similar conclusion that thiol groups are oxidized only to sulfenic acid, was drawn for 1,4-naphtoquinone-induced inhibition of urease [20]. In this inhibition, H_2O_2 resulting from redox cycling was found to bring about inactivation by oxidation along the arylation brought about by 1,4-naphtoquinone. If, however, due to the obvious complexity, the dehydroascorbic acid-Fe^{3+}- and 1,4-naphtoquinone–urease systems can be directly compared in respect of thiol oxidation states to the system with the direct addition of H_2O_2 studied here, remains to be elucidated.

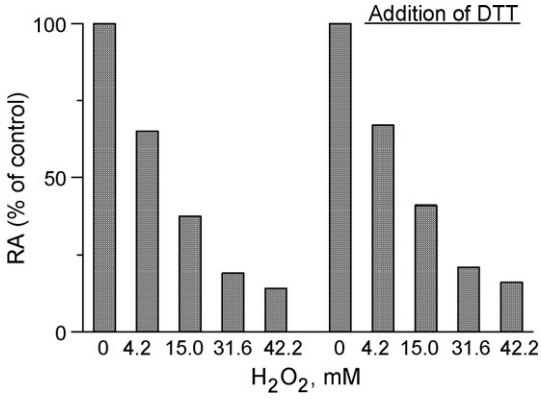


Fig. 7. Reactivation of H_2O_2-inactivated urease with DTT at pH 8.2. Urease (0.75 mg solid/mL) was incubated with different H_2O_2 of concentration for 20 min, after which the incubation mixtures had the excess H_2O_2 removed by catalase (268 U/mL, 10 min incubation). The mixtures were further incubated with 10 mM DTT for 10 min, and urease had its activity determined in the standard assay mixture. The RA values are reported as percent of the control activity.

Further to the thiol reactivation of H_2O_2-inactivated urease, the reactivation by dilution was checked at different times of incubation between 10 and 40 min. It was found that under no circumstances did the diluted samples regain activity (data not shown). This ensures that the modification of the enzyme thiols, whether at the early or later stage of inactivation, was stable and irreversible, supporting the DTT- and GSH-reactivation observations.

3.4. DTNB-spectroscopic quantification of thiol groups in H_2O_2-inactivated urease

To examine the reactivity and numbers of urease thiol groups involved in the reaction with H_2O_2, the relationship between urease activity and thiol modification following H_2O_2 treatment was assessed. For that, using DTNB titration, thiol groups were assayed under non-denaturating conditions in one urease preparation modified with 21.1 mM H_2O_2 at different times of incubation between 0 and 60 min, and in seven urease preparations modified with H_2O_2 at different concentrations from the range 1.05 to 84.4 mM incubated for one time interval of 20 min.

The spectroscopic time courses of the DTNB reactions with the urease preparations are presented in Fig. 8a and c, respectively. The curves for native urease showed a characteristic shape 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 active-site flap Cys592/molecule [44,45]. The curves for the modified urease preparations are similar in shape but are always located below the native enzyme, thereby revealing that the number of free thiol groups accessible in urease for DTNB through disulfide exchange, gradually diminished both in the course of inactivation (Fig. 8a) and by applying increasing H_2O_2 concentrations at one inactivation time (Fig. 8c).

In Fig. 8b and d the calculated numbers of modified thiols in urease are correlated with the residual activity of the enzyme. The correlations show that in both cases all 36 thiol groups available in urease under non-denaturating conditions were modified to achieve the complete inactivation of the enzyme, and that the modification consisted of two approximately linear phases, in each of which a 50% loss of the enzyme activity was observed. In the time-dependent experiment (Fig. 8b), the initial 50% loss occurred after ca. 12 min incubation, involving the modification the first 30 thiol groups. The completion of the inactivation occurred at 60 min incubation, the final phase involving the modification of the remaining six thiol groups. Thus, the initial phase was more rapid than the final one, from which it can be inferred that the 30 thiols oxidized in the initial phase are those highly reactive thiols, the other six being the less reactive active-site flap Cys-592s. Crucially, although urease quickly lost 50% of its activity by allowing 30 of its thiols to be modified, it was the slower modification of the six Cys-592s that abolished the activity. Similar results are seen in the concentration-dependent experiment (Fig. 8d). Namely, a concentration of about 15 mM H_2O_2 applied for 20 min brought about a 50% loss of urease activity involving again 30 thiol groups, but only higher concentrations of H_2O_2 gave rise to the oxidation of the remaining six

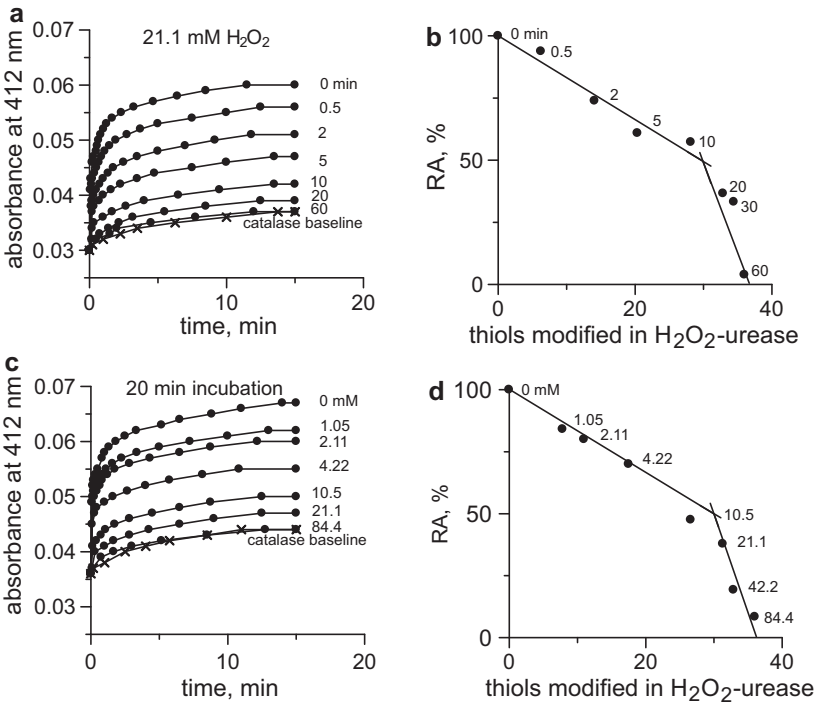


Fig. 8. DTNB titration of thiol groups in urease modified with H_2O_2 at pH 8.2. Spectroscopic time courses of DTNB reactions with H_2O_2-modified urease: (a) in the course of inactivation with 21.1 mM H_2O_2, and (c) by applying increasing H_2O_2 concentration at one inactivation time of 20 min. Enzyme residual activity (RA) as a function of number of enzyme thiol groups per urease molecule modified with H_2O_2: (b) in the course of inactivation with 21.1 mM H_2O_2, and (d) by applying increasing H_2O_2 concentration for 20 min. For (a) and (b) solutions of urease (3 mg solid/mL) and of H_2O_2 were mixed 1:1. In (a) at time intervals of incubation, and in (b) at 20 min incubation, two samples were withdrawn, one for the activity determination in the standard assay mixture and the other for DTNB titration. Prior to the titration, catalase (0.2 mL containing 268 units in 20 mM) was added for 10 min to the urease-H_2O_2 samples (1 mL) to remove H_2O_2. To perform the titration, 0.8 mL of 0.15 mM DTNB was added to 1.2 mL of the incubation mixture in a cuvette. The absorbance of the mixture was measured at 412 nm for 15 min. The background titration of catalase solution is included.

groups, the H_2O_2 concentration as high as 85 mM being required for the complete inactivation.

The observations made in these two DTNB-spectroscopic experiments confirm the earlier conclusion drawn from the urease protection experiments performed with the active-site binding inhibitors (Section 3.2), which substantiated the participation of the active-site cysteine in the inactivation of urease.

Interestingly, the results obtained are in line with those reported on thiol targeted inactivation of other ureases. For instance, in the inactivation of pigeon pea [44] and soybean [45] urease by –SH specific reagents, such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetamide, it was observed that the loss of half initial activity involved the modification of 30 enzyme thiol groups in a fast process, and the loss of another half, the modification of six thiol groups in a slower process.

4. Conclusions

Our previous studies indicated that in some urease inhibitor systems, H_2O_2 endogenously generated contributed to the observed inhibition. Herein the inhibition of urease by H_2O_2 was systematically investigated in an effort to assess its mechanism, kinetics and the inhibitory potency. It was found that the oxidative inactivation of urease by H_2O_2 was pH-dependent, growing with an increase in pH in the studied range of 6.2–8.2. This was interpreted as resulting from the deprotonation of the enzyme thiols to thiolate anions. The involvement of the groups was confirmed by the protection experiments performed with DTT and GSH, whereas the enzyme protection with active-site binding inhibitors proved the involvement of the active-site flap cysteinyl residues Cys-592. These residues, though not essential, when modified result in the loss of enzyme activity. The H_2O_2-modified urease failed to have its activity restored, which indicated that urease was oxidized by H_2O_2 irreversibly to sulfinic or sulfonic acid. Of the thiol groups available in urease under non-denaturating conditions, the oxidation of 30 thiol groups/molecule resulted in the loss of half the enzyme initial activity, while the oxidation of six active site Cys-592s/molecule completed the inactivation. H_2O_2 was assessed to be a comparatively weak urease inhibitor with IC_{50} = 11 mM at pH 8.2. The results obtained are of practical value for the description of urease inhibitory systems with the participation of H_2O_2 that may be exploited to combat the deleterious effects of urease activity in practical applications.

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