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Double mode of inhibition-inducing interactions of 1,4-naphthoquinone with urease: Arylation versus oxidation of enzyme thiols

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Abstract—In their inhibition-inducing interactions with enzymes, quinones primarily utilize two mechanisms, arylation and oxidation of enzyme thiol groups. In this work, we investigated the interactions of 1,4-naphthoquinone with urease in an effort to estimate the contribution of the two mechanisms in the enzyme inhibition. Jack bean urease, a homohexamer, contains 15 thiols per enzyme subunit, six accessible under non-denaturing conditions, of which Cys592 proximal to the active site indirectly participates in the enzyme catalysis. Unlike by 1,4-benzoquinone, a thiol arylator, the inactivation of urease by 1,4-naphthoquinone under aerobic conditions was found to be biphasic, time- and concentration-dependent with a non-linear residual activity-modified thiols dependence. DTT protection studies and thiol titration with DTNB suggest that thiols are the sites of enzyme interactions with the quinone. The inactivated enzyme had ~40% of its activity restored by excess DTT supporting the presence of sulfenic acid resulting from the oxidation of enzyme thiols by ROS. Furthermore, the aerobic inactivation was prevented in ~30% by catalase, proving the involvement of hydrogen peroxide in the process. When H_2O_2 was directly applied to urease, the enzyme showed susceptibility to this inactivation in a time- and concentration-dependent manner with the inhibition constant of H_2O_2 $K_i = 3.24$ mM. Additionally, anaerobic inactivation of urease was performed and was found to be weaker than aerobic. The results obtained are consistent with a double mode of 1,4-naphthoquinone inhibitory action on urease, namely through the arylation of the enzyme thiol groups and ROS generation, notably H_2O_2, resulting in the oxidation of the groups.

1. Introduction

Very common in nature, quinones play a variety of roles. Some of them play biologically essential role in mitochondrial respiration and plant photosynthesis, some are found toxic in foodstuffs and as environmental pollutants, and the toxicity of some of them has been exploited in anti-cancer drugs in chemotherapies.[1–6] In their interactions with biological molecules quinones primarily utilize two properties.[1–6] First, the electronegativity of oxygens in carbonyl groups, by lending partial positive charge to the ring, makes quinones electrophiles susceptible to nucleophilic attack, resulting in the covalent modifications of the nucleophilic sites on biological molecules, e.g., thiols in proteins. The reaction involved is a 1,4-reductive nucleophilic addition of thiols to quinones (Michael-type addition), also termed arylation of thiols. The product of the reaction is a hydroquinone thioether. Second, in the presence of reductants quinones may undergo one- or two-electron reductions, resulting in semiquinones or hydroquinones, respectively, of which the former being radicals may in turn in the presence of oxygen initiate a redox cycle. As a result, reactive oxygen species (ROS) are generated including semiquinone $SQ^{\cdot -}$, superoxide $O_2^{\cdot -}$ and hydrogen peroxide H_2O_2. These bring about inactivation of biological molecules by oxidation, e.g., of thiol groups of proteins. The role of quinones in the redox cycle is that of a catalyst transferring electrons in a one-electron process from a reductant to oxygen, which means that whether a quinone is capable of redox cycling depends in the first place on its one-electron reduction potential, E^0.[2,7] The estimates based on biological systems suggest that reduction potentials of quinones in the approximate range from −270 to −160 mV are conducive to redox cycling.[2,7–10] Accordingly, in their actions in biological systems quinones can preferentially act as pure arylators, pure redox cyclers or as both concomitantly. Thus, in cytotoxicity and enzyme inhibition studies 1,4-benzoquinone

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(E^0 = 78 mV[7]) has been mostly found to be a pure arylator,[7,8,11,12] 1,4-naphthoquinone (E^0 = −140 mV[7]) by contrast, both an arylator and redox cycler,[7,13–15] and for tetrachloro-1,4-benzoquinone (E^0 = 671 mV[7]) on the other hand it is very unlikely that it could act as a redox cycler at all. Enzymes are affected by quinones to various degrees[1] depending, in addition to the potential, also on molecular structure, pH, quinone/thiol ratio and the presence of oxygen.

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.[16–19] 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. Various urease inhibitors have been studied to be used to combat the undesirable effects brought about by urease activity. Among those are quinones proposed to be applied in combination with urea fertilizer to control soil urease activity to increase the overall efficiency of nitrogen utilization.[20,21] For its enzymatic hydrolysis urease utilizes an active site containing a binuclear nickel centre bridged by a carbamylated lysine and a hydroxide ion as was shown by the crystallographic structures resolved for bacterial ureases from *Klebsiella aerogenes*,[22] *Bacillus pasteurii*,[23] and *Helicobacter pylori*.[24] The ureases have a nearly superimposable active site, which implies that it is common to all ureases, including jack bean urease whose crystallographic structure has not been resolved to date. Jack bean urease is a homohexamer in which every subunit contains an active site. Proximally to the active site, in its flap is located Cys592, one of 15 cysteine residues per urease subunit.[25] Although determined not to be essential, Cys592 has been shown to be involved in the catalysis, namely when this cysteine is chemically modified urease becomes inactive.[25–27] Apart from Cys592, jack bean urease contains five other cysteine residues per subunit that are more reactive, all the six being accessible under non-denaturing conditions. Additional nine cysteine residues are disclosed only under denaturing conditions. The thiol groups of the cysteines are the most likely targets of quinone actions responsible for urease inhibition. Wide screening in field tests notwithstanding,[21] the inhibition of urease by quinones never has had its mechanism elucidated.

In our previous study[28] we investigated four quinones, 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, tetrachloro-1,4-benzoquinone, and 1,4-naphthoquinone, as inhibitors of jack bean urease. The first three quinones were found to share a common pattern of behaviour in this inhibition that we ascribed to the arylation of the urease thiols. We concluded that the arylation, by giving rise to conformational changes in the enzyme molecule, is responsible for the inhibition. In contrast, 1,4-naphthoquinone manifested a distinctly different pattern of behaviour supporting the suggestion that in this inhibition possible are other reactions, most likely redox cycling. In this work, we further examined possible contributions of the two mechanisms, arylation versus oxidation, in 1,4-naphthoquinone inhibition of urease. We demonstrate that the inhibitory action of 1,4-naphthoquinone towards urease has a mixed mechanism that comprises both arylation and oxidation of the enzyme thiol groups, the latter reaction mediated by hydrogen peroxide.

2. Results and discussion

2.1. Inactivation of urease by 1,4-NQ under aerobic conditions

As shown in Figure 1a, where residual activity (RA) of urease is plotted against time of enzyme incubation with 1,4-NQ, under aerobic conditions 1,4-NQ inactivated urease in a time- and concentration-dependent manner with a half-life of ~120 min at 10 μM 1,4-NQ, which reduced to a half-life of ~10 min at 20 μM inhibitor. The inset, being a semi-logarithmic plot (ln RA vs time), demonstrates that the activity of urease decreased linearly in a biphasic manner over incubation time, in each phase the inactivation reaction being of the first order. Accordingly, we described the overall inactivation curves as bi-exponential functions of time:[29,30]

$$RA = (100 - a) \cdot e^{-k_{fast} \cdot t} + a \cdot e^{-k_{slow} \cdot t} \quad (1)$$

The two rate constants are k_{fast} for the initial fast phase of the inhibition and k_{slow} for the subsequent slow phase. The parameter a reflects the contributions of the two components to the inhibition. The results are presented in Table 1. They show that the contribution of the fast phase to the inhibition was 42 ± 4% and that of a slow phase 58 ± 4%.

The biphasic inactivation reaction (Fig. 1a) indicates that there should be two types of 1,4-NQ–urease interactions, either involving two different types of enzyme groups or, which is more likely, thiol groups of two different reactivities or thiol groups being modified in two different ways, as will be argued later.

The residual activity (RA) data of Figure 1a are replotted in Figure 1b as RA vs 1,4-NQ concentration. Two curves are presented for 10 and 20 min inhibitor–urease incubation, which have IC_{50} values of ~40 and ~20 μM, respectively.

2.2. Protection of urease with reducing agents

The results of the protection experiments that consisted of the pre-incubation of 1,4-NQ with reducing agents, L-ascorbic acid, DTT and Na-dithionite, prior to the addition of urease are presented in Figure 2. Upon increasing the concentration of the agents we see the growing protection effect of urease against inactivation by 1,4-NQ. For DTT and Na-dithionite this effect begins at a concentration

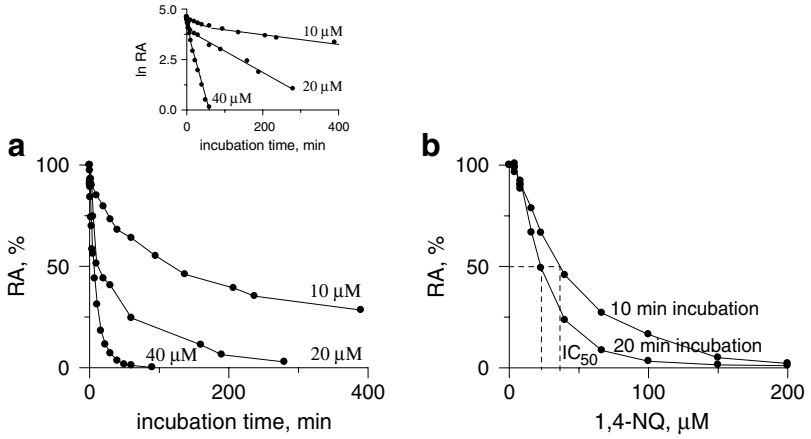


Figure 1. (a) Time courses of urease inactivation by 1,4-NQ under aerobic conditions (residual activity (RA) vs time); the inset presents a semi-logarithmic plot of RA vs time. The solutions of urease (2.0 mg solid/mL) and of 1,4-NQ were mixed 1:1. During the incubations, at time intervals 0.5 mL aliquots were transferred into the standard assay mixtures for enzyme residual activity determinations. (b) Urease residual activity as a function of 1,4-NQ concentration in solution for two incubation times, 10 and 20 min (data taken from Ref. 28).

Table 1. Inactivation of urease by 1,4-NQ: rate constants of the fast and slow phase of the inhibitor–enzyme reaction and a contribution of the slow phase to the process (*a*)

1,4-NQ concentration (μM)	k_{fast} (min^{-1})	k_{slow} (min^{-1})	*a* (%)
10	0.008	0.002	62
20	0.064	0.011	55
40	0.118	0.068	57

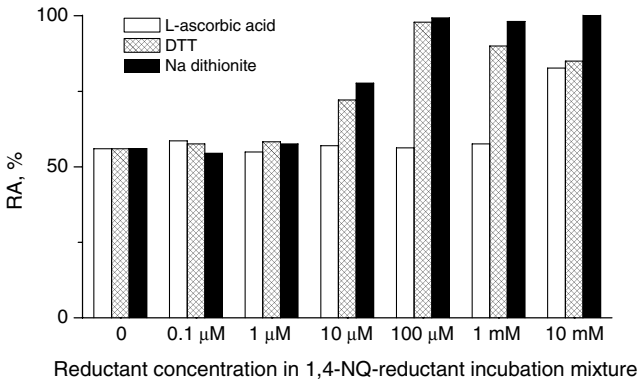


Figure 2. Protection of urease with reducing agents against inhibition by 1,4-NQ. The agents were pre-incubated with 40 μM 1,4-NQ for 10 min. Next, the pre-incubation mixtures were mixed with urease solutions (2.0 mg solid/mL), incubated for 20 min and had their activities measured in the standard assay mixtures.

of 10 μM to thoroughly prevent the inhibition at a concentration of 100 μM, this range corresponding to the concentration of 1,4-NQ in the pre-incubation mixture. For L-ascorbic acid the effect is less pronounced and the highest protection of 85% was achieved at a concentration of L-ascorbic acid of 10 mM. As shown, the magnitude of the protection effect of the three reductants grows in the order: L-ascorbic acid < DTT < Na-dithionite, and is consistent with their reduction potentials at pH 7, 58 mV, −330 and −527 mV, respectively.[31] Together, these observations provide evidence that responsible for this protection is the reduction of 1,4-naphthoquinone to 1,4-dihydroxynaphthalene, for stronger reductants, DTT and Na-dithionite, apparently in a stoichiometric manner, hence meaning that the oxidized state of the quinone is required for urease inhibition. Interestingly, the opinion was expressed as early as in 1933 that these are quinones present at trace levels as a result of oxidation in hydroquinones that are responsible for hydroquinones inhibition of urease.[32]

The DTT protection experiments that consisted of the pre-incubation of urease with excess DTT prior to the addition of 1,4-NQ on the other hand showed that DTT afforded complete protection of urease (data not shown). The action of DTT in the pre-incubation mixture apparently consisted of its covalent binding to urease thiol groups[25] thereby protecting them from the interactions with 1,4-NQ. This is an important observation in that it excludes the involvement of urease functional groups other than thiols from the interactions with 1,4-NQ responsible for the inhibition.

2.3. Reactivation of 1,4-NQ-inactivated urease

Reactivation of urease from the 1,4-NQ–urease complex with excess DTT, a nucleophilic-reducing agent, is presented in Figure 3. Intriguingly, the activity of urease was restored within 40 min only up to about 40%. This means that as a result of urease interactions with 1,4-NQ, thiol groups in urease were modified in two different ways, 40% of them could be reactivated with DTT, the remaining part being modified in a way that could not be reversed by the reaction with DTT. These results suggest that a 40% population of thiol groups in the inactivated urease was oxidized by ROS generated from redox cycling. Such an oxidation of –SH groups leads to sulfenic acid (–SOH) that can be reduced back to –SH by DTT, hence being the case observed, and subsequently to sulfinic ($–SO_2H$) and sulfonic acid ($–SO_3H$).[9,13,33] The incomplete restoration of enzyme activity could obviously be attributed to further oxidation of sulfenic acids to sulfinic and sulfonic acids, which would not be reversible by DTT, but given the electro-

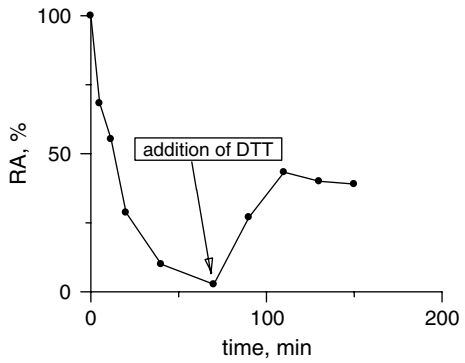


Figure 3. Reactivation of 1,4-NQ-modified urease by DTT. Urease (1.0 mg solid/mL) was incubated with 30 μM 1,4-NQ for 70 min, after which the incubation mixture was further incubated with 400 μM DTT. The activity recovery of the enzyme was measured over time in the standard assay mixtures.

philic properties common of all quinones it seems reasonable to assume that the other 60% population of thiol groups was arylated by 1,4-NQ producing thioethers, from which thiol groups could not be restored by DTT.[11] From the above results it follows that 1,4-NQ inhibits urease by a mixed mechanism that involves direct arylation of –SH groups and their oxidation. The latter, brought about by ROS, most likely H_2O_2, results from 1,4-NQ-catalyzed redox cycling. Strikingly, this redox cycling, whose effects were observed, took place without exogenously added reductant that is required to initiate the cycling. The reduction of 1,4-NQ that triggered the cycling was achieved, we think, most presumably by one or more reducing substances contained in the Sigma urease preparation applied in this study (see Section 4.1).

2.4. Thiols modification by 1,4-NQ under aerobic conditions assayed by DTNB titration

To further examine the mechanism of urease inactivation by 1,4-NQ under aerobic conditions we assessed the relationship between urease activity and thiol modification following quinone treatment. For that we assayed thiol groups using DTNB in one 1,4-NQ–urease complex (20 μM 1,4-NQ) at different times of incubation (Fig. 4a), and in six 1,4-NQ–urease complexes (2–200 μM 1,4-NQ), all of them incubated for 10 min (Fig. 4b).

As shown in Figure 4a and b the relationships between urease residual activity and the numbers of thiols modified are not linear, which is contrary to the behaviour of 1,4-BQ.[28] In Figure 4a we see that during the incubation the modification of about 20 of 36 thiols available in urease under non-denaturing conditions does not change the enzyme activity, whereas the modification of the remaining 16 thiols causes a steep activity drop leading to the complete activity loss.

The other correlation of urease residual activity with the number of modified thiols obtained for the same 10-min incubation at different 1,4-NQ concentrations (Fig. 4b) shows that low 1,4-NQ concentrations up to 4 μM modify about 12 thiols without considerably reducing the enzyme activity. Only higher 1,4-NQ concentrations modifying subsequent thiols up to all 36 groups are capable of inducing a quick inactivation of the enzyme. In an attempt to define which thiols, those modified by low or high 1,4-NQ concentrations, were arylated or oxidized, we compare our results with those we reported previously on the inhibition of urease by 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone and tetrachloro-1,4-benzoquinone.[28] From the correlations the quinones showed between enzyme residual activity, numbers of modified enzyme thiols and the enzyme concentration, we concluded that these are conformational changes in the enzyme molecule brought about by thiol arylation that are responsible for the inhibition. A similar interpretation was reported for the inhibition of glyceraldehyde-3-phosphate dehydrogenase by 1,4-benzoquinone.[11] In contrast to the arylation, oxidation of thiols to sulfenic, sulfinic or sulfonic acids, not introducing so overburdening molecules like aromatic rings to the enzyme molecule, should be less conducive to such drastic conformational changes. Consequently, it might be concluded that in our observations here (Fig. 4b), low concentrations of 1,4-NQ are responsible for oxidation of thiol groups, and higher concentrations for their arylation. The same might be extended to the other correlation of the residual activity with modified thiols recorded over the course of incubation (Fig. 4a), namely the first 20 thiol groups in the initial stage of incubation were oxidized, the others arylated, and this was the arylation that abolished the activity of urease. Furthermore, by combining this analysis with the results of the inactivation (Fig. 1a and Table 1) and reactivation (Fig. 3) experiments it can be surmised that in the initial fast phase of the inhibition, effective in ~40%, the oxidation of enzyme thiols takes place, the arylation, effective in ~60%, being the slow phase.

2.5. Participation of redox cycling in 1,4-NQ inhibition of urease—further evidence

The plots of urease inactivation by 1,4-NQ (Fig. 1a) and of thiol assays in 1,4-NQ-inactivated urease (Fig. 4) when set beside the corresponding plots obtained for 1,4-BQ,[28] strongly imply that in 1,4-NQ inhibition of urease, further to 1,4-nucleophilic addition characteristic of 1,4-BQ, operative should be other reactions. If these are redox processes induced by 1,4-NQ, as suggested in earlier sections, we performed the following experiments:

(i) inactivation of urease by 1,4-NQ under anaerobic conditions,
(ii) protection of urease by catalase under aerobic conditions,
(iii) quantification of hydrogen peroxide in 1,4-NQ–urease mixtures,
(iv) inactivation of urease by hydrogen peroxide.

(i) The inactivation curves of urease by 1,4-NQ were recorded for the same inhibitor concentrations under

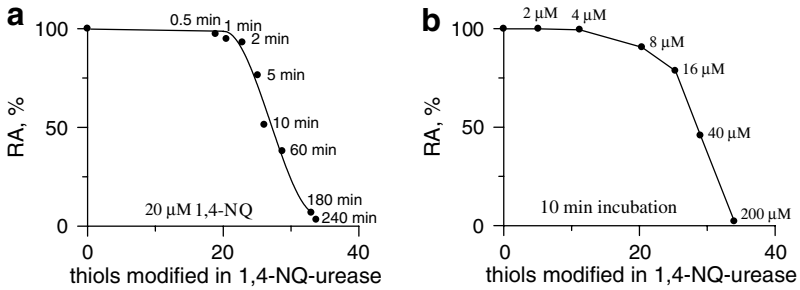


Figure 4. DTNB spectroscopic assay of thiol groups in 1,4-NQ-modified urease: (a) residual activity of urease as a function of number of thiols per urease molecule modified with 20 μM 1,4-NQ over the time of incubation; (b) residual activity of urease as a function of number of thiols per urease molecule modified with different 1,4-NQ concentrations at 10 min incubations (data taken from Ref. 28). For the former at time intervals of incubation, and for the latter at 10-min incubation with a given inhibitor concentration, two samples of the incubation mixtures were withdrawn, one for the activity determination in the standard assay mixture and the other for DTNB titration. For that 2.5 mL of the incubation mixture was transferred to a cuvette (light path 5 cm) and mixed with 2.5 mL of 0.15 mM DTNB. The absorbance of the mixture was measured at 412 nm for 15 min.

anaerobic and aerobic conditions. Representative curves are shown in Figure 5. The curves reveal that the inactivation of urease by 1,4-NQ is weaker in the absence of O_2. The shapes of the anaerobic curves imply that after a comparatively long incubation an equilibrium of the inhibitor–urease system was reached as was the case of 1,4-BQ[28] though observed at much shorter times. If we assume again that 1,4-BQ inhibition is attributable only to arylation, then accordingly, the anaerobic curves should represent the arylation contribution to the overall 1,4-NQ inhibition of urease illustrated by the aerobic curves. An increase in the inhibition in the presence of O_2 most likely reflects the ability of 1,4-NQ to act as a catalyst for the generation of ROS.

(ii) To further support the involvement of ROS in the aerobic inhibition of urease by 1,4-NQ, particularly of H_2O_2, the incubations were carried out under aerobic conditions in the presence of catalase, an efficient H_2O_2-decomposing enzyme. The results obtained for different 1,4-NQ concentrations in the incubation mixture are presented in Figure 6. As can be seen from

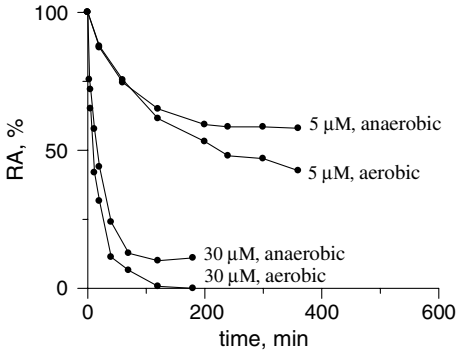


Figure 5. Time courses of urease inactivation by 1,4-NQ under aerobic and anaerobic conditions. The solutions of urease (2.0 mg solid/mL) and of 1,4-NQ were mixed 1:1. During the incubations, at time intervals 0.5 mL aliquots were transferred into the standard assay mixtures for enzyme residual activity determinations. For anaerobic inactivations the measurements were performed under nitrogen in a glove chamber, in which all the solutions were purged with nitrogen for 5 h prior to incubations and activity assays.

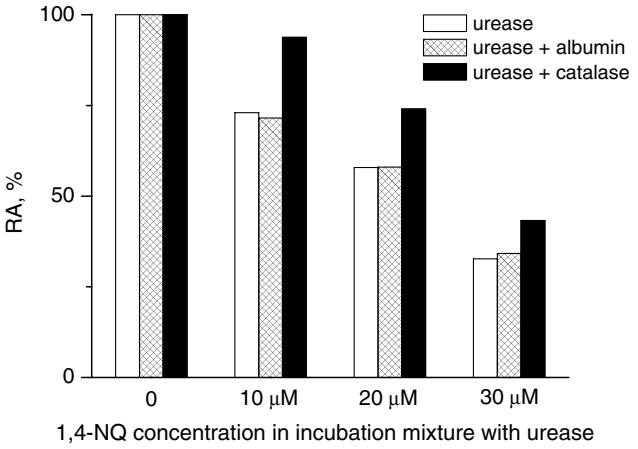


Figure 6. Protection of urease by catalase against 1,4-NQ inactivation under aerobic conditions. Catalase 100 U/mL and the corresponding amounts of albumin (control) were pre-incubated with urease (2.0 mg/mL) for 10 min. To the mixtures 1,4-NQ was added to give the final concentrations 10, 20, and 30 μM, the mixtures were incubated for 20 min and urease activity was determined in the standard conditions.

the figure, neither catalase nor egg albumin, used here as a control, had an impact on the free urease activity. By contrast, in 1,4-NQ–urease mixtures (10–30 μM 1,4-NQ) catalase prevented the inactivation of urease by about 30% in every case. Importantly, the effect of catalase could not be due to its conjugation with 1,4-NQ, as albumin did not prevent the inhibition.

(iii) To examine if H_2O_2, shown in the catalase experiment to be involved in 1,4-NQ inhibition of urease, is present in the inactivation mixture, we titrated the mixtures with $KMnO_4$. The titrations were quick at the beginning, but considerably slowed down towards the end point, giving the results with an estimated experimental error of ca. ±15%. Nonetheless, they provide an estimate of how much H_2O_2 was generated in the presence of the studied 1,4-NQ concentrations. The results are presented in Table 2.

(iv) To determine whether H_2O_2, shown to be present in the inactivation medium, inhibits urease we directly

Table 2. Estimates of hydrogen peroxide concentration in 1,4-NQ–urease incubation mixtures upon 20-min incubation

1,4-NQ concentration (μM)	RA (%)	H_2O_2 concentration (mM)
30	40	~0.8
50	24	~1.1
85	7	~1.2

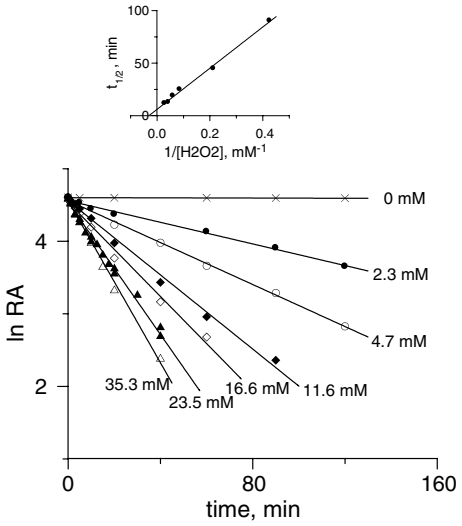


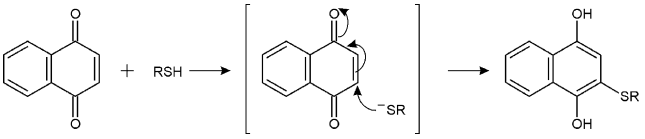
Figure 7. Inactivation of urease by hydrogen peroxide. The solutions of urease (2.0 mg solid/mL) and of H_2O_2 were mixed 1:1. During the incubations, at time intervals 0.5 mL aliquots were transferred into the standard assay mixtures for enzyme residual activity determinations. The inset presents half-lives of H_2O_2 inactivation of urease plotted against reciprocal of H_2O_2 concentration.

measured its inactivation of the enzyme over time (Fig. 7). We found that the inactivation was time- and concentration-dependent following a first-order decay with half-lives of 90, 45 and 15 min for 2.3, 4.7, and 23.5 mM H_2O_2, respectively. The inactivation rate constant and the inhibition constant for H_2O_2 obtained from the replot of the data in Figure 7 (see the inset) are equal to $k_i = 0.114\ min^{-1}$ and $K_i = 3.24$ mM. Of note is that to our knowledge these data are the first ones on the kinetics of urease inactivation by H_2O_2 in the literature.

Remarkably, the concentrations of H_2O_2 needed to inactivate urease (Fig. 7) match quite closely the concentrations estimated in 1,4-NQ–urease mixtures (Table 2). Consequently, the results of catalase and H_2O_2 experiments are soundly supportive of the hypothesis that it is the generation of H_2O_2 by redox cycling that accounts for a specific behaviour of 1,4-NQ in the inhibition of urease disparate from that of 1,4-BQ.

It is also worth mentioning that inhibitions by H_2O_2 of the same magnitude as observed here for urease were reported for other enzymes, e.g., for protein-tyrosine phosphatase α[9] and glyceraldehyde-3-phosphate dehydrogenase.[11] For the former it is also known that the oxidation of the catalytic cysteine to sulfenic acid takes place only at concentrations of H_2O_2 lower than 1 mM, with higher concentrations leading to sulfinic and sulfonic acids.[9] No such data have been reported for urease.

Together the findings of this study provide evidence that 1,4-naphthoquinone elicits its inhibition of urease through a mixed mechanism comprising arylation of enzyme thiols and their oxidation by redox cycling-generated ROS, mainly H_2O_2. The thiol arylation follows the equation[5]:



(2)

In the redox cycling on the other hand, a likely sequence of reactions is[5,9,34]:

$$\text{Red} + 1,4\text{-NQ} \rightarrow \text{Ox} + 1,4\text{-NQH}_2 \quad (3)$$

$$1,4\text{-NQ} + 1,4\text{-NQH}_2 \rightleftarrows 2\ 1,4\text{-NQ}^{\cdot -} + 2H^+ \quad (4)$$

$$1,4\text{-NQ}^{\cdot -} + O_2 \rightarrow 1,4\text{-NQ} + O_2^{\cdot -} \quad (5)$$

$$1,4\text{-NQH}_2 + O_2^{\cdot -} \rightarrow 1,4\text{-NQ}^{\cdot -} + H_2O_2 \quad (6)$$

The sequence is triggered by the reduction of 1,4-NQ (Eq. 3) followed by a comproportionation reaction resulting in the formation of semiquinone radical anions (Eq. 4). Once formed, these anion free radicals reduce molecular oxygen to superoxide anion radicals and regenerate the parent compound (Eq. 5). The superoxide in turn, by oxidizing the hydroquinone produces H_2O_2 (Eq. 6). The oxidative effects of H_2O_2 were observed in this study to contribute to the overall 1,4-NQ-induced inhibition of urease effectively in about 30–40% in the initial phase of the inhibition.

3. Conclusions

In this study, we examined the molecular mechanism by which 1,4-naphthoquinone inhibits urease. First, by mixing 1,4-NQ with reductants, L-ascorbic acid, DTT, and sodium dithionite, prior to the incubation with urease we showed that oxidized quinone is required for the inhibition, reduced quinone being unable to produce enzyme modification, be it through arylation or oxidation. Second, by performing a series of experiments under aerobic conditions that included 1,4-NQ-inactivation of the enzyme, thiol titration with DTNB of the quinone-modified enzyme, DTT-protection and reactivation, and in addition to that the anaerobic inactivation of urease that was found weaker than aerobic, we could in a stepwise way discover the mechanism of 1,4-NQ action on urease. The experiments showed that the mechanism is partly O_2-dependent meaning that in addition to the arylation of enzyme thiols with 1,4-NQ, it comprises the oxidation of the groups by ROS generated from 1,4-NQ-catalyzed redox cycling. Third, to prove that the latter was brought about by H_2O_2, we performed a catalase-protection study of urease and urease direct H_2O_2-inactivation.

Catalase was found to prevent the 1,4-NQ inactivation of the enzyme by ~30%, and H_2O_2 revealed the inhibitory potential for urease with a K_i of 3.24 mM. Together the observations are consistent with a double mode of 1,4-NQ action on urease that includes arylation of enzyme thiols and their oxidation by ROS, H_2O_2 in particular, generated from redox cycling.

4. Experimental

4.1. Materials

Urease (from jack beans, type III, activity 16 U/mg solid, total content of reducing substances 0.5 μg/unit), urea (for Molecular Biology), D,L-dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), catalase (from bovine liver, activity 1340 U/mg solid), egg albumin (grade V, electrophoretic purity 99%), L-ascorbic acid and sodium dithionite (Na-dithionite) were from Sigma. 1,4-Naphthoquinone (1,4-NQ) was from Fluka, and H_2O_2 from POCh, Poland. Phosphate buffer 50 mM, pH 7.8, was prepared by adjusting pH of phosphoric acid with NaOH. All the enzyme mixtures contained 2 mM EDTA.

4.2. Standard urease activity assay

The standard urease assay mixture contained 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[35] in samples withdrawn from the reaction mixture at 5 min reaction. The measurements were performed at ambient temperature.

4.3. Urease inactivation by 1,4-NQ

Solutions of urease (2.0 mg solid/mL) and of 1,4-NQ were mixed 1:1, the final concentration of 1,4-NQ being in the range 2–200 μM, after which the mixtures were incubated with occasional stirring. During the incubations, periodically 0.5 mL aliquots of the incubation mixture were transferred into the standard assay mixtures (25 mL) for enzyme residual activity determinations. All residual activities were normalized to the measured activity at time zero incubation. For aerobic inactivations the measurements were performed at ambient atmosphere. For anaerobic inactivations on the other hand, the measurements applying the same protocol as for aerobic conditions were performed under nitrogen in a glove chamber, in which all the solutions were purged with nitrogen for 5 h prior to incubations and activity assays.

4.4. Urease inactivation by H_2O_2

The same protocol as presented above for 1,4-NQ inactivations was used for H_2O_2 inactivation measurements. The concentration of H_2O_2 in the incubation mixtures applied was in the range from 2.3 to 35.3 mM in phosphate buffer, pH 7.8. The rate constant of the inactivation k_i and the inhibition constant K_i were calculated according to the method of Kitz and Wilson.[36,37]

4.5. Urease protection

In the urease protection experiments, in the final incubation mixture of urease with 1,4-NQ the concentration of urease always was 1.0 mg/mL (like in the inactivation experiments) and the concentration of 1,4-NQ was 20 μM, unless otherwise noted. The incubation time applied was 20 min.

For the protection studies by reducing agents, L-ascorbic acid, DTT and Na-dithionite, 1,4-NQ was pre-incubated for 10 min with different concentrations of the agents from the range between 0.1 μM and 10 mM. Next, the pre-incubation mixtures were mixed with urease solutions, incubated and had their activities measured.

For the protection studies by DTT, urease was incubated for 10 min with excess DTT (400 μM), followed by mixing with 1,4-NQ. Upon incubation of the mixture the urease activity was assayed.

For the protection experiments by catalase and egg albumin, catalase 100 U/mL and the corresponding amounts of albumin were pre-incubated with urease for 10 min. To the mixtures 1,4-NQ was added to give the final concentrations 10, 20, and 30 μM, the mixtures were incubated and urease activity was determined.

4.6. Reactivation of 1,4-NQ-inactivated urease

Reactivation was carried out with DTT. For that urease was incubated with 30 μM 1,4-NQ for 70 min, after which the incubation mixture was further incubated with excess DTT (400 μM). The activity recovery of the enzyme was measured over time.

4.7. Spectroscopic assays of thiol groups with DTNB in 1,4-NQ-inactivated urease

Two types of thiol assays in 1,4-NQ-inactivated urease were performed. In one, thiols were assayed over the time of enzyme incubation with 20 μM inhibitor, and in the other, urease was incubated for the same time of 10 min but with different inhibitor concentrations ranging from 2 to 200 μM. For the former at time intervals of incubation, and for the latter at 10 min incubation with a given inhibitor 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.[38] For that 2.5 mL of the incubation mixture was transferred to a cuvette (light path 5 cm) and mixed with 2.5 mL of 0.15 mM DTNB (prepared in 50 mM phosphate buffer, pH 7.8). The absorbance of the mixture was measured at 412 nm for 15 min. Prior to these measurements, background absorbances of the solutions in the proportions corresponding to the final DTNB–

urease reaction mixture were measured with and without the enzyme, and subtracted when necessary. The number of thiols in urease modified as a result of the interactions with 1,4-NQ was calculated according to the protocol proposed previously.[39] 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,[25] we converted the 15-min absorbances of 1,4-NQ-modified enzyme samples by direct absorbance/thiols proportion into the number of inhibitor-modified thiols per urease molecule.

4.8. Quantification of hydrogen peroxide in 1,4-NQ–urease mixtures

Hydrogen peroxide was quantified in 1,4-NQ–urease mixtures by titration with 0.004 M $KMnO_4$. Urease (2.0 mg/mL) was mixed with different concentrations of 1,4-NQ and incubated for 20 min. Upon incubation enzyme residual activity was determined in standard conditions, and the solutions were titrated with $KMnO_4$ in the presence of H_2SO_4. A blank titration of the urease mixture without the inhibitor was performed and subtracted from the results obtained for 1,4-NQ–urease mixtures.

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