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Quinone-induced inhibition of urease: Elucidation of its mechanisms by probing thiol groups of the enzyme

Wiesława Zaborska [a], Barbara Krajewska [a,*], Mirosława Kot [a], Waldemar Karcz [b]

[a] *Jagiellonian University, Faculty of Chemistry, 30-060 Kraków, Ingardena 3, Poland*
[b] *Silesian University, Faculty of Biology, 40-032 Katowice, Jagiellońska 28, Poland*

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Abstract

In this work we studied the reaction of four quinones, 1,4-benzoquinone (1,4-BQ), 2,5-dimethyl-1,4-benzoquinone (2,5-DM-1,4-BQ), tetrachloro-1,4-benzoquinone (TC-1,4-BQ) and 1,4-naphthoquinone (1,4-NQ) with jack bean urease in phosphate buffer, pH 7.8. The enzyme was allowed to react with different concentrations of the quinones during different incubation times in aerobic conditions. Upon incubation the samples had their residual activities assayed and their thiol content titrated. The titration carried out with use of 5,5′-dithiobis(2-nitrobenzoic) acid was done to examine the involvement of urease thiol groups in the quinone-induced inhibition. The quinones under investigation showed two distinct patterns of behaviour, one by 1,4-BQ, 2,5-DM-1,4-BQ and TC-1,4-BQ, and the other by 1,4-NQ. The former consisted of a concentration-dependent inactivation of urease where the enzyme–inhibitor equilibrium was achieved in no longer than 10 min, and of the residual activity of the enzyme being linearly correlated with the number of modified thiols in urease. We concluded that arylation of the thiols in urease by these quinones resulting in conformational changes in the enzyme molecule is responsible for the inhibition. The other pattern of behaviour observed for 1,4-NQ consisted of time- and concentration-dependent inactivation of urease with a nonlinear residual activity-modified thiols dependence. This suggests that in 1,4-NQ inhibition, in addition to the arylation of thiols, operative are other reactions, most likely oxidations of thiols provoked by 1,4-NQ-catalyzed redox cycling. In terms of the inhibitory strength, the quinones studied formed a series: 1,4-NQ $\approx$ 2,5-DM-1,4-BQ $<$ 1,4-BQ $<$ TC-1,4-BQ.

* Corresponding author. Fax: +48 12 6340515.
E-mail address: krajewsk@chemia.uj.edu.pl (B. Krajewska).

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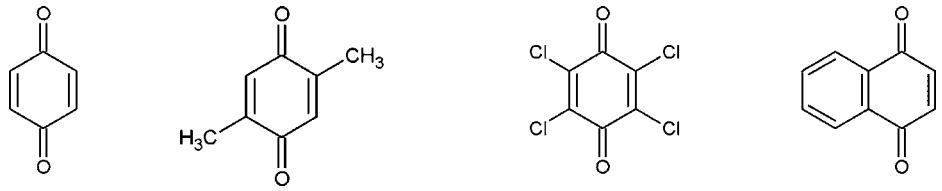
1. Introduction

Urease (urea amidohydrolase EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Urease is widely distributed in nature and is found in a variety of plants, algae, fungi and bacteria [1–4]. The structure, number and type of subunits, and the molecular weight depend on the enzyme origin. Despite these differences, the amino acid sequences of the active site are principally conserved in all known ureases, and the catalytic mechanism of their action is believed to be the same. Jack bean urease is a homohexamer with a molecular weight of 90.77 kDa reported for the subunit. The total thiol content in jack bean urease, determined by DTNB titration in the presence of 6 M guanidinum chloride, amounts to 15 thiol groups per subunit [5]. However, only 6 of them are accessible to the thiol reagent under non-denaturating conditions. Among the accessible thiols in urease, Cys592 located in the mobile flap of the active site is important in that, although reckoned not to be essential for the catalysis, it has been shown to be critical for the enzyme activity, most likely through structural interactions with other active-site amino acid residues [6].

Among a variety of substances studied as urease inhibitors are quinones. Their application in combination with urea fertilizers has been proposed for soil urease activity control [7,8]. Although widely screened in field tests [8], they never have had their inhibitory mechanism towards urease elucidated. Available are only few kinetic studies on quinone-induced inhibition of urease. Among those is one on polyhalogenated benzo- and naphthoquinones, in which the quinones were found to be non-competitive inhibitors of jack bean and bacterial ureases of different strengths [9]. In contrast, in our previous studies we showed that 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone [10] and tetrachloro-1,4-benzoquinone [11] are slow-binding inhibitors of jack bean urease, and their overall inhibition constants K_i^* were found 45 nM, 1.2 μM and 0.45 nM, respectively.

In their inactivation–inducing interactions with biological molecules quinones utilize two primary mechanisms [12–16]. One is a covalent modification of biological molecules at their nucleophilic sites, such as thiols in proteins, in which quinones act as electrophiles. The reaction involved is a 1,4-reductive nucleophilic addition of thiols to quinones, also referred to as 1,4-Michael addition or arylation of thiols. The product of the reaction is a thioether in which the quinone carbonyl groups have been reduced to hydroxyls. Alternatively, for quinones having all their electrophilic sites substituted, a nucleophilic substitution (addition–elimination) can operate, from which also a thioether results, whose carbonyl groups, however, are not reduced. The other chemical mechanism consists of redox cycling, in which in the presence of oxygen and a reductant reactive oxygen species (ROS) are generated. These bring about inactivation of biological molecules by oxidation, e.g. of protein thiol groups. Clearly, redox cycling induced by quinones is strongly dependent on their one-electron reduction potentials [13,17]. In their actions in biological systems quinones can act as pure arylators, pure redox-cycling agents or as both concomitantly.

In this work, we investigated the mechanism of inhibition of urease by four quinones, 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, tetrachloro-1,4-benzoquinone and 1,4-naphthoquinone, with the following molecular formulas, respectively:



For that we probed thiol groups of the enzyme involved in the process by their titration performed with 5,5′-dithiobis(2-nitrobenzoic) acid [18]. The elucidation of the mechanism of quinone–urease interactions is of significance for possible application of quinones as inhibitors controlling urease activity, mainly in agriculture.

2. Experimental

2.1. Materials

Urease (from jack beans, type III, activity 16 U/mg solid, total content of reducing substances 0.5 μg/U), urea (for Molecular Biology), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) were from Sigma. 1,4-benzoquinone (1,4-BQ), 2,5-dimethyl-1,4-benzoquinone (2,5-DM-1,4-BQ), tetrachloro-1,4-benzoquinone (TC-1,4-BQ) and 1,4-naphthoquinone (1,4-NQ) were from Fluka. Phosphate buffer 50 mM, pH 7.8, was prepared by adjusting pH of phosphoric acid with NaOH and 2 mM EDTA was added to all enzyme-containing solutions. pH 7.8 of phosphate buffer was chosen for the study for the following reasons: (1) to avoid interference of the inhibition of urease by phosphate known to be operative below pH 7.5 [19], with the inhibition by the quinones, (2) to facilitate quinone–enzyme reaction by 1,4-nucleophilic addition that is enhanced by deprotonation of protein thiols in alkaline pHs [14], and lastly because (3) DTNB titration of thiols in proteins requires alkaline pH [18].

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 [20] in samples withdrawn from the reaction mixture at 5-min reaction. The measurements were performed at ambient temperature.

2.3. Quinone-inactivations of urease

Solutions of urease (2.0 mg solid/mL) and of the quinones were mixed 1:1. The concentrations of the quinones used in the incubation mixtures were in the following ranges:

1–15 μM for 1,4-BQ, 0.05–0.4 mM for 2,5-DM-1,4-BQ, 0.015–1.9 μM for TC-1,4-BQ and 2–200 μM for 1,4-NQ. The mixtures prepared were incubated with occasional stirring. During the incubations, at time intervals 0.5-mL aliquots were transferred into the standard assay mixtures (25 mL) for enzyme residual activity determinations. The inactivations were carried out under aerobic conditions at ambient atmosphere. All residual activities determined were normalized to the measured activity at time zero incubation.

2.4. Spectroscopic assays of –SH groups with DTNB in quinone-modified urease

The samples of urease equilibrated with the quinones for the times required for the equilibrium, determined earlier for each individual quinone in the inactivation studies (Section 2.3), were subjected to the reaction with a cysteine-selective reagent DTNB. Notably for 1,4-NQ, the only quinone of the quinones studied here for which the equilibrium of the reaction with urease was not reached, 10 min incubation time was arbitrarily chosen for the DTNB titration. The incubations were carried out at different concentrations of the quinones. Upon incubation, the activity of urease was assayed under standard conditions, and at the same time the other sample of the incubation mixture was DTNB-titrated. For that 2.5 mL of the incubation 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 absorbance of the mixture was measured at 412 nm [18] 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. The number of thiols in urease modified as a result of the interactions with the quinones was calculated according to the protocol proposed previously [21]. 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-denaturating conditions), we converted the 15-min absorbances of quinone-modified enzyme samples by direct absorbance/thiols proportion into the number of inhibitor-modified thiols per urease molecule.

3. Results and discussion

3.1. Quinone-inactivation of urease (aerobic conditions)

The results of the inactivation of urease by the quinones investigated under aerobic conditions are presented in Fig. 1a and b for 1,4-BQ, Fig. 1c and d for TC-1,4-BQ and in Fig. 1e and f for 1,4-NQ. The results for 2,5-DM-1,4-BQ were very much like those for 1,4-BQ, which is why they are not presented but included in the discussion.

As shown in Fig. 1 the quinones studied revealed two distinct types of behaviour in the inactivation of urease, one was by 1,4-BQ, 2,5-DM-1,4-BQ and TC-1,4-BQ and the other by 1,4-NQ. For the former characteristic is that inactivation is primarily concentration-dependent (Fig. 1a and c), i.e. in incubation the activity of urease was reduced within a given time, not longer than 10 min, to a non-zero constant value which varied with inhibitor concentration, with the plateaus corresponding to the fraction of the uninhibited enzyme at equilibrium. For the latter, by contrast, characteristic is that the inhibitor, 1,4-NQ, inactivated urease in a time- and concentration-dependent manner (Fig. 1e) with a half-life of ~120 min at 10 μM 1,4-NQ, which reduced to half-life of 10 min at 20 μM

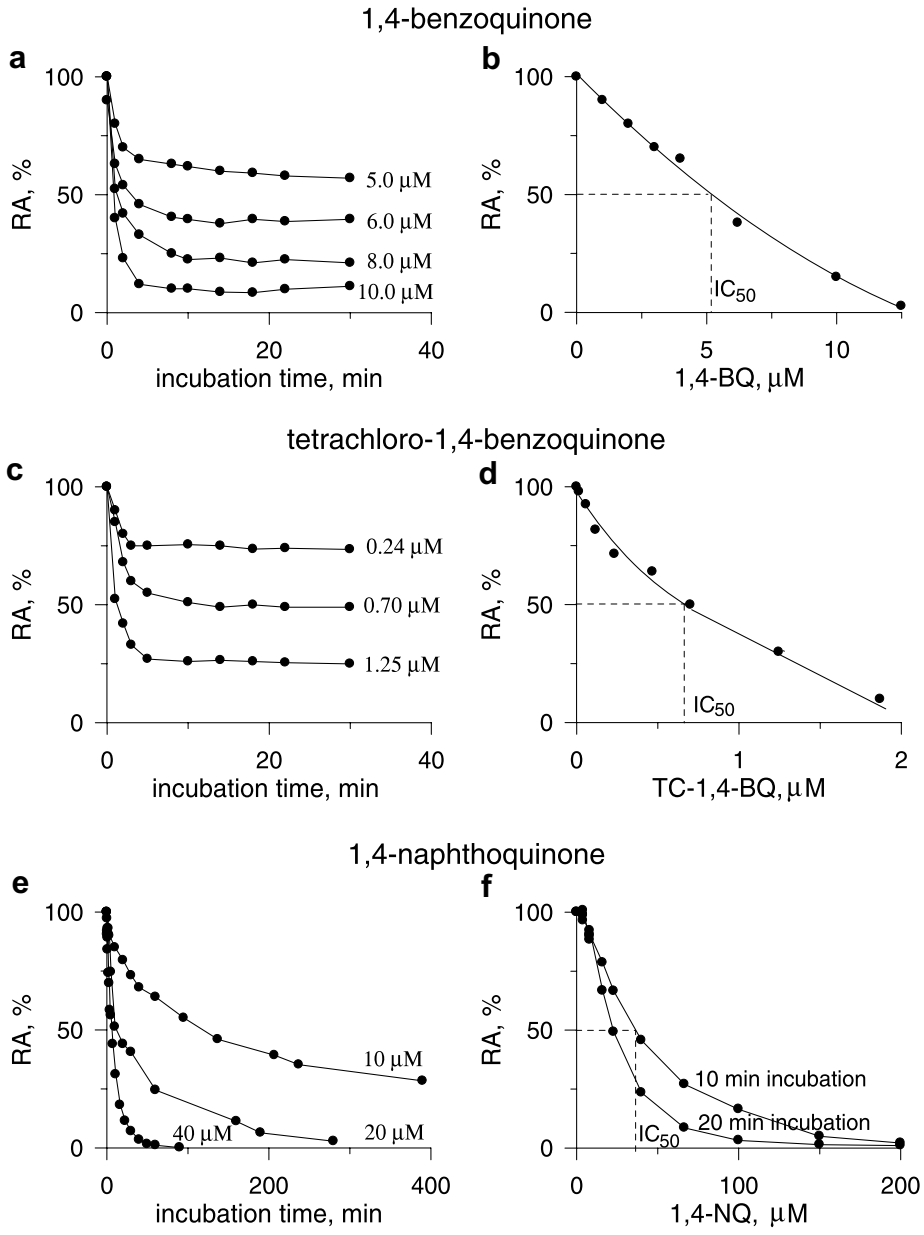


Fig. 1. Inactivation of urease by the quinones under study. Time courses of inactivation, enzyme residual activity (RA) over time of incubation with 1,4-benzoquinone (a), tetrachloro-1,4-benzoquinone (c) and 1,4-naphthoquinone (e). For that solutions of urease (2.0 mg solid/mL) and of the quinones were 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. Enzyme residual activities as a function of quinone concentrations in the incubation mixtures for 1,4-benzoquinone (b), tetrachloro-1,4-benzoquinone (d) and 1,4-naphthoquinone (f). *Note*: for 1,4-BQ and TC-1,4-BQ the equilibrium residual activities are presented, and for 1,4-NQ those corresponding to 10 and 20 min incubations.

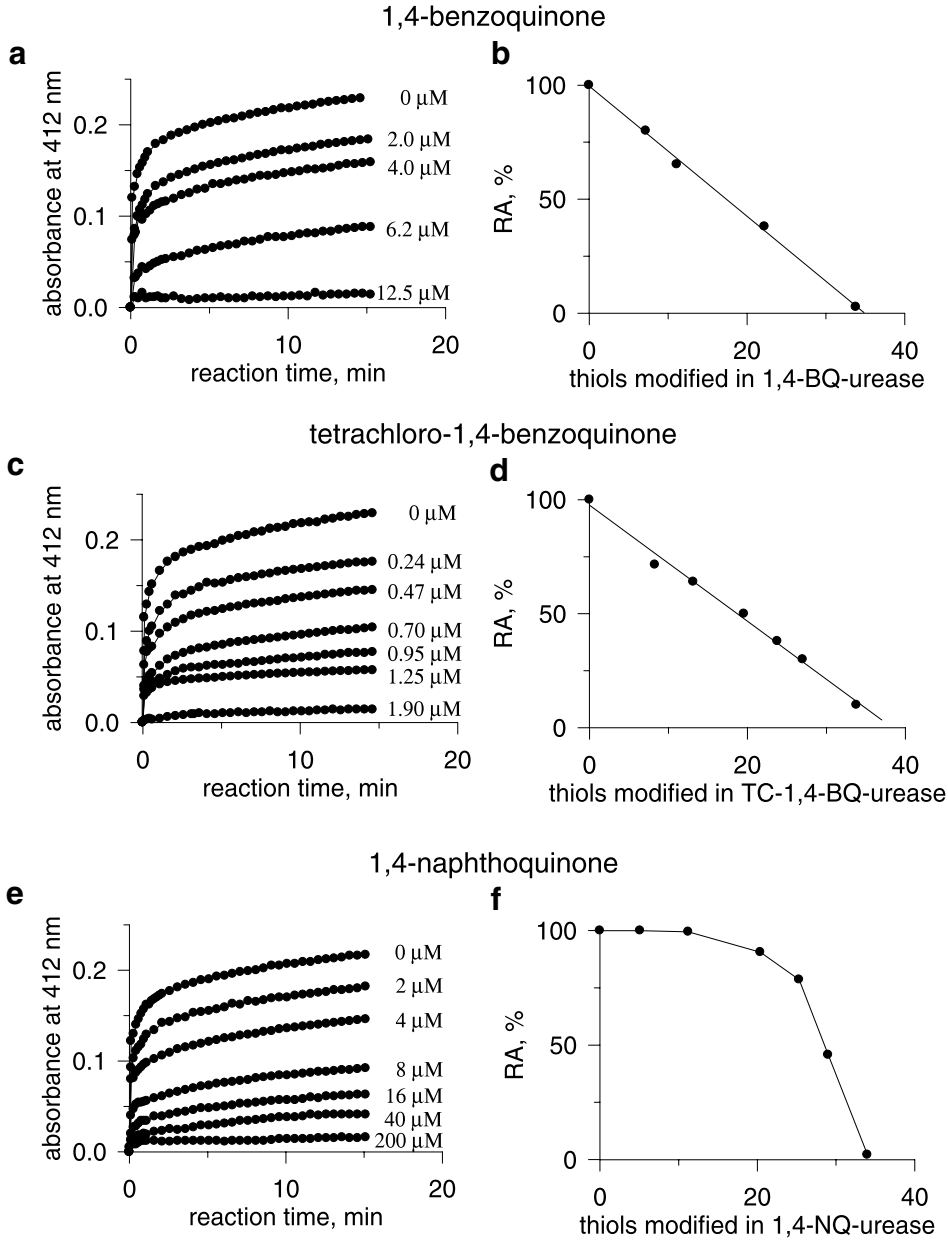


Fig. 2. DTNB titration of thiol groups in urease modified with the quinones under study. Spectroscopic time courses of DTNB reactions with urease modified with 1,4-benzoquinone (a), tetrachloro-1,4-benzoquinone (c) and 1,4-naphthoquinone (e). For that solutions of urease (2.0 mg solid/mL) and of the quinones were mixed 1:1. Upon equilibration (10 min) samples were subjected to the reaction with DTNB. 2.5 mL of the incubation mixture was transferred to a cuvette (light path 5 cm) and mixed with 2.5 mL 0.15 mM DTNB. The absorbance at 412 nm was recorded over 15 min. Enzyme residual activity as a function of number of enzyme thiols modified with 1,4-benzoquinone (b), tetrachloro-1,4-benzoquinone (d) and 1,4-naphthoquinone (f). *Note*: for 1,4-BQ and TC-1,4-BQ the equilibrium residual activities are presented, and for 1,4-NQ those corresponding to 10 min incubation.

inhibitor. In Fig. 1b, d and f, the urease residual activities are re-plotted against inhibitor concentrations. In these figures for the first group of quinones the equilibrium residual activities were used, whereas for 1,4-NQ residual activities corresponding to two times of incubation, 10 and 20 min, are presented. The resulting IC_{50} values, i.e. inhibitor concentration bringing about a 50 % inactivation of the enzyme are 5.5 μM for 1,4-BQ, 50 μM for 2,5-DM-1,4-BQ, 0.6 μM for TC-1,4-BQ and 40 μM for 1,4-NQ, the latter value corresponding to 10 min incubation. The results are consistent with our previous inhibition constants determinations (see Section 1). Thus, in terms of the inhibitory strength towards urease the quinones studied form the order: 1,4 NQ $\approx$ 2,5-DM-1,4-BQ $<$ 1,4-BQ $<$ TC-1,4-BQ.

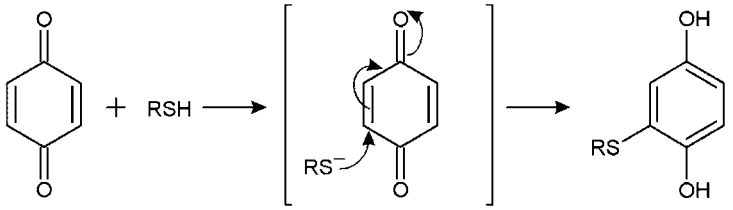
3.2. Spectroscopic assays of –SH groups with DTNB in quinone-modified urease

The spectroscopic titration of thiol-SH groups in quinone-modified urease carried out with the use of DTNB was done in an attempt to examine the involvement of urease thiol groups in the quinone-induced inhibition. In Fig. 2a, c and e; we present the time-courses of the reactions of DTNB with urease modified with different concentrations of the quinones, 1,4-BQ, TC-1,4-BQ and 1,4-NQ, respectively. Being very much like those of 1,4-BQ, the results of 2,5-DM-1,4-BQ are not shown.

The figures show that the number of the urease thiol groups available for DTNB decreases with the increasing quinone concentration used in the incubation mixtures. This provides evidence that the quinones inhibit urease by modifying the –SH groups of the enzyme. In Fig. 2b, d and f, the calculated numbers of modified thiols in urease are compared with the residual activity of the enzyme. Interestingly, like in the inactivation studies (Section 3.1) also here we see two patterns of behaviour, namely 1,4-BQ, 2,5-DM-1,4-BQ and TC-1,4-BQ show a linear correlation of the residual activity with the number of modified thiols, whereas for 1,4-NQ the correlation is non-linear. For the former group of quinones this indicates that upon increasing the quinone concentration, regardless of their reactivities, all enzyme thiols react with the quinones provoking progressive inhibition of the enzyme. The modification of all 36 –SH groups (obviously including the active-site flap Cys592) available in urease under non-denaturating conditions abolishes the enzyme activity. By contrast, for 1,4-NQ low concentrations up to 4 μM modify about 12 –SH groups without considerably altering the enzyme activity. Only higher inhibitor concentrations give rise to the modification of further –SH groups up to 36 –SHs inducing a quick drop in the enzyme activity. Apparently, Cys 592 is among the 24 thiols of the activity drop.

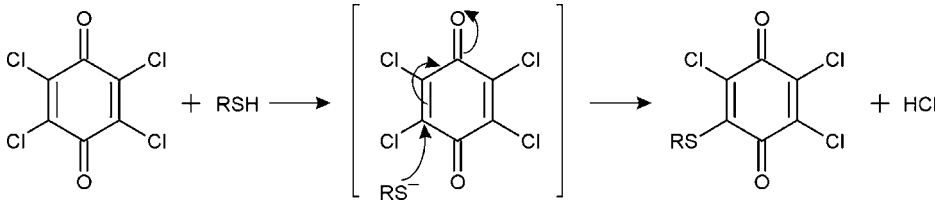
3.3. Molecular mechanisms of quinone inhibition of urease

From the results of the quinone-inactivation studies of urease under aerobic conditions (Section 3.1) and of the thiols titration in the quinone-modified urease (Section 3.2) it can be inferred that 1,4-BQ, 2,5-DM-1,4-BQ and TC-1,4-BQ have one common feature that characterizes their interactions with urease responsible for their one pattern of inhibitory behaviour observed, in contrast to 1,4-NQ that interacts with urease in a distinctly different manner. Taking into account that 1,4-BQ inhibitions of enzymes derive primarily from the actions of the quinone as an electrophile in 1,4-reductive nucleophilic addition of enzymes thiol groups [17], according to the following equation:



(1)

we can assume that the common feature in the behaviour of this group of quinones with urease is a covalent modification of urease thiol groups leading to the formation of thioethers. Importantly, basic pHs are conducive to this reaction (our pH was 7.8) by helping thiols to deprotonate, owing to which they become stronger nucleophiles. Interestingly, for 2,5-DM-1,4-BQ the inhibition of urease was found to be weaker than that of 1,4-BQ as judged by their IC_{50} values (see Section 3.1). This is due to the fact that 2,5-DM-1,4-BQ is a weaker electrophile than 1,4-BQ, the reason being that methyl groups are electron-donating substituents by whose action the electrophilicity of the quinone ring is diminished. Remarkably, having all four *ortho*- and *meta*-positions occupied by chlorines, TC-1,4-BQ is not capable of acting as an electrophile in the 1,4-nucleophilic addition. The reaction taking place in this case is a nucleophilic substitution of chlorines by the urease thiols according to the equation:



(2)

in which also a thioether is formed though no reduction of ketone groups of the quinone to hydroxyls takes place. Among the quinones studied, TC-1,4-BQ was found to be the strongest urease inhibitor. This is due to the fact that in addition to mono-thioethers like the one presented in Eq. (2), TC-1,4-BQ readily forms di-, tri- and tetra-conjugates with thiols, thereby being capable of bridging thiol groups in the enzyme. This fact was observed by Van Ommen et al. in their study of glutathione *S*-transferase [22]. The authors demonstrated that the higher was the number of chlorines in chlorine-substituted 1,4-BQs the higher became their inhibitory strength towards the enzyme [23]. It was proposed that it is the enhanced electrophilicity of *ortho*- and *meta*-positions of the quinoid ring resulting from multiple substitution of the ring with chlorines that is responsible for this effect. Also very importantly, the authors observed that one conjugation of the quinone with one thiol facilitates a subsequent conjugation of the same quinone at another position in its ring with another protein thiol, thereby allowing the quinone to cross-link a protein [24,25]. The above not only explains the strongest inhibition of TC-1,4-BQ towards urease, but also corroborates the hypothesis that the direct cause of the inhibition of urease by the quinones are conformational changes resulting from the covalent conjugation of the quinones to the protein, as argued below. Of note is that the arylation of urease thiols, be it their 1,4-nucleophilic addition or nucleophilic substitution, responsible for the inhibition by 1,4-BQ, 2,5-DM-1,4-BQ and TC-1,4-BQ, is consistent with the magnitude of one-electron reduction

potentials of these quinones, $E_{1,4\text{-BQ}} = 78$ mV, $E_{2,5\text{-DM-}1,4\text{-BQ}} = -67$ mV and $E_{\text{TC-}1,4\text{-BQ}} = 671$ mV [17] that practically preclude them from acting as redox cyclers [17].

For further analysis of the mechanism of urease inhibition by the arylating quinones, we chose the strongest one, TC-1,4-BQ. With its inhibition constant as low as 0.45 nM [11], lower than 1.9 nM for Hg^{2+} ions [21], it might be assumed that TC-1,4-BQ reacts with urease in a stoichiometric fashion. Needed for the analysis, the concentration of urease in the incubation mixtures was assessed from the absorbance exhibited by free urease in the DTNB titration (Fig. 2) using TNB molar absorbance 13,600 M^{-1} cm^{-1} [18] and assuming that the absorbance recorded corresponds to 36 thiol groups in urease. The concentration thus obtained amounts to 0.0944 μM urease, corresponding to 3.40 μM urease thiol groups, and is indeed comparable to the concentration of TC-1,4-BQ. Importantly, this concentration includes both active and inactive forms of the enzyme that apparently participated both in the reaction with TC-1,4-BQ and DTNB. The results of the TC-1,4-BQ inactivation analysis are presented in Fig. 3 where urease residual activity (Fig. 3a) and the number of urease free thiols in the quinone-urease conjugates (Fig. 3b) are correlated with the TC-1,4-BQ/urease ratio. The results indicate that TC-1,4-BQ inactivates urease at a molar TC-1,4-BQ/urease ratio of 15 ± 2. This means that one TC-1,4-BQ molecule reacts with 2.4 thiol groups on average, dithioethers being apparently the predominant form resulting from the reaction. In Fig. 3b, a consequential characteristic of the correlation is the slope of the linear part of the curve, here of approximately −2 as yielded by linear regression analysis. If interpreted in terms of the concept of "half-of-the-sites" vs "all-of-the-sites reactivity" [26–28], this value is indicative of the inhibition that arises from active site alkylation with reagents capable of inducing strong conformational changes that encompass intersubunit interactions. As a consequence, it may reasonably be concluded that in the case of TC-1,4-BQ and the other arylating quinones in our study, responsible for the inhibition brought about by arylation of the thiols in urease are conformational changes in the molecule of the enzyme. A similar interpretation was reported for the inhibition of glyceraldehyde-3-phosphate dehydrogenase by 1,4-benzoquinone [26]. The above conclusion also explains why TC-1,4-BQ, among other quinones studied, revealed the highest effectiveness in the inhibition of

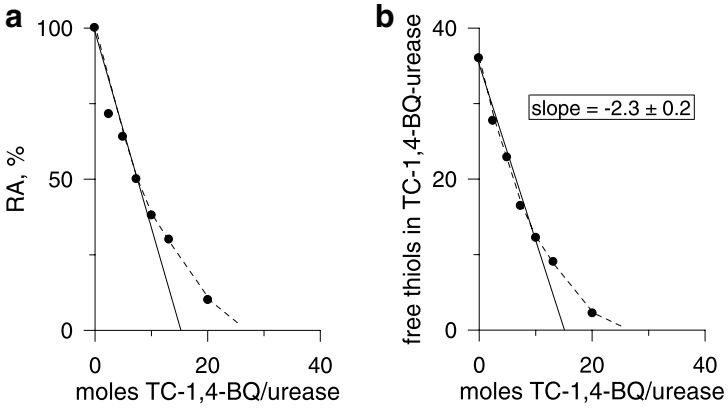


Fig. 3. Inactivation of urease by TC-1,4-BQ. (a) Urease residual activity plotted as a function of molar TC-1,4-BQ/urease ratio in the incubation mixture. (b) Number of free thiol groups in urease conjugated with TC-1,4-BQ, plotted as a function of molar TC-1,4-BQ/urease ratio in the incubation mixture.

urease. As shown earlier, this effectiveness is due to the formation of di-conjugates of this quinone with urease thiols that can result in cross-links between thiols, and these apparently introduce the most damaging conformational changes to the enzyme structure.

Having its 2- and 3-positions free, 1,4-NQ should also be able to arylate thiols. In its interactions with urease, however, it manifested a different behaviour, which strongly suggests that in addition to the same 1,4-nucleophilic addition typical of 1,4-BQ, operative in this inhibition should be other reactions. These most likely are oxidations of some –SH groups by reactive oxygen species generated by redox cycling catalyzed by 1,4-NQ, apparently less inhibitory than the 1,4-addition. This idea, substantiated by the magnitude of the one-electron reduction potential of 1,4-NQ equal to −140 mV [17], hence conducive to redox cycling, as compared to 68 mV of 1,4-BQ, a typical arylator, is extensively discussed in [29].

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