Interactions of Quinones with Thioredoxin Reductase

A CHALLENGE TO THE ANTIOXIDANT ROLE OF THE MAMMALIAN SELENOPROTEIN*

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Mammalian thioredoxin reductases (TrxR) are important selenium-dependent antioxidant enzymes. Quinones, a wide group of natural substances, human drugs, and environmental pollutants may act either as TrxR substrates or inhibitors. Here we systematically analyzed the interactions of TrxR with different classes of quinone compounds. We found that TrxR catalyzed mixed single- and two-electron reduction of quinones, involving both the selenium-containing motif and a second redox center, presumably FAD. Compared with other related pyridine nucleotide-disulfide oxidoreductases such as glutathione reductase or trypanothione reductase, the $k_{cat}/K_m$ value for quinone reduction by TrxR was about 1 order of magnitude higher, and it was not directly related to the one-electron reduction potential of the quinones. A number of quinones were reduced about as efficiently as the natural substrate thioredoxin. We show that TrxR mainly cycles between the four-electron reduced ($E_2$) and two-electron reduced ($E_1$) states in quinone reduction. The redox potential of the $E_2/E_1$ couple of TrxR calculated according to the Hal dane relationship with NADPH/NADP + was $E_0 = -0.294$ V at pH 7.0. Antitumor azidinylbenzoquinones and daunorubicin were poor substrates and almost inactive as reversible TrxR inhibitors. However, phenanthrene quinone was a potent inhibitor (approximate $K_i = 6.3 \pm 1 \mu M$). With other flavoenzymes, quinones could confer superoxide-producing NADPH oxidase activity to mammalian TrxR. A unique feature of this enzyme was, however, the fact that upon selenocysteine-targeted covalent modification, which inactivates its normal activity, reduction of some quinones was not affected, whereas that of others was severely impaired. We conclude that interactions with TrxR may play a considerable role in the complex mechanisms underlying the diverse biological effects of quinones.

Thioredoxin reductase (TrxR, EC 1.8.1.9) catalyzes NADPH-dependent reduction of the redox-active disulfide in thioredoxin (Trx), which serves a wide range of functions in cellular proliferation and redox control (1, 2). Thioredoxin reductases are homodimeric proteins that differ in properties between different classes of organisms. Low $M_1$ (34-kDa subunit) TrxRs of prokaryotes, plants, or yeast contain FAD and a redox-active disulfide/dithiol active site and display narrow substrate specificities. High $M_2$ (54–58 kDa) TrxRs of animals have in contrast remarkably wide substrate specificities, explained by an additional easily accessible C-terminal redox center. This redox center is either a dithiol/dithiols as in TrxR of Plasmodium falciparum or Drosophila melanogaster or a selenocysteine-containing selenenylsulfide/selenolthiol motif as found in TrxRs of mammals (3–6). In recent years, the catalytic mechanism of mammalian TrxR has been unraveled in significant detail. The three-dimensional crystal structure of rat TrxR is similar to that of glutathione reductase, including conserved FAD and NADP(H)-binding domains, but TrxR has a 16-residue C-terminal extension carrying the catalytic Cys-497/Sec-498 couple that in essence substitutes for glutathione as a substrate of the N-terminally located active site disulfide/dithiol motif (7). In the catalytic cycle of mammalian TrxR, NADPH first reduces FAD, which subsequently passes redox equivalents to the redox-active disulfide with formation of a dithiol, located within a conserved -CVNVGC- sequence. Finally, this dithiol reduces the selenenylsulfide formed by the Cys and Sec residues in a -GCUG sequence located at the C-terminal end of the other subunit in the dimeric enzyme (5, 7, 8). The so-formed selenolthiol is the proper active site of mammalian TrxR, reducing Trx or other substrates such as lipoic acid, ascorbic acid, or the synthetic model substrate, 7,8-dihydroxy-2,5-dimethyldibenzo[a]phenanthrene (DZQ) (9). Consecutive reduction of the three redox-active motifs of mammalian TrxR, i.e. the FAD, the N-terminal disulfide, and the C-terminal selenenylsulfide, gives two-, four-, and six-electron reduced states of the enzyme, with specific spectral properties that are well characterized (5, 8). It is believed that during normal catalysis, mammalian TrxR cycles between the two- and four-electron reduced states with the two or four electrons shared mainly between the catalytic disulfide and the selenenylsulfide (5). The disulfide/dithiol motif also forms a charge transfer complex with the FAD (8). Recently, this mechanism was demonstrated also for D. melanogaster TrxR where, however, a...
dithiol motif substitutes for the role of the selenolthiol in the mammalian enzyme (3, 6).

Many factors make mammalian TrxR an important target of drugs and xenobiotics, as recently reviewed (10). The broad substrate specificity of mammalian TrxR is expected to provide antioxidant function at several levels, coupled to reduction of cytosolic lipoate, selenium compounds, hydroperoxides, and ubiquinone-10 (see Refs. 11–15); this would be perturbed if TrxR becomes inhibited in cells. Another important antioxidant system that would suffer from TrxR inhibition is that of the mammalian peroxiredoxins, which all require functional Trx for regeneration (16). Furthermore, cytokine functions of extracellular Trx, as well as increased Trx and TrxR levels in some tumor cell lines and in synovial fluid or tissue of patients suffering from rheumatoid arthritis (10, 17), argue for targeting of TrxR as a possible therapeutic approach in certain diseases. In fact, TrxR is inactivated by several compounds in clinical use, including 1,3-bis-(2-chloro-ethyl)-1-nitrosourea (BCNU) (18), anticancer platinum compounds (19), antiarthritic gold compounds (20, 21), and immunostimulatory dinitrohalobenzenes (22). Modification of TrxR by dinitrohalobenzenes yields covalently linked dinitrophenyl groups that seem to act as mediators between the enzyme-bound FAD and oxygen, thus enhancing the superoxide-producing NADPH oxidase activity of TrxR, while inhibiting the normal function of the enzyme (22). This may play a role in the provoked inflammation seen upon topical application of dinitrohalobenzenes on skin, as discussed elsewhere (2).

Quinones are a widespread group of oxygen-substituted, often biologically active, aromatic compounds. They are found as naturally occurring substances, as synthetic drugs for clinical use, or as components of environmental pollutants. The antitumor, cytotoxic, and antiparasitic activities of clinically used quinones are mainly believed to stem from effects due to redox cycling of their free radical or hydroquinone states, derived from reduction by flavoenzymes, or from covalent modifications of DNA and other cellular nucleophiles (see Refs. 23–25). Reduction of quinones by pyridine nucleotide-disulfide reductases related to TrxR, i.e. glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase, has been studied in significant detail (26–28). The interactions with quinones may also concomitantly inhibit the normal reactions of these enzymes (29). Previously, studies of mammalian TrxR regarding interactions with quinones include the demonstrated reduction of ubiquinone-10, 2-methyl-1,4-naphthoquinone, and alloxan (14, 30). Inactivation of reduced TrxR by antitumor aziridinylbenzoquinones and anthracyclines has also been reported (31).

However, no systematic analysis of the reactions of quinones with mammalian TrxR has yet been performed. Here we examined the interactions of recombinant rat TrxR (32) with a number of structurally diverse quinones, including aziridinylbenzoquinones DZQ, RH1, MeDZQ, BZQ (25), anthracycline daunorubicin (Fig. 1), and a number of model compounds for both partially and fully substituted quinones. We show that significant quinone reduction seems to occur not at the FAD but at the selenolthiol motif, which is unique in comparison to the quinone reduction by non-selenoprotein pyridine nucleotide-disulfide oxidoreductases. Furthermore, we show that cer-
tain specific quinone compounds, e.g. juglone, may bypass this selenothiol motif yet be efficiently reduced by the enzyme. Finally, we show that the four-electron reduced form of TrxR participates in the quinone reduction, and we have determined the previously unknown redox potential of the enzyme.

MATERIALS AND METHODS

Enzymes and Proteins—Recombinant rat TrxR1 was essentially prepared as described (32), with slight methodological improvements to be reported elsewhere. The enzyme was pure as judged by Coomassie-stained SDS-PAGE and had a specific activity of 22 units/mg in the model DTNB assay (9). The enzyme concentration was determined from the absorbance of FAD, ε2₄₅₀ = 11.3 mm⁻¹ cm⁻¹ (8). Chlamydomonas reinhardtii thioredoxin was prepared as described (33), and its concentration was determined using ε₂₄₅₀ = 10.9 mm⁻¹ cm⁻¹ (34).

Reagents—NADPH, NADP⁺, cytochrome c, superoxide dismutase, catalase, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), gold thioglucose, and BCNU were obtained from Sigma and were used without additional purification. Daunorubicin was obtained from Reakhim (Russia); 2-methyl-3-glutathionyl-5-hydroxy-1,4-naphthoquinone was a generous gift of Dr. Karin Olinger (Linköping University, Sweden); 2,5-Diaziridinyl-1,4-benzoquinone (DZQ), 2,5-diaziridinyl-3-hydroxy-4-methyl-1,4-benzoquinone (RH1), 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone (MeDZQ), and BZQ were synthesized according to established procedures (35–37), and their purity was checked by melting point, elemental analysis, and IR and NMR spectra. Other quinones were obtained from Sigma and used without additional purification.

Experimental Procedures—All experiments were carried out in 0.1 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA, at 25 °C. Rapid reaction studies were performed using a DX.17MV stopped-flow spectrophotometer (Applied Photophysics) under aerobic conditions. Steady-state reaction rates were monitored spectrophotometrically, using a Hitachi-557 spectrophotometer. Typically, 100–1000 μM NADPH was used, and the rate of DTNB reduction (0.15–1.5 μM) by TrxR was measured following the increase in absorbance at 412 nm (Δε₁₄₅₀ = 13.6 mm⁻¹ cm⁻¹), considering that one molecule of DTNB (and NADPH) produces two TNB anions (9). Steady-state NADPH-quinone reductase activity of TrxR was monitored following the rate of NADPH oxidation (Δε₅₄₆₀ = 6.2 mm⁻¹ cm⁻¹) using 100 μM NADPH. In separate experiments, 50 μM cytochrome c was added into the reaction mixture, and its quinone-mediated reduction was monitored at 550 nm (Δε₅₅₀ = 20 μM⁻¹ cm⁻¹). The reaction rate was corrected for the background (direct) reduction of cytochrome c by TrxR. In separate experiments, this was found to be 0.33 mol of cytochrome c reduced per s per mol TrxR subunit under the utilized assay conditions. When C. reinhardtii Trx (10–80 μM) was the electron acceptor, reaction rates were monitored by following NADPH oxidation. In the reverse reaction of TrxR, reduction of NADP⁺ (50–500 μM) was monitored at 340 nm in the presence of Trx (10–80 μM) and 5 μM DTT as reducing agent (38). This activity was corrected for the background reaction (direct reduction of NADP⁺ by TrxR in the presence of 8 mM DTT but in the absence of Trx), which was 0.2 mol NADP⁺/s/mol subunit. The kinetic parameters of reactions, i.e., the catalytic constant (kcat), and the bimolecular rate constant (kcat/Km), correspond to the reciprocal intercepts and slopes of the Lineweaver-Burk plots, [E]/v versus 1/[S], where v is reaction rate, [E] and [S] are the enzyme and substrate concentrations, respectively. The kcat/Km corresponds to molecules of NADPH oxidized (or NADP⁺ reduced) by enzyme-active sites, i.e., the dimeric TrxR holoenzyme has twice the activity.

The TrxR inhibition experiments were performed in two ways. In studies of reversible inhibition, substrates and inhibitor were introduced into the spectrophotometer cell first, at which point reaction was started by TrxR addition. The inhibition constants were calculated from Cleland plots, plotting the dependence of reciprocal kcat/Km values at variable substrate versus inhibitor concentration (Ki), or the dependence of plot intercept with y axis versus inhibitor concentration (Ki). In the irreversible inhibition experiments, TrxR (0.6–6.0 μM) was incubated in the presence of NADPH (200 μM) and inhibitor at 25 °C. After the incubation for the indicated period, an aliquot of the reaction mixture was introduced into a spectrophotometer cell (with a 100–200-fold factor of dilution), and the rate of DTNB reduction was monitored in the presence of 50 μM NADPH and 1.5 mM DTNB. Alternatively, quinone reductase activity of TrxR was monitored by following the oxidation rate of 50 μM NADPH in the presence of quinone.

RESULTS

Reactions of TrxR with Disulfide Substrates—Before analysis of quinone reduction catalyzed by the recombinant mammalian TrxR preparation used throughout this study, we probed its reduction of the two model disulfide substrates DTNB and Trx. As reported before (21), at fixed NADPH concentrations (6–60 μM) and varied DTNB (0.15–1.5 mM), the Lineweaver-Burk plots showed a series of parallel lines, thus pointing to a “ping-pong” scheme (data not shown). The same ping-pong patterns were observed using C. reinhardtii Trx instead of DTNB as substrate (oxidant). The catalytic constants (kcat) and the steady-state bimolecular rate constants of oxidative and reductive half-reactions (kcat/Km) as determined here are given in Table I. The kcat values of both reactions were marginally lower than those catalyzed by native purified mammalian TrxR using DTNB (4000 min⁻¹ for dimeric native TrxR, i.e., 33 s⁻¹ per active site) or mammalian (3300 min⁻¹; 27.5 s⁻¹) and E. coli (3000 min⁻¹; 25 s⁻¹) Trx, as summarized from the literature (9). It can be noted that C. reinhardtii Trx with Km = 19 μM was a slightly more efficient oxidant for the recombinant enzyme than E. coli Trx with Km = 35 μM for native mammalian TrxR (9), which in the latter case gives kcat/Km = 0.714 μM⁻¹ s⁻¹ to be compared with 1.06 μM⁻¹ s⁻¹ for C. reinhardtii Trx found here (Table I). We also assessed the reverse reaction of TrxR, i.e., reduction of NADP⁺ by C. reinhardtii reduced Trx utilizing 8 mM DTT as reducing agent. This reaction also followed ping-pong kinetics (data not shown) with a kcat close to that of the forward reaction (see Table I).

By using the data presented in Table I, the redox potential of TrxR can be calculated. According to the Haldane relationship, the ratio of the bimolecular rate constants of forward and reverse reactions gives the equilibrium constant of the reaction (K), which in turn is related to the difference in the standard redox potential of the reactants (ΔE° = 29.5 mV × log K for a two-electron transfer). This relationship, based on the rates of enzyme reactions with NADPH (E° = −0.320 V), has been used for calculating E°₂₀ of yeast (39) and P. falciparum glutathione reductases (40). For Reaction 1, the ratio of kcat/Km for NADPH oxidation and NADP⁺ reduction (Table I) gives K = 8.07 ± 0.5, and E°₂₀ of Trx, = −0.294 ± 0.003 V.

NADPH + TrxROX ↔ NADP⁺ + Trxred

REACTION 1

Alternatively, for Reaction 2, the ratio of kcat/Km for reduced and oxidized Trx (Table I)

Trx-(SH)₂ + TrxROX ↔ Trx-S₂ + Trxred

REACTION 2

gives K = 2.04 ± 0.2. Because the E°₂₀ of C. reinhardtii Trx has been determined to be −0.290 ± 0.01 V (41), this would give
E'0 of TrxR of −0.281 ± 0.01 V, in good agreement with the value obtained using NADPH.

We next analyzed the effect of NADP⁺ on the NADP⁺-dependent reduction of DTNB by TrxR. At fixed concentrations of DTNB and varied NADP⁺, the reaction product NADP⁺ acted as a mixed inhibitor with respect to NADPH, increasing both slopes and intercepts in Lineaweaver-Burk plots (Fig. 2A). The same mixed inhibition pattern was observed using fixed NADPH and varied DTNB concentrations (Fig. 2B). At [NADPH] = 150 μM, the Kᵢ of NADP⁺ determined according to the slopes of Lineaweaver-Burk plots (Kᵢ/slopes) was equal to 270 ± 40 μM, whereas the Kᵢ value determined according to the plot intercepts with the y axis (Kᵢ/intercepts) was equal to 1200 ± 200 μM. This inhibition of TrxR by NADP⁺ may possibly play a physiological role in cases of intracellular NADPH/NADP⁺ ratios, e.g. in hepatocytes upon ethanol intoxication (42).

Quinone Reduction by TrxR—Pyridine nucleotide-disulfide reductases like lipoamide dehydrogenase, glutathione reductase, and trypanothione reductase, as well as low Mₛ-type TrxR of Arabidopsis thaliana, perform mixed single- and two-electron reduction of quinones with reactivity typically increasing with an increase in the single-electron reduction potential (E'1/2) of the quinone substrate (26–28, 38). The E'1/2 values of the quinones examined in the present study are given in Table II.

In reactions with mammalian TrxR, we found that 9,10-phenanthrene quinone and 5-hydroxy-1,4-naphthoquinone (juglone or walnut toxin), which have low redox potentials (compounds 6 and 8, Table II), led to excess NADPH oxidation over quinone reduction, whereas 1,4-benzoquinone, which has a high redox potential (compound 1, Table II), oxidized more or less a stoichiometric amount of NADPH (Fig. 3A). This showed that juglone and 9,10-phenanthrene quinone participated in redox cycling with TrxR. The reduction of these quinones could be coupled to reduction of cytochrome c, added in separate experiments. The juglone- and phenanthrene quinone-mediated cytochrome c reduction rates by TrxR were 2- and 1.5-fold higher than the NADPH oxidation rates, respectively, and both were decreased by 20–25% upon addition of 30 μM superoxide dismutase. This strongly suggested that the reduction of quinones by TrxR was accompanied by an aerobic redox cycling with formation of superoxide. Such reaction could, however, be consistent with both single- and two-electron reduction mechanisms, because the hydroquinone forms of these quinones may either reduce cytochrome c directly (with transient formation of semiquinones), or may rapidly autoxidize with formation of superoxide that in turn reduces cytochrome c (43, 44). Quantitatively, the percentage of single-electron flux may be estimated using 1,4-benzoquinone as substrate, because the hydroquinone form of this quinone cannot reduce cytochrome c at pH ≤ 7.2, whereas the benzoquinone derivative rapidly reduces cytochrome c (k = 1 μM⁻¹ s⁻¹) (45). Making use of this property, we found that the percentage of single-electron flux in the TrxR-catalyzed reduction, expressed as the ratio of the doubled rate of cytochrome c reduction to NADPH oxidation rate at the expense of 1,4-benzoquinone (44), was 6.5 ± 1%. Hence, TrxR reduces 1,4-benzoquinone mainly with a two-electron reduction mechanism.

One should note that the rate of TrxR-catalyzed NADPH oxidation by 1,4-benzoquinone sharply decreased after 15–20 s (Fig. 3A), and the same feature was observed in reactions with other partially substituted benzoquinones (data not shown). In contrast, reactions with juglone (Fig. 3A) or other partially substituted naphthoquinones (data not shown) proceeded at the same or even an increased rate with time. We hypothesized that covalent modification of reduced TrxR by partially substituted quinones, known as potent alkylating agents targeting thiol groups and other nucleophiles (24), may over time affect the activity when the enzyme becomes exposed to quinones. Indeed, incubation of reduced TrxR with 50 μM 1,4-naphthoquinone or its 5-hydroxy- and 5,8-dihydroxy-derivatives even for as little as 15 s resulted in a 50–60% decrease of the DTNB reduction rate. Addition of 50 μM 1,4-benzoquinone or 2,3-dichloro-1,4-naphthoquinone decreased the activity by 80–85%. Superoxide dismutase and catalase (30 μg/ml) could not protect TrxR from this inactivation. In contrast, activity of reduced TrxR was unchanged after incubation with 50 μM of the fully substituted 9,10-phenanthrene quinone for at least 3–4 min. Due to the rapid modification of reduced TrxR by partially substituted quinones, it became important to assess whether TrxR was able to reduce those compounds before modification occurred. We therefore monitored the quinone-mediated cytochrome c reduction using stopped-flow spectrophotometry, which revealed that both juglone and 9,10-phenanthrene quinone reduction began immediately after mixing, demonstrating that both compounds were substrates of the unmodified enzyme (Fig. 3B). The kinetic parameters of juglone reduction determined for the 0–5-s time interval were kᵦ = 4.5 ± 0.2 s⁻¹ and kᵦ/Kᵦ = 1.6 ± 0.1 μM⁻¹ s⁻¹, which was close to the same parameters determined under steady-state conditions (Table II). These results indicated that reduced TrxR could reduce partially substituted quinones before becoming modified and, notably, that the modified enzyme retained the ability to reduce quinones in solution (Fig. 3A). This property resembles the reactivity of mammalian TrxR modified with dinitrohalobenzenes (22), and it may be of significance for the mechanisms of cytotoxicity of quinones, as is further discussed below.

In Table II, the kᵦ and kᵦ/Kᵦ values of the different quinones analyzed here for reduction by mammalian TrxR are...
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### Table II

| No. | Quinone                                      | $E'_1$ $\mu V$ | $k_{cat}$ $s^{-1}$ | $k_{cat}/K_m$ $\mu M^{-1} s^{-1}$ | $\%$ of Trx $^d$ | $\%$ of Trx $^e$ |
|-----|----------------------------------------------|----------------|-------------------|-----------------------------------|-----------------|-----------------|
| 1.  | 1,4-Benzoquinone                             | -0.09          | 4.8 ± 0.5         | 0.39 ± 0.10                       | 24%             | 12%             |
| 2.  | 2-Methyl-1,4-benzoquinone                    | -0.01          | 2.8 ± 0.3         | 0.106 ± 0.011                     | 14%             | 7%              |
| 3.  | 2,3-Dichloro-1,4-naphthoquinone              | -0.035         | 9.6 ± 0.3         | 6.1 ± 0.8                         | 48%             | 57%             |
| 4.  | 2,5-Diaziridinyl-1,4-benzoquinone (DZQ)      | -0.054         | 0.15 ± 0.01       | 0.0055 ± 0.0007                   | 0.7%            | 0.5%            |
| 5.  | 2,5-Dimethoxy-1,4-benzoquinone               | -0.07          | 1.8 ± 0.1         | 0.19 ± 0.01                       | 9%              | 18%             |
| 6.  | 5-Hydroxy-1,4-naphthoquinone (juglone)       | -0.09          | 4.9 ± 0.2         | 2.1 ± 0.1                         | 24%             | 19%             |
| 7.  | 5,8-Dihydroxy-1,4-naphthoquinone             | -0.11          | 7.4 ± 0.2         | 0.23 ± 0.02                       | 37%             | 22%             |
| 8.  | 9,10-Phenanthrene quinone                    | -0.12          | 17.3 ± 0.8        | 1.9 ± 0.2                         | 86%             | 179%            |
| 9.  | 1,4-Naphthoquinone                           | -0.15          | 4.6 ± 0.2         | 0.44 ± 0.03                       | 23%             | 42%             |
| 10. | 2-Methyl-5-hydroxy-1,4-naphthoquinone        | -0.16          | 3.2 ± 0.2         | 0.021 ± 0.002                     | 16%             | 2%              |
| 11. | 2-Methyl-3-glutathionyl-5-hydroxy-1,4-naphthoquinone | -0.16          | 0.11 ± 0.02       | 0.007 ± 0.001                     | 0.5%            | 0.7%            |
| 12. | 2,5-Diaziridinyl-5-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RHI) | -0.18          | 0.13 ± 0.02       | 0.001 ± 0.0001                    | 0.6%            | 0.9%            |
| 13. | 2-Methyl-1,4-naphthoquinone                  | -0.20          | 1.5 ± 0.1         | 0.012 ± 0.002                     | 7.5%            | 1.1%            |
| 14. | 2,5-Dimethyl-3,6-diaziridinyl-1,4-benzoquinone (MeDZQ) | -0.23          | 0.16 ± 0.02       | 0.0044 ± 0.0003                   | 0.8%            | 0.4%            |
| 15. | Tetramethyl-1,4-benzoquinone                 | -0.26          | 0.12 ± 0.01       | 0.0006 ± 0.0005                   | 0.6%            | 0.6%            |
| 16. | Daunorubicin                                  | -0.34          | <0.03             | <0.015%                          | <0.00045        | <0.04%          |
| 17. | 2,5-Di(2-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone (BZQ) | -0.38          | >0.02             | (<0.1%)                          | <0.00025        | (<0.02%)        |
| 18. | 2-Hydroxy-1,4-naphthoquinone                 | -0.41          | 0.04 ± 0.01       | 0.0007 ± 0.0001                   | 0.2%            | 0.7%            |

$^a$ from Refs. 24 and 47.
$^b$ Percent of $k_{cat}$ using C. reinhardtii Trx (see Table I).
$^c$ Percent of $k_{cat}/K_m$ using C. reinhardtii Trx as oxidant (see Table I).
$^d$ $E'_1$ calculated assuming that aziridine and methyl groups may similarly influence the $E'_1$, value, and that the substitution of a methyl group by hydroxymethyl may increase the $E'_1$, by 0.05 V (47).

J. Butler, personal communication.

![Graph A](image1.png)

**Fig. 3.** TrxR-catalyzed reduction of quinones under aerobic conditions. A, oxidation of NADPH (300 μM) by 50 μM 1,4-benzoquinone (curve 1), 50 μM 5-hydroxy-1,4-naphthoquinone (curve 2), and 50 μM 9,10-phenanthrene quinone (curve 3) in the presence of 40 nM TrxR. The time of enzyme addition is indicated with an arrow. B, cytochrome c reduction accompanying quinone reduction by TrxR and NADPH, monitored in a stopped-flow spectrophotometer. The first syringe contained TrxR and cytochrome c, and the second contained NADPH and quinone. Concentrations after mixing were as follows: TrxR, 0.5 μM; cytochrome c, 50 μM; and NADPH 50 μM, using either juglone 50 μM (curve 1) or 9,10-phenanthrene quinone 20 μM (curve 2).

given. It can be noted that several quinones were highly efficient substrates for the enzyme. It should also be noted that although the log $k_{cat}/K_m$ of the quinone reduction somewhat increased with an increase in quinone $E'_1$ (Fig. 4), the correlation was poor ($r^2 = 0.5631$) and much less evident than seen in reduction of these quinones by other pyridine-nucleotide disulfide reductases (26–28, 38).

To probe further the details of the catalysis, we next attempted to identify the redox state(s) of TrxR responsible for quinone reduction. Four-electron reduced (EH$_2$) TrxR has the typical spectrum of the FAD-thiolate charge-transfer complex with $\Delta E_{540} = 2.8$ m$\mu$M$^{-1}$ cm$^{-1}$ (8), whereas the two-electron reduced state (EH$_1$) has about 50% of that absorbance. Evidently, in the EH$_1$ state of TrxR, two electrons are shared between the N-terminal catalytic disulfide and the C-terminal motif having the Cys-Sec couple, which therefore decreases the proportion of enzyme molecules having the FAD-thiolate charge transfer at any given moment. We obtained the EH$_2$ spectrum by addition of 1 eq of NADPH under aerobic conditions, whereas the EH$_4$ state spectrum could be obtained by use of an NADPH regeneration system such as 5.0 mM glucose 6-phosphate and 5 μg/ml glucose-6-phosphate dehydrogenase, or addition of 3–5 equivalents of NADPH (data not shown). In the presence of the NADPH-regenerating system under aerobic conditions, however, we failed to observe complete six-electron reduction of TrxR that would be characterized by a disappearance of the 460–540-nm absorbance (8). By having determined the spectra of the EH$_2$ and EH$_4$ species, we subsequently

![Graph B](image2.png)

**Fig. 4.** Correlation between quinone redox potential and reduction by TrxR. The correlation between $k_{cat}/K_m$ for quinone reduction by TrxR and the quinone single-electron reduction potential ($E'_1$) is shown in this plot, with values and numbering of compounds as given in Table II.
probed the turnover of TrxR upon addition of NADPH and quinone by monitoring the 540-nm absorbance in stopped-flow experiments. Because the solubility of 9,10-phenanthrene quinone, the most efficient quinone oxidant in terms of $k_{cat}$ (Table II) is limited (50 μM) and other fully substituted quinones oxidize TrxR at low rates (Table II), we used juglone in these experiments. The mixing of TrxR with a 5–10-fold excess of NADPH resulted in a rapid rise in the absorbance at 540 nm, which was completed in 20 ms, reaching an amplitude that corresponded to formation of $E_{H4}$ (curve I, Fig. 5A). This signal was stable for at least 200 s due to the low inherent NADPH oxidase activity of TrxR (<0.005 s$^{-1}$). In a separate experiment, juglone was added to the syringe containing NADPH. Simultaneous addition of juglone and NADPH to TrxR resulted in a rapid 540-nm absorbance rise closely reaching the level of $E_{H4}$, whereupon the fall in absorbance suggested the presence of enzyme species cycling between the $E_{H4}$ and $E_{H2}$ states (0.008 ± Δ$A_{540}$ ± 0.025, curves 2 and 3, Fig. 5A) until final oxidation to the fully oxidized state occurred upon NADPH exhaustion. Analyzing the final part of the kinetic curves (Δ$A_{540}$ < 0.025, Fig. 5A) using single-exponential fit yielded a pseudo first-order rate constant ($k_{cat}$), which exhibited a linear dependence on juglone concentration (Fig. 5B). The calculated second-order rate constant, 0.012 ± 0.002 μM$^{-1}$s$^{-1}$, was 2 orders of magnitude lower than the $k_{cat}$/Km of juglone in the steady-state reaction (Table II). This strongly suggests that complete oxidation into $E_{H4}$ species is an unlikely event in reactions of TrxR with juglone in the presence of NADPH and, furthermore, that during reduction of juglone TrxR seems to cycle between the $E_{H4}$ and $E_{H2}$ species, in analogy to its reduction of disulfide substrates.

The TrxR reaction with the most active fully substituted quinone substrate, phenanthrene quinone (Table II), followed a typical ping pong pattern (data not shown) with a $k_{cat}$/Km for NADPH of 2.8 ± 0.2 μM$^{-1}$s$^{-1}$, which was close to the $k_{cat}$/Km value of NADPH for DTNB and Trx reduction (Table I). At [NADPH] = 150 μM, NADP$^+$ acted as a mixed inhibitor with regard to phenanthrene quinone, increasing both the slopes and intercepts in Lineweaver-Burk plots (Fig. 6A) with $K_{i_{(slope)}} = 300 ± 30 μM$ and $K_{i_{(intercept)}} = 1100 ± 200 μM$; this was close to the corresponding parameters obtained for DTNB reduction (see above). This finding further strengthened the notion that mammalian TrxR can reduce quinones with a catalytic mechanism analogous to its reduction of disulfide substrates.

It has been reported that anthracyclines and aziridinylbenzoquinones are competitive inhibitors with regard to DTNB in steady-state reduction by TrxR, and that incubation of reduced TrxR in their presence may lead to covalent binding and irreversible inactivation (31). In view of the rapid TrxR inactivation by partially substituted quinones (see above), we therefore performed more detailed inhibition studies of TrxR using fully substituted quinones, including the anthracycline compound daunorubicin. Phenanthrene quinone was found to inhibit DTNB reduction, with kinetics compatible with a competitive inhibitor having $K_i = 6.3 ± 1.0 μM$ (Fig. 6B). In contrast, the anticancer quinone compounds tetramethyl-1,4-benzoquinone, MeDzQ, RH1, BZQ, and daunorubicin (Fig. 1) were surprisingly weak as competitive inhibitors, displaying $K_i = 400 μM$ in the DTNB assay. After 15 and 30 min incubation of TrxR in the presence of 200 μM NADPH and 50 μM quinone, the TrxR activity had decreased by 50 ± 5 and 70 ± 7% (daunorubicin), 39 ± 4 and 48 ± 5% (MeDzQ), 25 ± 3 and 33 ± 4% (RH1), and 58 ± 5 and 72 ± 5% (BZQ), respectively, using 1.5 mM DTNB as electron acceptor. We also found that a 30-min incubation of TrxR with only NADPH in the absence of quinones resulted in a loss of 26 ± 2% of activity. These activity losses could not be prevented by addition of catalase and superoxide dismutase (30 μg/ml).

**TrxR Inactivation by Gold Thiogluco- and BCNU—**Reduced mammalian TrxR is readily inactivated by close to stoichiometric concentrations of gold compounds such as gold thiogluco- and auranofin, which presumably form an irreversible covalent bond with the Sec residue (21). BCNU, which alkylates thiol groups and presumably Sec, also inhibits the enzyme but at millimolar concentrations (8). In agreement with earlier studies, we found that incubation of reduced TrxR (0.6 μM) with a slight excess of gold thiogluco (1.0 μM) led to a rapid and almost complete loss of activity with regard to reduction of DTNB. We also found that 9,10-phenanthrene quinone reduc-
The results of this work show that quinones in essence can turn mammalian TrxR into a superoxide-producing NADPH oxidase. We found that some quinones were highly efficient substrates for TrxR and could function as competitive inhibitors with respect to other substrates of the enzyme, whereas other quinones were weak substrates and/or inhibitors. Furthermore, separate classes of quinones reacted with TrxR according to different patterns, with their reduction being either inhibited or nearly unaffected by covalent modification of the selenolthiol motif.

Like other related pyridine-nucleotide disulfide oxidoreductases, TrxR could perform mixed single- and two-electron reduction of quinones. The single-electron flux as determined here, 6.5%, was intermediate between the corresponding values for glutathione reductase (3.6% (27)) and trypanothione reductase (40% (26)). However, quinones were significantly more efficient substrates (oxidants) for TrxR than for the other enzymes, because \(k_{cat}/K_{m}\) values (Table II) were about 1 order of magnitude higher than in the corresponding reactions catalyzed by glutathione reductase or trypanothione reductase (26, 27). Notably, some quinones analyzed here were as efficient as thioredoxin or DTNB as substrates for TrxR (cf. Tables I and II).

We find unequivocal evidence that the \(EH_4\) state of TrxR, which performs the reduction of disulfide substrates (3, 5, 6), is also responsible for the major part of quinone reduction. (i) The transient 540 nm absorbance data (Fig. 5A) demonstrates TrxR cycling between the \(EH_1\) and \(EH_2\) states in juglone reduction. Only after NADPH exhaustion did the oxidation of \(EH_2\) to \(E_{ox}\) take place, and it occurred with a rate constant 2 orders of magnitude lower than the juglone \(k_{cat}/K_{m}\) value in steady-state reduction (Fig. 5B and Table II). (ii) The NADPH \(k_{cat}/K_{m}\) for the reduction of phenanthrene quinone was close to that seen in the reduction of both DTNB and Trx. (iii) NADPH− inhibited phenanthrene quinone reduction in a similar manner and with similar \(K_I\) values as it inhibited DTNB reduction (Figs. 2B and 6A). Thus, based on these findings, we feel secure to conclude that the same redox state of TrxR was responsible for the major part of reduction of DTNB, Trx, and with similar \(K_I\) values as it inhibited DTNB reduction (Fig. 5B and Table II).
however, that the reaction of quinones with glutathione reductase, trypanothione reductase, and lipoamide dehydrogenase mainly involves the reduced flavin and not the active site dithiol (26, 27), as is true for the $E_{H4}$ state of mammalian TrxR containing the FADH$_2$ instead of a dithiol motif due to the high potential of single-electron oxidation of thiols, which is usually $\pm 1.0$ V unless affected by neighboring residues (47). Naturally, we had expected that quinones would have accepted electrons mainly from the equilibrium form of the $E_{H4}$ state of TrxR containing the FADH$_2$. Unexpectedly, however, TrxR treatment with gold thioglucose almost completely suppressed reduction of phenanthrene quinone as well as of DTNB (Fig. 7A). Even more surprising was the finding that juglone reduction was little affected under the same conditions, whereas both DTNB and juglone reductions by TrxR were inhibited by BCNU, albeit somewhat less in the case of juglone. Collectively, these results demonstrate that juglone is strikingly more efficient and also somewhat less in the case of juglone. Reduction of quinones by mammalian TrxR proceeded with one-electron transfer mechanisms. This proposed scheme for reactions of quinones with mammalian TrxR is based both upon the results presented herein and on the previously characterized reactions of mammalian TrxR with natural substrates (5, 8) and dinitrohalobenzenes (22). See text for further details.

![Proposed model for reduction of quinones by mammalian TrxR.](image)

**Fig. 8.** Proposed model for reduction of quinones by mammalian TrxR. The FAD of the fully oxidized $E_{ox}$ form of mammalian TrxR (denoted species 1 in this figure) is reduced by NADPH to FADH$_2$ in species 2. The electrons may then be transferred to the N-terminal disulfide to form a flavin-thiolate charge transfer complex (species 3) and subsequently to the C-terminal selenenylsulfide of the other subunit to form a selenolthiol motif (species 4). Collectively, enzyme species 2–4 form the two-electron reduced $E_{H2}$ state of TrxR. A second molecule of NADPH may subsequently reduce the enzyme to the $E_{H4}$ state (species 5 and 6). The selenolthiol motif reduces typical substrates of the enzyme, such as Trx and DTNB, thereby forming a selenenylsulfide and converting TrxR from the enzyme-bound FAD. These divergent properties of different quinones should probably involve different accessibility of the individual quinones to the FAD. Our proposed mechanism for quinone reduction by mammalian TrxR is shown in Fig. 8 and is explained further in the figure legend. The parallel participation of FADH$_2$ and the reduced selenolthiol in quinone reduction by TrxR should explain the poorly expressed dependence of $k_{cat}/K_m$ versus $E^1_7$ of quinones (Fig. 4), which clearly contrasts with the parabolic plots of log $k_{cat}/K_m$ versus $E^1_7$ characteristic for the FADH$_2$-mediated quinone reduction by lipoamide dehydrogenase, glutathione reductase, as well as trypanothione reductase (26–28).

Our data on the mixed inhibition exerted by NADP$^+$ taken together with the high rate of the reverse reaction of TrxR may provide valuable information on the thermodynamic properties of human TrxR. To our knowledge, this is the first report showing that forward and reverse reactions of TrxR may proceed at similar rates. Because NADP(H) and Trx (or DTNB) interact with spatially separated domains of TrxR (7), the enzyme shares the “hybrid ping-pong” mechanism seen in glutathione reductase (48, 49). In accordance with such a mechanism, one would expect a competitive inhibition of NADP$^+$ toward NADPH and an uncompetitive inhibition toward disulfide substrates (48), which contradicts the findings shown in Fig. 2, A and B. In contrast, the reverse reaction of glutathione reductase, i.e. the reduction of NADP$^+$ by GSH, is only $\leq 10\%$ of the forward reaction rate (39). The mixed type NADP$^+$ inhibition was probably observed due to the fast TrxR-
catalyzed reoxidation by NADP\(^+\) (Fig. 2, A and B) resulting from competition between NADP\(^+\) and disulfide oxidant for the same redox state of enzyme in the reoxidation reaction (39, 48, 50). The redox potential of the \(E_H/E_H^+\) couple of TrxR determined here to \(-0.294\) V was clearly more negative than the redox potential of the \(E_D/E_D^+\) couple of yeast glutathione reductase, which is \(-0.255\) V (39), and slightly more negative than the \(E_D/E_H^+\) couple of pig heart lipoamide dehydrogenase, which is \(-0.280\) V (51).

How important are the reactions of quinones with mammalian TrxR as a mechanism explaining their biological effects? Naturally, the answer to that question should be complex and dependent on quinone species as well as TrxR expression and function in a particular cell. Nonetheless we would like to emphasize some features that may be of importance. Because quinones, as substrates for TrxR, may be reduced in both one- and two-electron redox cycling reactions producing superoxide, this may lead to increased oxidative stress. This may be especially true for quinones with relatively stable semiquinone states and thereby hydroquinone forms that rapidly autoxidize, e.g. hydroxy-1,4-naphthoquinones and phenanthrene quinone (24, 43, 44). However, quinones with an unstable semiquinone state may also act as antioxidants when regenerated by TrxR, as in reduction of cytosolic ubiquinone that yields ubiquinol with antioxidant properties (14). Whether the redox cycling of a quinone compound with TrxR leads to antioxidant effects or an increased oxidative stress may therefore not be immediately concluded. However, a second effect of quinone interference with the antioxidant functions of human TrxR would derive from their different degrees of inhibition of reduction of other disulfide substrates, notably Trx and following that also the peroxiredoxins. This could contribute to an oxidative stress as part of the cytotoxic effects displayed by many quinone compounds. Importantly, we showed here that partially substituted quinones may rapidly modify reduced TrxR, causing deviations from the expected steady-state course of the NADPH: quinone reductase reactions and thereby suppressing the disulfide reduction capacity. In view of the rapid modification of the selenolthiol motif by alkylating agents such as 1,3-dinitro-4-chlorobenzene (22), this C-terminal motif is the likely candidate for modification by quinones as well. Further studies of this mechanism for quinone cytotoxicity should certainly be carried out at a cellular level.

We also need to discuss the possible importance of TrxR inhibition and inactivation by fully substituted quinones, although this type of inhibition cannot directly involve irreversible covalent modification. The efficient competitive inhibition of TrxR by phenanthrene quinone with regard to DTNB (Fig. 6B) should most probably be due to efficient function of the quinone as an alternative oxidant (Table II), thereby diverting electron flux from DTNB. That interpretation is strengthened by the fact that the \(K_m\) value of phenanthrene quinone was close to its \(K_m^\text{ox}\) value in NADPH oxidation. Such inhibition might be important in the cytotoxicity of the phenanthrene quinone compound, which is a component of exhaust gases and an important environmental pollutant (24). In relation to such inhibition, we find it important to note that we found the anticancer aziridinylbenzoquinones MeDQz, RH1, and BQz (23, 25) as well as daunorubicin (Fig. 1) to be almost inactive as direct competitive inhibitors, in contrast to the efficient inhibition by these analogues of purified mammalian TrxR reported previously (31). This discrepancy, also observed by others (10), suggests that alternative mechanisms than direct TrxR inhibition may be of importance for the anticancer effects of these quinones (52). We did note a time-dependent inactivation of reduced TrxR by aziridinylbenzoquinones and daunorubicin, but also this property was less efficient than what was reported earlier (31). Because these quinones are unable to react with cysteine or selenocysteine directly, the inactivation should have been due to alkylating reactions of their reduced products, the quinomethide of daunorubicin or aziridinyl-substituted hydroquinones (23, 25). These may be formed during the TrxR-catalyzed reduction. Thus, daunorubicin treatment may with time inhibit TrxR through formation of other metabolites, but daunorubicin does not directly inhibit this enzyme.

To conclude, we have here characterized the reactions of quinones with mammalian TrxR and found unique features in these reactions not shared by other related pyridine nucleotide-disulfide oxidoreductases. These reactions involve the selenocysteine residue of TrxR and should profoundly interfere with the important physiological functions that TrxR plays in mammalian cells. The data presented here could therefore provide a molecular basis for further studies aiming at elucidating the possible underlying reactions mediating biological effects of quinone compounds.

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