

EFFECT OF GLUTATHIONE ON THE REDOX TRANSITIONS OF NAPHTHOHYDROQUINONE DERIVATIVES FORMED DURING DT-DIAPHORASE CATALYSIS

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The oxidation of GSH coupled to the redox transitions of 1,4-naphthoquinone derivatives during *DT*-diaphorase catalysis was examined. The quinones studied included 1,4-naphthoquinone and its dimethoxy- and hydroxy derivatives and were selected according to their different ability to undergo nucleophilic addition with GSH and the dual effect of superoxide dismutase on hydroquinone autoxidation.

GSH was oxidized to GSSG during the redox transitions of the above quinones, regardless of their substitution pattern. This effect was accompanied by an increase of total O₂ consumption, indicating the ability of GSH to support quinone redox cycling. The values for the relationship $[O_2]_{consumed}/[GSSG]_{formed}$ were, with every quinone examined, above unity, thus pointing to the occurrence of autoxidation reactions other than those involved during GSSG formation.

These results are discussed in terms of the functional group chemistry of the quinones and the thermodynamic properties of the reactions involved in the reduction of the semi- to the hydro-quinone by GSH.

KEY WORDS: Naphthoquinones, autoxidation, superoxide dismutase, glutathione, *DT*-diaphorase.

INTRODUCTION

Electron-transfer reactions as well as the generation and reactivity of free radicals in biological systems are controlled by thermodynamic, kinetic, and environmental factors.^{1,2} The redox chemistry of hydro- and semi-quinones is to a large extent determined by the physicochemical properties of the molecule, such as the reduction potential and the influence on it of the substitution pattern, and by environmental factors, such as pH, solvent cage, solvation energy, and medium polarity. Kinetic factors can allow a reaction – otherwise thermodynamically unlikely – to proceed by exerting a modification on its equilibrium.² Energetically unfavourable reactions,

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which proceed to completion by the removal of reaction products, are exemplified by the acceleration of semiquinone autoxidation by superoxide dismutase^{2,3} and the oxidation of thiols coupled to the reduction of *N*-acetyl-*p*-benzoquinone imine,⁴ naphthoxyl-,⁵ aminopyrine-,² and alloxan^{6,7} radicals, and during the course of the peroxidase-catalyzed oxidation of several drugs.^{8,9} At the cellular level, quantification of some of the products of the reaction between menadione and GSH pointed out that the major route of removal of GSH in this reaction was *via* oxidative processes.¹⁰

Two-electron transfers to quinonoid compounds are brought forward by *DT*-diaphorase catalysis and nucleophilic addition, e.g., reaction with sulfur nucleophiles such as GSH. The former activity is formally understood in terms of a hydride transfer from the flavoprotein to a two-electron acceptor,¹¹ whereas the reaction between quinonoid compounds and nucleophiles is a 1,4-reductive addition of the Michael type.¹² Hydroquinones — formed by both processes — undergo one-electron oxidations to the semiquinone form, transition that can be accomplished by way of autoxidation, oxidation by O₂⁻, disproportionation, cross-oxidation and oxidation by metals. The contribution of each individual reaction to the overall process is determined by the factors cited above. The relative participation of redox cycling and thiol nucleophilic addition to naphthoquinone toxicity has been recently discussed¹³ as well as the thiol reactivity towards several methyl-substituted naphthoquinone bioreductive alkylating agents¹⁴ and the autoxidation associated with GSH reductive addition.^{15,16}

This study addresses the question of the role of GSH on the redox transitions of naphthohydroquinones following the two-electron reduction of the corresponding quinones by *DT*-diaphorase. In order to elucidate the radical chain reactions involved in quinone/GSH interactions, three compounds from the naphthoquinone series (1,4-naphthoquinone and its methoxy — and aromatic-ring, — OH-substituted derivatives), which can serve as substrates for *DT*-diaphorase, were selected based on (a) their different abilities to undergo GSH reductive addition and (b) the dual effect of superoxide dismutase on the autoxidation of the hydroquinone formed.

MATERIALS AND METHODS

Chemicals and biochemicals

1,4-Naphthoquinone, 5-hydroxy-1,4-naphthoquinone, H₂O₂ and *p*-hydroxyphenylacetic acid were from Aldrich-Chemie (Steinheim, FRG). Cu, Zn-superoxide dismutase, NADPH, horseradish peroxidase, GSH, and GSSG were from Boehringer (Mannheim, FRG). Mn-superoxide dismutase was from Sigma Chemical Co. (St. Louis, Mo, USA). 2,3-Dimethoxy-1,4-naphthoquinone was a gift from Prof. G.M. Cohen (Department of Pharmacology, University of London, UK). *DT*-Diaphorase was purified from rat liver¹⁷ and had a specific activity of 2100 μmoles NADH_{oxidized} × min⁻¹ × mg protein⁻¹, measured with 2-methyl-1,4-naphthoquinone as electron acceptor.

Assay conditions

The standard incubation mixture consisted of 20 μM quinone compound, 200 μM NADPH, and 9.1- or 27.3 ng *DT*-diaphorase × ml⁻¹ in air-saturated 0.25-M sucrose/0.1-M potassium phosphate buffer, pH 7.5. The reaction was started upon addition of *DT*-diaphorase. Assay temperature was 37°C.

Spectrophotometric assays

NADPH oxidation was followed at 340–400 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) with a dual-wavelength, double-beam spectrophotometer (model UV-3000; Shimadzu Corporation, Kyoto, Japan). H_2O_2 formation was measured fluorometrically coupled to *p*-hydroxyphenylacetate dimerization ($\lambda_{\text{excitation}} = 315 \text{ nm}$; $\lambda_{\text{emission}} = 410 \text{ nm}$).¹⁸ GSH was measured by spectrophotometric¹⁹ – and HPLC²⁰ methods.

HPLC assays

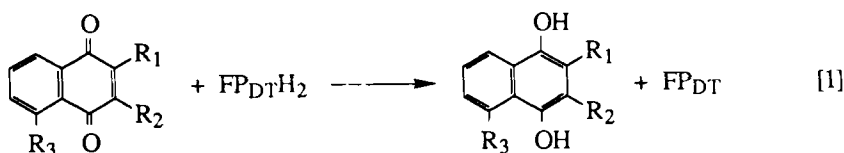
Glutathione (GSH) and glutathione disulfide (GSSG) were measured by dinitrophenol derivatives²⁰ using UV detection ($\lambda = 365 \text{ nm}$; absorbance detector model 441; Millipore AB, Waters Chromatography Division, Stockholm, Sweden) after separation by reverse-phase, ion-exchange HPLC (automatic sample injection system model WISPTM 710B; Millipore AB, Waters Chromatography Division, Stockholm, Sweden) connected to a solvent delivery system (model 8700; Spectra physics). Flow rate was $1.5 \text{ ml} \times \text{min}^{-1}$ and injection volume of the derivatization sample was $100 \mu\text{l}$.

Oxygen uptake

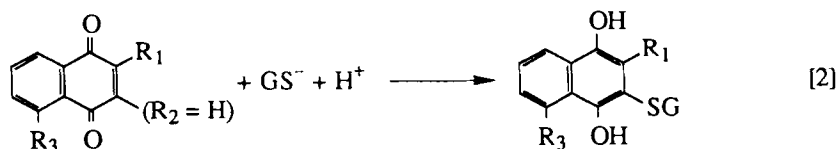
Oxygen consumption was measured with a Clark oxygen electrode (model 53; Yellow Spring Instruments).

RESULTS

In order to evaluate the various redox reactions of GSH during autoxidation of hydroquinones formed during *DT*-diaphorase catalysis (reaction 1), three naphthoquinones- 2,3-dimethoxy-1,4-naphthoquinone, 1,4-naphthoquinone, and



5-hydroxy-1,4-naphthoquinone- were selected according to their different ability to undergo nucleophilic addition reactions with GSH (reaction 2) and the dual effect of superoxide dismutase on the autoxidation of the hydroquinones generated during *DT*-diaphorase catalysis.

*2,3-Dimethoxy-1,4-naphthoquinone*

2,3-Dimethoxy-1,4-naphthoquinone is reduced efficiently by *DT*-diaphorase²¹ and the autoxidation of its hydroquinone form is inhibited by superoxide dismutase.²² The

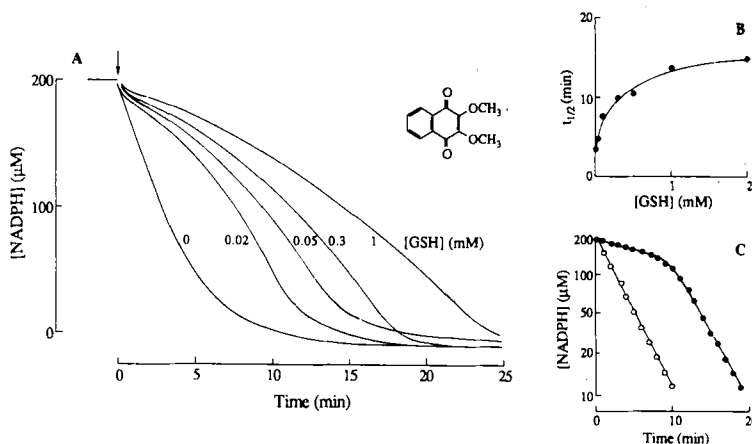


FIGURE 1 Effect of GSH on NADPH oxidation during 2,3-dimethoxy-1,4-naphthoquinone reduction by *DT*-diaphorase. (A) Time course of NADPH oxidation during *DT*-diaphorase-catalyzed reduction of 2,3-dimethoxy-1,4-naphthoquinone in the absence and presence of GSH. Assay conditions: 20 μM 2,3-dimethoxy-1,4-naphthoquinone and 200 μM NADPH in 0.25-M sucrose/0.1-M potassium phosphate buffer, pH 7.5, were supplemented with 27.3 ng *DT*-diaphorase $\times \text{ml}^{-1}$ to initiate the reaction (indicated by the arrow). Numbers beside the traces indicate GSH concentration. (B) Dependence of half-time ($t_{1/2}$) (time required to oxidize NADPH to half-maximal value) on GSH concentration. (C) Semilogarithmic plot of [NADPH] oxidation versus time obtained from traces as in (A) in the absence (\circ) and presence (\bullet) of 0.3 mM GSH.

electron donating properties of the $-\text{OCH}_3$ substituents at R_1 and R_2 , however, prevent the reductive addition of sulfur nucleophiles,¹² such as GSH.

Figure 1A shows the time course of NADPH oxidation coupled to the reduction and redox cycling of 2,3-dimethoxy-1,4-naphthoquinone during *DT*-diaphorase catalysis in the absence and presence of GSH. In the former instance, NADPH oxidation proceeded at a initial rate of $28.9 \pm 1.4 \mu\text{M} \times \text{min}^{-1}$, and the semilogarithmic plot of [NADPH] versus time (Figure 1C) indicated a monophasic relationship involving a single component with a slope of $31.6 \mu\text{M} \times \text{min}^{-1}$. In the latter instance, the rate of NADPH oxidation showed a kinetic lag, the duration of which was dependent on GSH concentration (Figure 1A): half-maximal effect was obtained with 180–200 μM GSH (Figure 1B). Three components could be distinguished in the semi-logarithmic plot of [NADPH] versus time (Figure 1C) in the presence of GSH: a first slow phase which decayed with a slope of $6.6 \mu\text{M} \times \text{min}^{-1}$ (corresponding to the kinetic lag), followed by a transition phase which progressed to a rate of $\sim 32 \mu\text{M} \times \text{min}^{-1}$, similar to that observed for reactions in the absence of the thiol.

Autoxidation is more accurately evaluated in terms of H_2O_2 production than O_2 uptake, for the former reflects the fraction of O_2 molecules reduced during the autoxidation process. Addition of catalase to the reaction mixture in the O_2 electrode cell led to recovery of about 50% of the O_2 consumed. Unfortunately, the spectrofluorimetric method utilized¹⁸ led to an underestimation of H_2O_2 apparently because free GSH in the reaction mixture could compete efficiently with the fluorescent dye for peroxidase compound I. Figure 2A shows that O_2 consumption following the two-electron enzymic reduction of the dimethoxy-naphthoquinone proceeded at a rate of $28 \pm 2.1 \mu\text{M} \times \text{min}^{-1}$. The effect of GSH on O_2 uptake was twofold: [a] it slowed

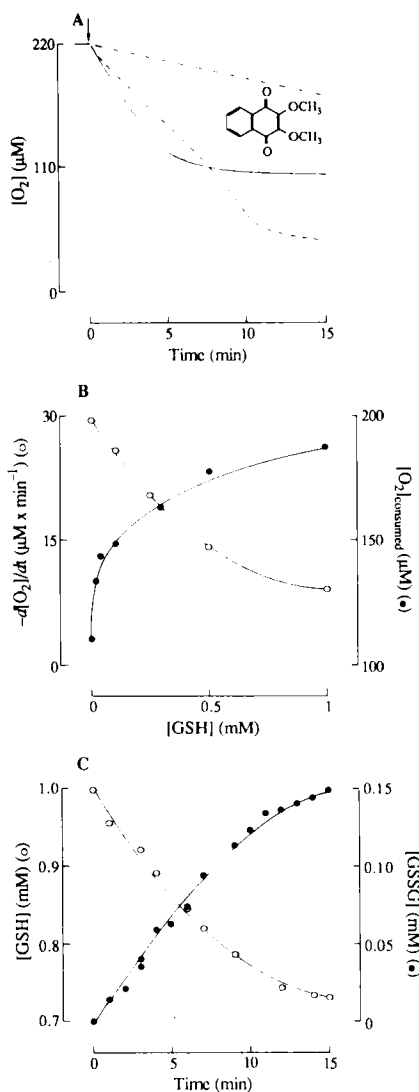
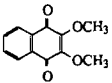




FIGURE 2 Effect of GSH and superoxide dismutase on the autoxidation of 2,3-dimethoxy-1,4-naphthohydroquinone formed during *DT*-diaphorase catalysis. **(A)** Time course of O_2 uptake during the autoxidation of 2,3-dimethoxy-1,4-naphthohydroquinone. Assay conditions as in Figure 1A.: (—) control; (---) plus 0.5 mM GSH; (-·-·) plus 0.5 mM GSH + 1 μM Mn-superoxide dismutase. **(B)** Effect of GSH concentration on the rate of O_2 uptake (○) and total O_2 consumed (●) during the autoxidation of 2,3-dimethoxy-1,4-naphthohydroquinone. **(C)** Time course of GSH consumption and GSSG formation coupled to the redox transitions of 2,3-dimethoxy-1,4-naphthohydroquinone during *DT*-diaphorase catalysis. Assay conditions as in Figure 1A in the presence of 1 mM GSH.

TABLE I

Effect of GSH and superoxide dismutase on the autoxidation of the hydroquinone forms of 1,4-naphthoquinone and its methoxy- and hydroxy derivatives during *DT*-diaphorase catalysis. Assay conditions as in Figures 1–4. When present, concentrations of GSH and SOD (Cu,Zn- or Mn) were 1 mM and 1 μ M, respectively. Values for NADPH oxidation and O₂ consumption were calculated from linear relationships against *DT*-diaphorase concentration ($n = 4$).

			
Redox transitions			
<i>DT</i> -diaphorase catalysis	yes	yes	yes
GSH-reductive addition	no	yes	yes
Effect of SOD on QH ₂ autoxidation	–	–	+
Reduction potentials			
		mV	
E(Q/Q ^{•-})	– 183	– 140	– 93
E(Q ^{•-} /Q ²⁻)	+ 27*	+ 270	+ 157§
Autoxidation			
		$\mu\text{M} \times \text{min}^{-1}$	
$-d[\text{NADPH}]/dt$			
Control	28.9 \pm 1.4	9.3 \pm 0.4	51.3 \pm 4.1
+ GSH	6.9 \pm 0.3	5.5 \pm 0.3	23.2 \pm 2.1
+ GSH + SOD ^o	2.3 \pm 0.1	2.4 \pm 0.1	21.0 \pm 0.8
$-d[\text{O}_2]/dt$			
Control	28.1 \pm 2.1	2.3 \pm 0.4	54.5 \pm 1.3
+ GSH	7.5 \pm 0.3	13.8 \pm 1.9	36.6 \pm 0.8
+ GSH + SOD ^o	3.5 \pm 0.3	4.8 \pm 1.4	35.2 \pm 1.8
$[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$			
+ GSH	1.24	1.28	1.31
+ GSH + SOD ^o	1.26	1.22	1.17

Symbols (+) and (–) indicate enhancement and inhibition of hydroquinone autoxidation by superoxide dismutase, respectively (ref. 22). Reduction potential values are from refs. 30–33. *Calculated from E_{1/2} values from ref. 21. §From ref. 33 (at pH 3). ^oThe extent of inhibition by Cu,Zn-SOD or Mn-SOD was the same.

the rate of O₂ consumption (Figure 2A, B), effect which — on line with that observed with NADPH oxidation — was expressed as a lag phase; [b] increasing concentrations of GSH (over the range studied, 20–1000 μ M) enhanced the total amount of O₂ consumed (Figure 2B), thus indicating the occurrence of a redox cycling process, albeit at slower rates. In the presence of both GSH (1 mM) and superoxide dismutase (2 μ M) of either the Mn- or Cu, Zn-containing enzyme) (Figure 2A), O₂ consumption was strongly inhibited (3.5 \pm 0.3 $\mu\text{M} \times \text{min}^{-1}$).

The redox transitions of 2,3-dimethoxy-1,4-naphthoquinone during *DT*-diaphorase catalysis were coupled to GSH oxidation to its disulfide (Figure 2C; $+d[\text{GSSG}]/dt = 14.1 \mu\text{M} \times \text{min}^{-1}$). At a concentration of 1 mM GSH and after 20-min incubation about 152 \pm 23 μM GSSG was formed and 188 \pm 14 μM O₂ was consumed, thus yielding a value of 1.24 for the relationship $[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$.

Comparison of the integral of the time courses of O₂ consumption (Figure 2A) and GSSG formation (Figure 2C) showed that the kinetic lag observed during the former process was accompanied by an initial high rate of GSSG formation. Superoxide dismutase (either the Mn- or the Cu,Zn-containing enzyme) inhibited the autoxidation of the dimethoxy-naphthohydroquinone (Figure 2A, Table I) by suppressing O₂^{•-}

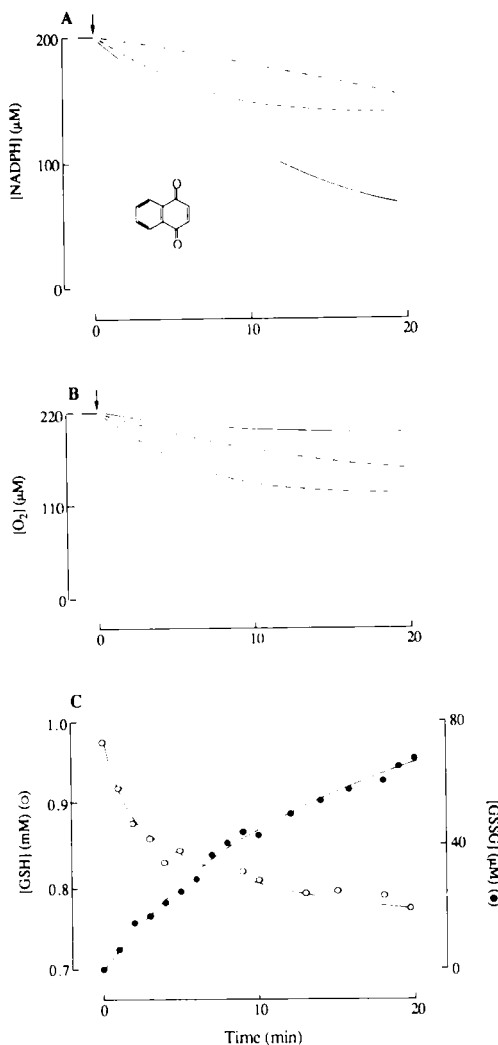


FIGURE 3 Effect of GSH and superoxide dismutase on the autoxidation of 1,4-naphthoquinone formed during *DT*-diaphorase catalysis. Assay conditions: 20 μM 1,4-naphthoquinone and 200 μM NADPH in 0.25-M sucrose/0.1-M potassium phosphate buffer, pH 7.5, were supplemented with 9.1 ng *DT*-diaphorase $\times \text{ml}^{-1}$ to initiate the reaction (indicated by the arrow). (A) and (B) Time courses of NADPH oxidation and O₂ uptake, respectively. (—), control; (---) in the presence of 1 mM GSH; (-·-·-), in the presence of 1 mM GSH plus 1 μM Mn-superoxide dismutase. (C) Time course of GSH consumption and GSSG formation. Assay conditions as above in the presence of 1 mM GSH.

dependent chain reactions, regardless whether O₂⁻ originated from either semiquinone autoxidation or thiol-dependent processes (see below).

1,4-Naphthoquinone

1,4-Naphthoquinone is reduced efficiently by *DT*-diaphorase²¹ and the slow autoxida-

tion of the hydroquinone product is inhibited by superoxide dismutase.^{22,23} 1,4-Naphthoquinone undergoes rapid nucleophilic addition with GSH with formation of a glutathionyl-naphthohydroquinone conjugate,²⁴ which participates in one-electron transfer reactions with the formation of a glutathionyl-naphthosemiquinone intermediate.^{25,26}

The redox transitions of naphthohydroquinone formed during *DT*-diaphorase catalysis are summarized in Figure 3, where the concentration of the enzyme (within the linear range in the plots of $-d[\text{NADPH}]/dt$ versus [*DT*-diaphorase]) was 3-fold lower ($9.1 \text{ ng} \times \text{ml}^{-1}$) than that used with the other naphthoquinones. NADPH (present in 10 molar excess over the quinone) was completely oxidized (initial rate: $9.3 \pm 0.4 \mu\text{M} \times \text{min}^{-1}$) during enzymic catalysis of naphthoquinone (Figure 3A), effect explained in terms of a redox cycling involving the one-electron transitions of the hydroquinone: comproportionation and autoxidation. The latter process contributed by about 24% to total NADPH oxidation, as indicated by measurements of O_2 uptake ($2.3 \pm 0.4 \mu\text{M} \times \text{min}^{-1}$; Figure 3B).

The effect of GSH on the above redox transitions can be summarized as follows:

- 1) The thiol inhibited the rate of NADPH oxidation by about 41% ($5.5 \pm 0.3 \mu\text{M} \times \text{min}^{-1}$) (Figure 3A and Table I) and the time course did not show a kinetic lag as that observed with the dimethoxy derivative (Figure 1A, C).
- 2) O_2 uptake was enhanced by GSH ($13.8 \pm 1.9 \mu\text{M} \times \text{min}^{-1}$; Figure 3B), effect which revealed the contribution to autoxidation of reactions other than those linked to NADPH oxidation.
- 3) The redox transitions of the unsubstituted naphthoquinone during *DT*-diaphorase catalysis were coupled to GSH oxidation ($+d[\text{GSSG}]/dt = 4.8 \mu\text{M} \times \text{min}^{-1}$, for the conditions in Figure 3C). The value for the relationship $[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$ was 1.28.
- 4) The rate of GSH consumption was higher than that of GSSG formation at early incubation times (Figure 3C). This can be explained in terms of two processes contributing to GSH consumption: on the one hand, the thiol nucleophilic addition to the quinone (reaction 2) and, on the other, thiol oxidation (see below).
- 5) Mn- or Cu,Zn-superoxide dismutase inhibited by 55–65% the NADPH oxidation (Figure 3A; Table I) and O_2 consumption (Figure 3B; Table I) linked to the redox transitions of GSH. The total amount of GSSG formed was slightly decreased by superoxide dismutase, thus yielding a value of 1.22 for the relationship $[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$.

5-Hydroxy-1,4-naphthoquinone

5-Hydroxy-1,4-naphthoquinone or juglone is — alike the other two quinones — reduced by *DT*-diaphorase²¹ but the autoxidation of the hydroquinone product is enhanced by superoxide dismutase,²² as also observed with other aromatic-ring, hydroxy-substituted naphthoquinones.²⁷ 5-Hydroxy-1,4-naphthoquinone undergoes rapid nucleophilic addition with GSH,²¹ and the —SG substituent is situated at C_3 , in position α to the carbonyl group adjacent to the —OH substituent (R_3) in the aromatic ring.²⁸ Similar to the case of glutathionyl-1,4-naphthoquinone, the thioether derivative of 5-hydroxy-1,4-naphthoquinone yields a glutathionyl-semiquinone intermediate, presumably formed through cross-oxidation reactions.²⁵

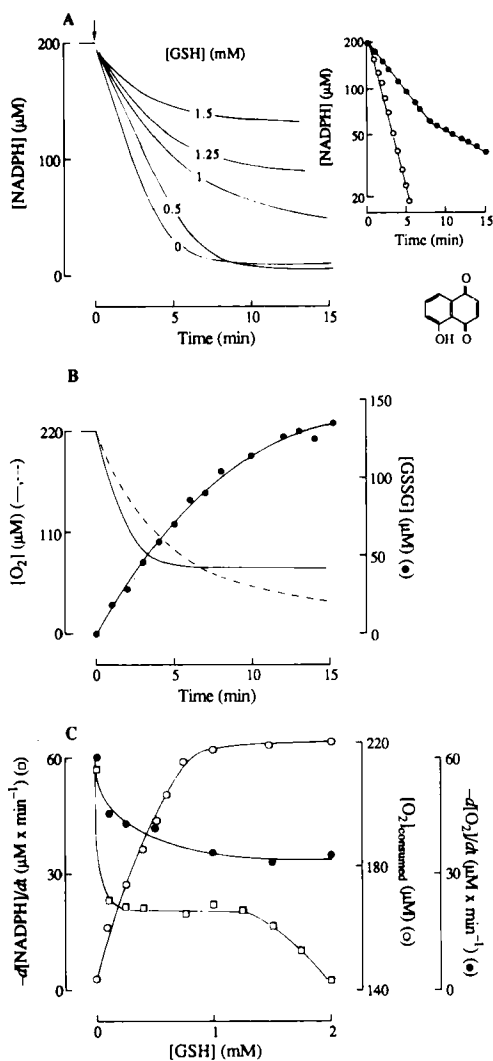


FIGURE 4 Effect of GSH on the redox transitions of 5-hydroxy-1,4-naphthoquinone during *DT*-diaphorase catalysis. **(A)** Effect of GSH on the time course of NADPH oxidation during the reduction of 5-hydroxy-1,4-naphthoquinone by *DT*-diaphorase. Assay conditions: 20 μM 5-hydroxy-1,4-naphthoquinone and 200 μM NADPH in 0.25-M sucrose/0.1-M potassium phosphate buffer, pH 7.5, were supplemented with 27.3 ng *DT*-diaphorase $\times \text{ml}^{-1}$ to initiate the reaction (indicated by the arrow). Numbers beside the traces indicate different concentrations of GSH. Insert: Semilogarithmic plot of NADPH oxidation versus time from data in Figure 4A in the absence (\circ) and presence (\bullet) of 1 mM GSH. **(B)** Time course of O₂ consumption and GSSG formation: (—) and (---) O₂ uptake in the absence and presence of 1 mM GSH, respectively. Assay conditions as in Figure 4A in the presence of 1 mM GSH. **(C)** Dependence of the rate of NADPH oxidation and O₂ consumption and total O₂ consumed on GSH concentration. Assay conditions as in Figure 4A.

At the [GSH]/[5-hydroxy-naphthoquinone] ratios used in this study (ranging $5 \Rightarrow 400$), the GSH reductive addition to the quinone (reaction 2) takes place rapidly with formation of a glutathione conjugate. The latter is reduced by *DT*-diaphorase at a slower (2.9-fold) rate than the parent compound.²¹ The results shown below correspond to the effect of GSH on the redox transitions of 3-glutathionyl-5-hydroxy-1,4-naphthoquinone during *DT*-diaphorase catalysis (Figure 4).

In the absence of GSH, NADPH was oxidized at a rate of $51.3 \pm 4.1 \mu\text{M} \times \text{min}^{-1}$ (Figure 4A), this being reflected as a single component in the semilogarithmic plot (Figure 4A, insert). In the presence of GSH, the semilogarithmic plot of [NADPH] *versus* time showed a biphasic response: a first phase with a slope of $17.1 \mu\text{M} \times \text{min}^{-1}$ and the second one with a slope of $4 \mu\text{M} \times \text{min}^{-1}$. The inhibition of NADPH oxidation was dependent on GSH concentration: 42% inhibition was observed between 50 μM and 1 mM GSH, whereas above the latter value inhibition increased up to 90% (2 mM GSH) (Figure 4C).

The O_2 uptake accompanying the *DT*-diaphorase catalysis of 5-hydroxy-1,4-naphthoquinone ($54.4 \pm 1.3 \mu\text{M} \times \text{min}^{-1}$) was inhibited by GSH in a concentration-dependent manner, whereas total O_2 consumed was 1.55-fold enhanced (Figure 4C). It is worth noting that the rates of O_2 uptake shown in Figure 4B, C and Table I are higher than the corresponding rates of NADPH oxidation. This was expected since three individual reactions contribute to O_2 consumption in the presence of the thiol and only one (the enzymic catalysis) is dependent on NADPH: firstly, the autoxidation subsequent to the GSH nucleophilic addition to the hydroxyquinone; secondly, the O_2 consumption coupled to the thiol redox transitions, and lastly, the autoxidation following the *DT*-diaphorase catalysis of the glutathionyl-hydroxyquinone. GSSG formation proceeded at a rate of $19.3 \mu\text{M} \times \text{min}^{-1}$ for the assay conditions in Figure 4B and the $[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$ value obtained was 1.31.

The slight effect of either Cu,Zn- or Mn-superoxide dismutase on the rates of NADPH oxidation and O_2 consumption in the presence of GSH as well as on the $[\text{O}_2]_{\text{consumed}}/[\text{GSSG}]_{\text{formed}}$ ratio (Table I) reflects the particular chemistry of aromatic-ring, —OH-substituted naphthoquinones, in which the semiquinone species is stabilized by internal hydrogen bonding thereby displacing towards the left the equilibrium of: $2\text{Q}^{\cdot-} \rightleftharpoons \text{Q} + \text{Q}^{2-}$. Hence, comproportionation reactions are central to the redox chemistry of —OH-substituted naphthoquinones. It can be hypothesized that superoxide dismutase affected in a similar manner the equilibrium of the reactions in which the semiquinone can participate, i.e., $\text{Q}^{\cdot-} \Rightarrow \text{Q}$ and $\text{Q}^{\cdot-} \Rightarrow \text{QH}_2$, coupled to O_2 reduction and GSH oxidation, respectively (see DISCUSSION section and Figure 5).

DISCUSSION

General mechanistic considerations

Table I summarizes the main features of the naphthoquinones examined here in terms of [a] their ability to serve as substrates for two-electron transfer processes, [b] their reduction potentials, and [c] the effect of GSH on the redox transitions of the quinones during *DT*-diaphorase catalysis.

GSH oxidation coupled to the reduction of the semiquinone (reaction 3) (as shown for aminopyrine cation radical²) is — for the quinones examined here — thermodynamically unfavourable, since the $E(\text{GS}^{\cdot}, \text{H}^+/\text{GSH})$ value ($= +840 \text{ mV}$)²⁹ is

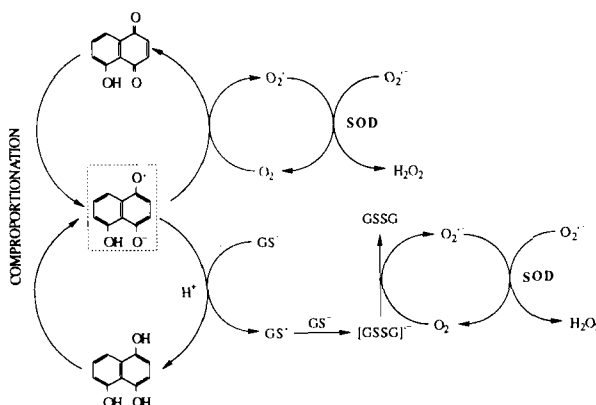
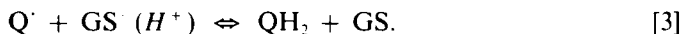


FIGURE 5 Importance of comproportionation reactions and the effect of GSH and superoxide dismutase on the redox transitions of the semiquinone form of 5-hydroxy-1,4-naphthoquinone.

far more positive than the individual $E(Q^{\cdot-}/Q^{2-})$ values (Table I). (The latter were calculated from the relation $E(Q^{\cdot-}/Q^{2-}) = 2E(Q/Q^{2-}) - E(Q/Q^{\cdot-})$ ³⁰ and taking $E(Q/Q^{\cdot-})$ values³¹⁻³³ listed in the table).



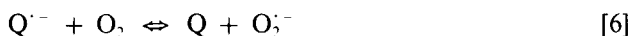
However, reaction 3 can be controlled kinetically upon removal of the thiyl radical by either dimerization² (reaction 4) ($k_4 = 9.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$)³⁴ or conjugation with GS^- to form the disulfide anion radical (reaction 5) ($k_5 = 6.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$).³⁴



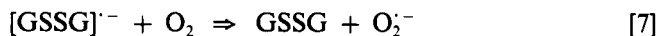
The former, reaction 4, is not likely to be important, unless $GS^{\cdot-}$ builds up locally to relatively high concentrations (for a discussion, see ref.³⁵). The latter, reaction 5, is dependent upon pH, because of the dissociation of: $GSH \rightleftharpoons GS^- + H^+$ ($pK \sim 9$). At pH 7.5 and $[GSH] = 1 \text{ mM}$, $[GS^-]/[GSH] = (1 + 10^{pK - pH})^{-1} = 0.3$ (see ref.³⁵). GS^- is not only relevant to the completion of reaction 5 but also to the action of the thiol as a nucleophile (reaction 2).

The general features pertaining the concurrent oxidation of GSH to GSSG during the autoxidation of the above hydroquinones can be summarized as follows:

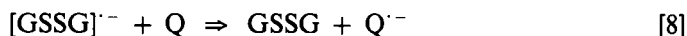
- [1] The rate of NADPH oxidation is not completely arrested by GSH, thus implying that a fraction of the semiquinone is not reduced as in reaction 3, but it is continuously oxidized *via* disproportionation and/or autoxidation; the quinone thus formed is a substrate for DT-diaphorase, hence account for the slow rate of NADPH oxidation. The competition between GSH (reaction 3) and O_2 (reaction 6) for the semiquinone is controlled by the actual concentration of reactants and the reduction potentials of the couples involved. Consideration of the latter parameter – i.e. $E(Q/Q^{\cdot-})$ values in Table I, $E(O_2/O_2^{\cdot-}) = -155 \text{ mV}$, and $E(GS^{\cdot-}, H^+/GSH) = +840 \text{ mV}$ – supports the view that a fraction of the semiquinone decays *via* autoxidation.



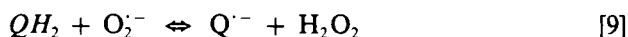
- [2] O_2 uptake can be accounted for in terms of two O_2 -consuming reactions: [a] the autoxidation of the semiquinone (reaction 6) and [b] the one-electron transfer from $[GSSG]^-$ to O_2 (reaction 7) ($k_7 = 1.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$).^{34,36} That a fraction of the O_2 consumed is accounted



for by pathways other than that in reaction 7 is substantiated by the observation that the values for the relation $[O_2]_{\text{consumed}}/[GSSG]_{\text{formed}}$ are above unity. The competition between O_2 (reaction 7) and the quinone (reaction 8) for $[GSSG]^-$ should be negligible, for any quinone formed through reaction 6 will be readily reduced by *DT*-diaphorase (reaction 1).



- [3] Reaction 9 brings forward the oxidation of the hydroquinone to the semiquinone and H_2O_2 and constitute the main propagation reaction in hydroquinone autoxidation. An analogous



reaction has been studied with catechols and ascorbic acid leading to the postulation of a common mechanism *via* a sequential proton-hydrogen transfer.³⁷ Thus, O_2^- is the propagating species in the free radical chain involved in hydroquinone autoxidation (reaction 9) and it can originate from semiquinone (reaction 6)- and $[GSSG]^-$ (reaction 7) autoxidation.

Specific comments

The oxidation of GSH coupled to the reduction of a semiquinone, as accomplished through the model reactions described above, applies to those model quinones, such as 2,3-dimethoxy-1,4-naphthoquinone, which cannot undergo nucleophilic addition and the autoxidation of its hydroquinone form involves O_2^- as the free radical propagating species (reaction 9). However, generalization is difficult, specially when the redox chemistry of the quinone is complicated by [a] its ability to undergo nucleophilic addition with GSH (e.g., 1,4-naphthoquinone and its —OH-substituted derivative) and/or [b] the presence of a —OH substituent in the aromatic ring (e.g., 5-hydroxy-1,4-naphthoquinone).

In the former instance, the reactions subsequent to the GSH nucleophilic addition to the quinone seem to contribute largely to autoxidation; these reactions are not entirely linked to NADPH oxidation and are effected differently by GSH.

In the latter instance, the intramolecular hydrogen bonding between the —OH substituent at C_5 and the —C=O at C_4 leads to stabilization of the semiquinone transient species involving displacement over to the left of the reaction:³⁸ $2Q^- \rightleftharpoons Q + Q^{2-}$. The stability of the semiquinone along with its high reactivity towards oxygen³⁸ makes reaction 6 — rather than reaction 9 — the main autoxidation reaction following the two-electron reduction of the quinones by *DT*-diaphorase. This explains the enhancement of autoxidation of this hydroquinone by superoxide dismutase,²² at variance with the reported inhibition of autoxidation of other hydroquinones.^{22,39,40} Of note, in the presence of GSH, superoxide dismutase accelerates — by catalyzing the disproportionation of O_2^- — opposite redox transitions, i.e., $Q^- \Rightarrow Q$

and $Q^{\cdot-} \Rightarrow Q^{2-}$, which are coupled to O_2 reduction and GSH oxidation, respectively, as summarized in the scheme in Figure 5.

Because of its high intracellular concentration, reactions with GSH should be considered as realistic when evaluating the metabolic pathways of quinonoid compounds. However, the effect of GSH on the $Q^{\cdot-} \Leftrightarrow Q^{2-}$ transition cannot be generalized within a single bimolecular reaction, but should be analyzed in terms of the physico-chemical properties of the quinonoid compound, which are an expression of the functional group chemistry.

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