Regular paper

Two-electron reduction of quinones by *Enterobacter cloacae* PB2 pentaerythritol tetranitrate reductase: quantitative structure-activity relationships

Lina Misevičienė[1], Žilvinas Anusevičius[1], Jonas Šarlauskas[1], Richard J. Harris[2], Nigel S. Scrutton[2] and Narimantas Čėnas[1✉]

[1]Institute of Biochemistry, Vilnius, Lithuania, [2]Faculty of Life Sciences, The University of Manchester, Manchester, UK

In order to clarify the poorly understood mechanisms of two-electron reduction of quinones by flavoenzymes, we examined the quinone reductase reactions of a member of a structurally distinct old yellow enzyme family, *Enterobacter cloacae* PB2 pentaerythritol tetranitrate reductase (PETNR). PETNR catalyzes two-electron reduction of quinones according to a 'ping-pong' scheme. A multiparameter analysis shows that the reactivity of quinones increases with an increase in their single-electron reduction potential and pK_a of their semiquinones (a three-step (e⁻,H⁺,e⁻) hydride transfer scheme), or with an increase in their hydride-transfer potential (E_7(H⁻)) (a single-step (H⁻) hydride transfer scheme), and decreases with a decrease in their van der Waals volume. However, the pH-dependence of PETNR reactivity is more consistent with a single-step hydride transfer. A comparison of X-ray data of PETNR, mammalian NAD(P)H:quinone oxidoreductase (NQO1), and *Enterobacter cloacae* nitroreductase, which reduce quinones in a two-electron way, and their reactivity revealed that PETNR is much less reactive, and much less sensitive to the quinone substrate steric effects than NQO1. This may be attributed to the lack of π-π stacking between quinone and the displaced aromatic amino acid in the active center, e.g., with Phe-178' in NQO1.

Keywords: pentaerythritol tetranitrate reductase, quinones, electron-transfer, reduction mechanism

INTRODUCTION

The therapeutic action and/or toxicity of quinoidal drugs and environmental pollutants is frequently attributed to their flavoenzyme-catalyzed reduction. Single-electron reduction of quinones to their anion-radicals initiates their redox cycling under aerobic conditions, and leads to the oxidative stress (O'Brien, 1991). Typically, these reactions are performed by flavoenzymes dehydrogenases-electrontransferases, e.g., NADPH:cytochrome P-450 reductase (EC 1.6.4.2)

and ferredoxin:NADP⁺ reductase (EC 1.18.1.2), which contain stabilized neutral flavin semiquinones, and possess natural single-electron accepting oxidants (Iyanagi, 1990, and references therein). These reactions follow an 'outer-sphere' electron transfer with a weak electronic interaction between the reactants (Marcus & Sutin, 1985), and are characterized by a linear or parabolic dependence of log (rate constant) on the single-electron reduction potential of quinones, E^1_7, with an absence of pronounced effects of quinone structure (Čėnas *et al.*, 1994; Anusevičius *et al.*, 1997).

Two-electron reduction of quinones frequently contributes to their detoxification, because the relatively stable hydroquinones undergo conjugation, and are readily excreted from organism (O'Brien, 1991, and references therein). However, due to very few mechanistic studies, the mechanism(s) of two-electron (hydride) reduction of quinones by flavoenzymes are unclear, except for the requirement of the instability of anionic flavin semiquinone in the enzyme active center (Tedeschi *et al.*, 1995; Koder *et al.*, 2002, and references therein). In the most thoroughly studied reactions of NAD(P)H:quinone oxidoreductase (NQO1, DT-diaphorase, EC 1.6.99.2) and *Enterobacter cloacae* nitroreductase (NR, EC 1.6.99.7), the reactivity of quinones in general increases upon an increase in their electron (hydride)-accepting potency, but it is also strongly influenced by their structural peculiarities (Anusevičius *et al.*, 2002; Nivinskas *et al.*, 2002). Thus, it is unclear whether the two-electron reduction of quinones requires their specific interaction(s) with the flavoenzyme active center as well, e.g., the π-π stacking of quinone with the displaced aromatic amino-acid residue, like Phe-178′ in NQO1 (Li *et al.*, 1995). For this reason, it is necessary to extend mechanistic studies of two-electron reduction using flavoenzymes of different groups and families.

Pentaerythritol tetranitrate reductase (PETNR) from *E. cloacae* strain PB2 is NADPH-oxidizing flavoenzyme containing one noncovalently bound flavin mononucleotide (FMN) per 40 kDa monomer (Binks *et al.*, 1996), and belongs to the structurally distinct old yellow enzyme (OYE) family of flavoenzymes. PETNR possesses broad oxidant specificity, performing the reductive denitration of aliphatic nitroesters such as the explosives pentaerythritol tetranitrate and glycerol trinitrate (Binks *et al.*, 1996), reduction of α/β unsaturated compounds such as 2-cyclohexenone, codeinone, steroids (French *et al.*, 1996; Barna *et al.*, 2001), and nitroaromatic compounds (French *et al.*, 1998; Khan *et al.*, 2002). The midpoint potential of FMN at pH 7.0 ($E_{m,7}$) is –0.267 V (Khan *et al.*, 2004) with less than 1% of anionic semiquinone (FMN$^-$·) stabilized at equilibrium (Khan *et al.*, 2002). There exist some data on the reduction of quinones by OYE (Massey & Schopfer, 1986), however, their reactions with PETNR have not been studied.

In this study, we have obtained a quantitative structure-activity relationship in the reduction of quinones by PETNR, and distinguished the effects of the electron/hydride-accepting potency of quinones and their steric parameters.

MATERIALS AND METHODS

E. cloacae PB2 PETNR was purified as described previously (French *et al.*, 1996). The enzyme concentration was determined spectrophotometrically using ε_{464}=11.3 mM^{-1} cm^{-1}. 2-Methyl-3-glutathionyl-5-hydroxy-1,4-naphthoquinone and 2,3-diglutathionyl-1,4-naphthoquinone were a generous gift from Dr. K. Ollinger (Linkoping University, Sweden). AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone) and BZQ (2,5-bis(2′-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone) were synthesized according to previously described procedures (Chou *et al.*, 1976). All other compounds were obtained from Sigma or Aldrich and used as received.

All the kinetic measurements were carried out in 0.05 M K-phosphate (pH 7.0), containing 1 mM EDTA at 25°C, using a Hitachi-557 spectrophotometer. The initial rates of PETNR-catalyzed oxidation of NADPH by quinones were determined by monitoring NADPH oxidation ($\Delta\varepsilon_{340}$=6.2 mM^{-1} cm^{-1}). The kinetic parameters of reactions, i.e., the catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m) of quinone reduction correspond to the reciprocal intercepts and slopes of the Lineweaver-Burk plots, [E]/v *vs.* 1/[Q], where v is the reaction rate, [E] is the enzyme concentration, and [Q] is quinone concentration. The k_{cat} corresponds to the number of molecules of NADPH oxidized by the enzyme active center per second. The quinone substrate inhibition constants (K_{iq}) at fixed NADPH concentration were obtained from the intercepts of plots [E]/v *vs.* [Q] with the x axis. 1,4-Benzoquinone-mediated reduction of cytochrome c by PETNR was monitored according to $\Delta\varepsilon_{550}$=20 mM^{-1} cm^{-1} (concentrations of 1,4-benzoquinone and cytochrome c, 50 μM, concentration of NADPH, 150 μM).
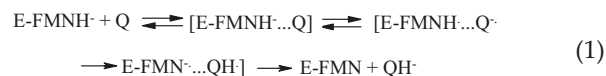
The quantum chemical calculations of the van der Waals volumes (VdWvol) of quinones were performed using Spartan'04 Pro software (Wavefunction, Inc.). The multiparameter regression analysis was performed using Statistica (version 4.3, Statsoft Inc., 1993).

RESULTS

Monitoring 1,4-benzoquinone-mediated reduction of cytochrome c by PETNR at pH 7.0, we found that the rate of cytochrome c reduction was equal to 5% of the NADPH enzymatic oxidation rate by benzoquinone, determined in the absence of cytochrome c. This shows that in the reduction of 1,4-benzoquinone single-electron flux constitutes about 2.5% (the ratio of double cytochrome c reduction rate and NADPH oxidation rate), because 1,4-benzosemiquinone reduces cytochrome c rapidly ($k \sim 10^6$ M^{-1}s^{-1}), whereas benzohydroquinone reduces it at a negligible rate at pH < 7.2 (Iyanagi, 1990, and refer-

ences therein). Thus, PETNR reduces quinones in a two-electron way to hydroquinones.

Like in our previous studies of two-electron transferring NQO1 and *E. cloacae* NR (Anusevičius *et al.*, 2002; Nivinskas *et al.*, 2002), one should consider two possible hydride transfer mechanisms from reduced PETNR (E-FMNH⁻) to quinones. The first is a three-step (e⁻,H⁺,e⁻) mechanism:

$$E\text{-}FMNH^- + Q \rightleftharpoons [E\text{-}FMNH^-...Q] \rightleftharpoons [E\text{-}FMNH^-...Q^{\cdot}$$
$$\longrightarrow E\text{-}FMN^{\cdot-}...QH^{\cdot}] \longrightarrow E\text{-}FMN + QH^- \qquad (1)$$

In this case, quinone reactivity should increase with an increase in their single-electron reduction potential (E^1_7) and pK_a of semiquinone ($pK_a(QH\cdot)$) (Fukuzumi *et al.*, 1989). Alternatively, in a single-step transfer,

$$E\text{-}FMNH^- + Q \rightleftharpoons [E\text{-}FMNH^-...Q] \longrightarrow E\text{-}FMN + QH^- \qquad (2)$$

quinone reactivity should increase with an increase in their hydride-transfer potential (($E_7(H^-)$), i.e., the potential of the quinone/anionic hydroquinone (Q/QH⁻) redox couple (Carlson & Miller, 1985). The latter is expressed as

$$E_7(H^-) = E_{m,7} - 0.03\ V \times (pK_a(QH_2) - 7.0), \qquad (3)$$

where $E_{m,7}$ is the midpoint potential of the quinone/hydroquinone couple at pH 7.0, and $pK_a(QH_2)$ is pK_a of hydroquinone. If $pK_a(QH_2) \leq 7.0$, then $E_7(H^-) \cong E_{m,7}$. We examined the reactivity of PETNR with structurally diverse quinoidal compounds possessing a broad range of E^1_7, $pK_a(QH\cdot)$, $E_7(H^-)$, and van der Waals volumes (VdWvol) (Table 1).

In the reactions of PETNR with 5-hydroxy-1,4-napthoquinone (Fig. 1A), 5,8-dihydroxy-1,4-naphthoquinone, 2,6-dimethyl-, trimethyl- and tetramethyl-1,4-benzoquinones (not shown), we obtained a series of parallel plots in Lineweaver-Burk coordinates at varied concentrations of quinones and several fixed NADPH concentrations, with an upward curvature at high quinone concentrations. This points to a 'ping-pong' scheme with quinone substrate inhibition. The quinone substrate inhibition constants (K_{iq}) increased with an increase in NADPH concentration (Fig. 1B). This shows that quinones bind to the oxidized enzyme form, thus acting as competitive inhibitor towards NADPH. The kinetic parameters of reaction, k_{cat} and k_{cat}/K_m for quinone reduction, and K_{iq} of quinones are given in Table 1. We were unable to determine k_{cat}/K_m of 1,4-benzoquinone, because its K_m was below 1.0 μM ($k_{cat} \sim 25$ s⁻¹ at [NADPH]=150 μM). The k_{cat}/K_m values for NADPH oxidation, determined using several quinone oxidants, did not depend on their nature, being in the range of $4.2\times10^5 - 2.9\times10^5$ M⁻¹ s⁻¹. The k_{cat} values for the most active quinones were close to 34 s⁻¹ (Table 1), i.e., close to the maximal rate of the reduction of PETNR by NADPH, determined in the presteady-state kinetics (Basran *et al.*, 2003). Thus, in these cases, the overall reaction rate becomes limited by the reductive half-reaction, and not by the oxidation of reduced PETNR by quinones.

The data of Table 1 show that the reactivity of quinones in general increases with an increase in their electron accepting potency. Analyzing quinone reactivity in terms of a three-step (Eqn. 1) hydride transfer, we found that the linear relationship between log k_{cat}/K_m and E^1_7 is poorly expressed
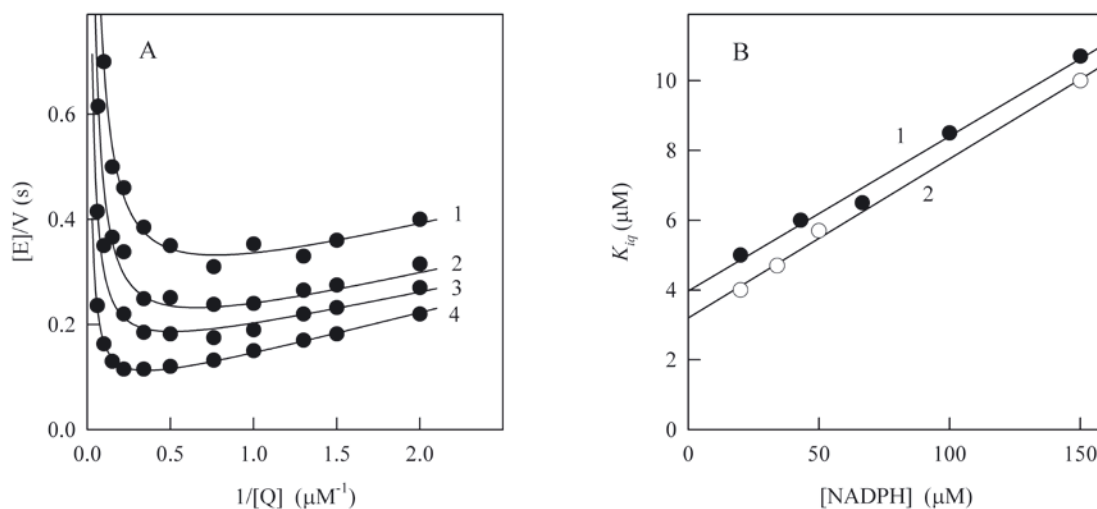
Figure 1. Kinetics of PETNR-catalyzed quinone reduction.
A. The rates of PETNR-catalyzed oxidation of NADPH by various concentrations of 5-hydroxy-1,4-naphthoquinone, presented in Lineweaver-Burk coordinates. Concentration of NADPH, 20 μM (1), 33 μM (2), 50 μM (3), and 150 μM (4). B. Dependence of the quinone substrate inhibition contants (K_{iq}) of 5,8-dihydroxy-1,4-naphthoquinone (1) and 5-hydroxy-1,4-naphthoquinone (2) on the concentration of NADPH.

Table 1. Catalytic (k_{cat}) and bimolecular rate constants (k_{cat}/K_m) of reduction of quinones by PETNR, their substrate inhibition constants (K_{iq}), single-electron reduction potentials (E^1_7), hydride transfer potentials ($E_7(H^-)$), pK_a of semiquinones (pK_a(QH·)), and van der Waals volumes (VdWvol).

The values of E^1_7, $E_7(H^-)$, and pK_a(QH·) are taken from Wardman (1989) and Čėnas *et al.* (2004).

| No. Compound	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}s^{-1})	K_{iq} (µM)	E^1_7 (V)	$E_7(H^-)$ (V)	pK_a(QH·)	VdWvol (Å^3)
1. 2-Methyl-1,4-benzoquinone[a]	30.3 ± 3.9	9.3 ± 1.8×10^6	80.0	0.01	0.120	4.45	142.5
2. AZQ[a]	29.2 ± 1.2	1.4 ± 0.1×10^6		−0.07	−0.046	6.30	360.6
3. 2,6-Dimethyl-1,4-benzoquinone[b]	26.9 ± 1.9	1.1 ± 0.2×10^7	56.0	−0.08	0.058	4.75	160.6
4. 5-Hydroxy-1,4-naphthoquinone[b]	16.0 ± 2.0	1.4 ± 0.1×10^7	3.0	−0.09	−0.060	3.65	179.7
5. Methylene blue[a]	25.6 ± 0.4	1.3 ± 0.1×10^6		−0.10	0.00		297.6
6. 5,8-Dihydroxy-1,4-naphthoquinone[b]	25.0 ± 3.0	5.0 ± 0.7×10^7	4.0	−0.11	−0.084	2.80	192.3
7. 9,10-Phenanthrene quinone[a]	30.0 ± 3.0	1.2 ± 0.1×10^7	25.0	−0.12	−0.034		218.3
8. 1,4-Naphthoquinone[a]	34.1 ± 2.9	1.8 ± 0.3×10^7	44.4	−0.15	−0.029	4.10	168.1
9. 2,3-Diglutathionyl-1,4-naphthoquinone[a]	7.2 ± 1.4	1.2 ± 0.1×10^5		−0.15	−0.029[c]		684.0
10. 2-Methyl-5-hydroxy-1,4-naphthoquinone[a]	19.6 ± 2.0	9.6 ± 0.3×10^6	46.0	−0.16			199.6
11. 2-Methyl-3-glutathionyl-5-hydroxy-1,4-naphthoquinone[a]	32.1 ± 2.5	2.0 ± 0.5×10^6	115	−0.16			455.6
12. 2,3,5-Trimethyl-1,4-benzoquinone[b]	32.6 ± 2.0	8.8 ± 0.5×10^6	>100	−0.17	0.00	5.00	178.5
13. 2-Methyl-1,4-naphthoquinone[a]	30.7 ± 3.9	1.9 ± 0.4×10^7	19.6	−0.20	−0.114	4.50	187.2
14. 9,10-Anthraquinone-2,6-disulfonate[a]	11.1 ± 0.1	1.2 ± 0.1×10^5		−0.25	−0.21	3.00	314.5
15. Tetramethyl-1,4-benzoquinone[b]	22.0 ± 3.0	5.0 ± 1.5×10^5	30	−0.26	−0.086	5.10	197.5
16. Riboflavin[a]	4.4 ± 0.05	2.6 ± 0.1×10^4		−0.318	−0.21	8.50	422.7
17. 1,8-Dihydroxy-9,10-anthraquinone[a]	4.0 ± 0.1	2.0 ± 0.1×10^5		−0.325		3.95	245.5
18. BZQ[a]	0.4 ± 0.05	1.8 ± 0.1×10^3		−0.385	−0.32		308.5
19. 1-Hydroxy-9,10-anthraquinone[a]	4.7 ± 0.2	1.1 ± 0.1×10^5		−0.385		4.60	231.5
20. 2-Hydroxy-1,4-naphthoquinone[a]	1.1 ± 0.1	9.2 ± 1.2×10^4		−0.41	−0.20	4.70	179.7
21. 2-Methyl-3-hydroxy-1,4-naphthoquinone[a]	1.1 ± 0.3	2.5 ± 0.5×10^4		−0.46	−0.24		199.6

[a]Kinetic parameters and K_{iq} were determined at fixed NADPH concentration (150 µM); [b]k_{cat} determined at saturating NADPH concentration, K_{is} determined by extrapolation to [NADPH]=0; [c]Assumed to be the same as for 1,4-naphthoquinone.

(r^2=0.6233, F(1,19)=31.17, not shown), as well as the parabolic (second order) relationship (r^2=0.6352, F(2,18)=15.60, Fig. 2A). The omission of bulky glutathione conjugates (compounds 9,11 (Table 1)) slightly improved the regressions, giving r^2=0.6777, F(1,17)=35.60 (linear), and r^2=0.7015, F(2,16)=18.86 (parabolic). The data of Fig. 2B show that the reactivity of several quinones with similar E^1_7 values decreases with an increase in their VdWvol. Using VdWvol of quinones as an additional parameter, the second order regression was markedly improved (r^2=0.8538, F(3,17)=31.50):

$$\log k_{cat}/K_m = (8.00 \pm 0.38) - (1.35 \pm 3.57)\, E^1_7 - (18.49 \pm 7.34)\,(E^1_7)^2 - (0.45 \pm 0.09) \times 10^{-2}\, \text{VdWvol}, \ (n=21) \quad (4)$$

The introduction of pK_a(QH·) (Table 1) as a third variable did not improve the regression significantly (r^2=0.9103, F(4,9)=19.04):

$$\log k_{cat}/K_m = (7.94 \pm 0.43) - (2.50 \pm 3.02)\, E^1_7 - (19.09 \pm 6.96)\,(E^1_7)^2 - (0.69 \pm 0.13) \times 10^{-2}\, \text{VdWvol} + (0.07 \pm 0.08)\, \text{p}K_a(\text{QH·}), \ (n=14) \quad (5)$$
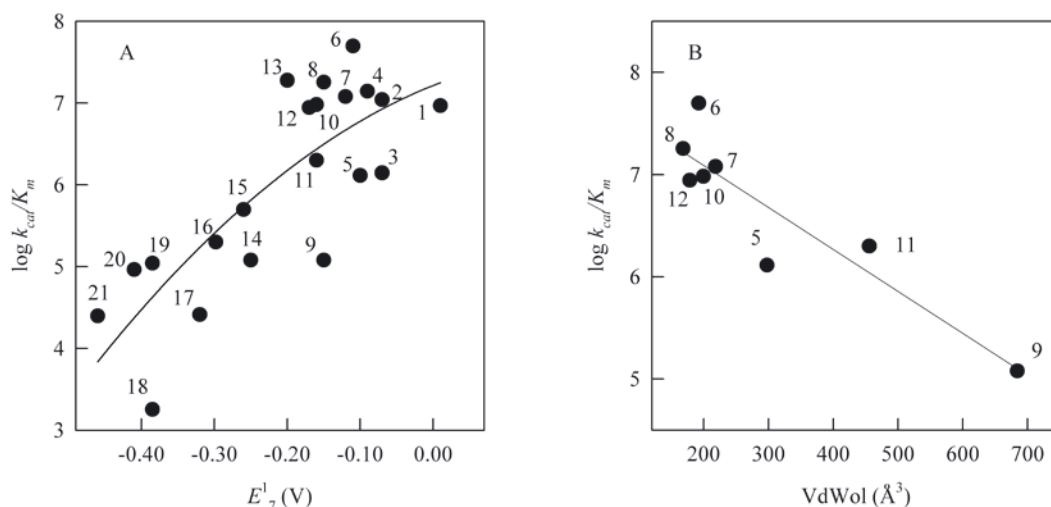
Figure 2. Dependence of the reactivity of PETNR on the electron-accepting potency and steric parameters of quinones.
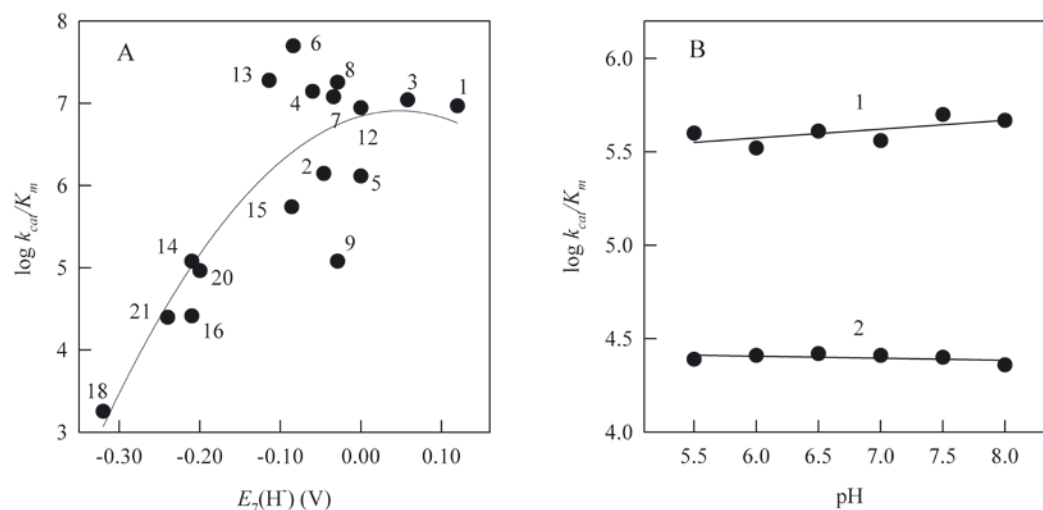A. Dependence of k_{cat}/K_m of quinones on their single-electron reduction potentials (E^1_7). B. Dependence of k_{cat}/K_m of quinones on their Van der Waals volume (VdWvol). The numbers of compounds are taken from Table 1.

giving an uncertain dependence of quinone reactivity on the proton-accepting potency of their radicals (Eqn. 5). We failed to improve the regressions by the introduction of (VdWvol)^2 as an additional variable.

Alternatively, analyzing quinone reactivity in terms of a single-step hydride transfer (Eqn. 2), we found that their log k_{cat}/K_m increased with an increase in $E_7(H^-)$ (Table 1), although the regressions were poorly expressed (r^2=0.5897, F(1,15)=21.51 (linear, not shown), and r^2=0.6979, F(2.14)=16.04 (parabolic, Fig. 3A)). Using VdWol of quinones as an additional parameter, the regression was considerably improved (r^2=0.8621, F(3,13)=27.09):

$$\log k_{cat}/K_m = (7.81\pm0.30) + (1.03\pm2.23)\, E_7(H^-) - (32.66\pm8.96)\, (E_7(H^-))^2 - (0.40\pm0.10)\times10^{-2}\, \text{VdWvol}$$
$$(n=17). \tag{6}$$

The obtained structure-activity relationships (Eqns. (4–6)) are equally well consistent with both three-step and single-step hydride transfer (Eqns. 1, 2), demonstrating similar negative coefficients Δlog (k_{cat}/K_m)/ΔVdWvol in both cases. In order to define the reaction mechanism, we studied the pH-dependence of the reactivity of riboflavin and tetramethyl-1,4-benzoquinone (Fig. 3B). This approach, which has been used in the studies of *E. cloacae* NR (Nivinskas *et al.*, 2002), is based on the pH-independent

Figure 3. Dependence of the reactivity of PETNR on the hydride-accepting potency of quinones and on pH.
A. Dependence of k_{cat}/K_m of quinones on their hydride-transfer potential ($E_7(H^-)$). The numbers of compounds are taken from Table 1. B. pH dependence of k_{cat}/K_m of tetramethyl-1,4-benzoquinone (1) and riboflavin (2).

single-electron reduction potential of tetramethyl-1,4-benzoquinone at pH 5.5–8.0 ($pK_a(QH·)=4.6$, Table 1), and the negative dependence of E^1 of riboflavin on pH with $\Delta E^1/\Delta pH=-0.059$ V ($pK_a(QH·)=8.8$, Table 1). In the case of a three-step hydride transfer with the partly rate-limiting first electron transfer (Eqn. 1), an increase in pH should relatively decrease the reactivity of riboflavin with respect to the reactivity of tetramethyl-1,4-benzoquinone. However, the log k_{cat}/K_m values of both compounds were pH-independent (Fig. 3B). This is consistent with a single-step hydride transfer (Eqn. 2), because the pH-dependence of hydride-transfer potentials of both compounds is the same, $\Delta E(H^-)/\Delta pH=-0.030$ V.

DISCUSSION

Our data show that the reactivity of quinones towards two-electron transferring PETNR is relatively insensitive to their structural peculiarities, and depends mainly on the energetics of charge transfer (Figs. 2A, 3A, Eqns. 4–6). In this aspect, PETNR differs from NQO1, whose reactivity is much more sensitive to the steric effects of quinones with the coefficients $\Delta log\ (k_{cat}/K_m)/\Delta VdWvol=-1.46–1.69 \times 10^{-2}$ (Anusevičius *et al.*, 2002; Čėnas *et al.*, 2004), which distort the reactivity dependence on the quinone reduction potential. PETNR also differs from *E. cloacae* NR, which is relatively insensitive to the VdWvol of quinones, but shows a pronounced specificity towards 2-hydroxy-1,4-naphthoquinone structure (Nivinskas *et al.*, 2002). A similar parabolic log k_{cat}/K_m *vs*. E^1_7 dependence for a limited number of quinones has been demonstrated in their two-electron reduction by *Clostridium kluyveri* diaphorase (Čėnas *et al.*, 1987). Thus, our data provide further evidence that in certain cases the two-electron (hydride) reduction of quinones by flavoenzymes may not be significantly influenced by the specific interactions, similar to their single-electron reduction by flavoenzymes dehydrogenases-electrontransferases. Thus, the flavin semiquinone instability may be a sufficient factor for the two-electron (hydride) reduction of quinones. Although the reduction of quinones by PETNR is interesting mainly as a model reaction, it has been recently shown that a member of the OYE family is the main enzyme in *Trypanosoma cruzi* responsible for the NADPH-dependent bioreductive activation of trypanocidal quinones and aromatic nitrocompounds (Kubata *et al.*, 2002).

It is of certain interest to compare the 'intrinsic' reactivities of two-electron transferring flavoenzymes, i.e., their k_{cat}/K_m of quinone reduction at $\Delta E_7(H^-)$ about 0. Because *E. cloacae* NR contains anionic $FMNH^-$ at pH 7.0, its $E_7(H^-)=E_{m,7}=-0.190$ V (Koder *et al.*, 2002). X-Ray studies also suggest the presence of anionic reduced flavins in NQO1 and PETNR (Li *et al.*, 1995; Barna *et al.*, 2001). Thus, their $E_7(H^-)$ may be equal to or below their $E_{m,7}$ (Eqn. 3, i.e., –0.159 V (NQO1, Tedeschi *et al.*, 1995), and –0.267 V (PETNR, Khan *et al.*, 2004). At $\Delta E_7(H^-) \sim 0$, the quinone reduction rate constant by NQO1 may reach 10^7 M^{-1} s^{-1} (Anusevičius *et al.*, 2002). In *E. cloacae* NR the rate constant varies between about 10^7 M^{-1} s^{-1} (2-hydroxy-1,4-naphthoquinone) and about 10^5 M^{-1} s^{-1} (other compounds) (Nivinskas *et al.*, 2002). In contrast, the 'intrinsic' reactivity of PETNR is lower, being close to 10^4 $M^{-1}s^{-1}$ (Table 1, Fig. 3A). This may point to a less efficient electronic interaction between quinones and $FMNH^-$ isoalloxazine ring in PETNR. Indeed, the binding of quinones in the active center of NQO1 involves their π-π stacking with the flavin isoalloxazine ring, with the displacement of the aromatic residues of Tyr-128' and Phe-178', the latter stacking behind the quinone ring (Li *et al.*, 1995). The binding of benzoate to *E. cloacae* NR involves some displacement of Phe-124 and Tyr-123, however, these residues do not stack with benzoate (Haynes *et al.*, 2002). In contrast, the binding of steroid or nitroaromatic ligands to PETNR involves only small perturbations of Tyr-351, Tyr-186 and/or Trp-102 (Barna *et al.*, 2001; Khan *et al.*, 2002; 2004), without any stacking interactions. These data enabled us to conclude that the active center of PETNR is flexible, and is able to accommodate large ligands without significant aminoacid displacements. This property is most probably responsible for the less efficient electronic interaction between quinones and $FMNH^-$ in PETNR, and, in parallel, for the absence of pronounced discrimination of bulky oxidants.

Acknowledgements

REFERENCES

Anusevičius Ž, Martinez-Julvez M, Genzor CG, Nivinskas H, Gomez-Moreno C, Čėnas N (1997) Electron transfer reactions of *Anabaena* PCC 7119 ferredoxin:NADP⁺ reductase with nonphysiological oxidants. *Biochim Biophys Acta* **1320**: 247–255.

Anusevičius Ž, Šarlauskas J, Čėnas N (2002) Two-electron reduction of quinones by rat liver NAD(P)H:quinone oxidoreductase: quantitative structure–activity relationships. *Arch Biochem Biophys* **404**: 254–262.

Barna TM, Khan H, Bruce NC, Barsukov I, Scrutton NS, Moody PCE (2001) Crystal structure of pentaerythritol tetranitrate reductase: "flipped" binding geometries for steroid substrates in different redox states of the enzyme. *J Mol Biol* **310**: 433–447.

Basran J, Harris RJ, Sutcliffe MJ, Scrutton NS (2003) H-tunneling in the multiple H-transfers of the catalytic cycle

of morphinone reductase and in the reductive half-reaction of the homologous pentaerythritol tetranitrate reductase. *J Biol Chem* **278**: 43973–43982.

Binks PR, French CE, Nicklin S, Bruce NC (1996) Degradation of pentaerythritol tetranitrate by *Enterobacter cloacae* PB2. *Appl Environ Microbiol* **62**: 1214–1219.

Carlson BW, Miller LL (1985) Mechanism of the oxidation of NADH by quinones. Energetics of one-electron and hydride routes. *J Am Chem Soc* **107**: 479–485.

Čėnas NK, Vienožinskis JV, Kulys JJ (1987) Kinetic regularities of *Cl. kluyveri* diaphorase. *Biokhimiya* **52**: 91–96 (in Russian).

Čėnas N, Anusevičius Ž, Bironaitė D, Bachmanova GI, Archakov AI, Ollinger K (1994) The electron transfer reactions of NADPH:cytochrome P450 reductase with nonphysiological oxidants. *Arch Biochem Biophys* **315**: 400–406.

Čėnas N, Anusevičius Ž, Nivinskas H, Misevičienė L, Šarlauskas J (2004) Structure–activity relationships in two-electron reduction of quinones. *Methods Enzymol* **382B**: 258–277.

Chou F, Khan AH, Driscoll JS (1976) Potential central nervous system antitumor agents. Aziridinylbenzoquinones. 2. *J Med Chem* **19**: 1302–1308.

French CE, Nicklin S, Bruce N (1996) Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. *J Bacteriol* **178**: 6623–6627.

French CE, Nicklin S, Bruce N (1998) Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Appl Environ Microbiol* **64**: 2864–2868.

Fukuzumi S, Ishikawa M, Tanaka T (1989) Acid-catalysed reduction of *p*-benzoquinone derivatives by an NADH analoque, 9,10-dihydro-10-methylacridine. The energetic comparison of one-electron *vs.* two-electron pathways. *J Chem Soc Perkin Trans* **2**: 1811–1816.

Haynes CA, Koder RL, Miller A-F, Rodgers DW (2002) Structures of nitroreductase in three states. Effects of inhibitor binding and reduction. *J Biol Chem* **277**: 11513–11520.

Iyanagi T (1990) On the mechanism of one-electron and two-electron reduction of quinones by microsomal flavin enzymes — the kinetic analysis between cytochrome-B5 and menadione. *Free Radic Res Commun* **8**: 259–268.

Khan H, Harris RJ, Barna T, Craig DH, Bruce NC, Munro AW, Moody PCE, Scrutton NS (2002) Kinetic and structure basis of reactivity of pentaerythritol tetranitrate reductase with NADPH, 2-cyclohexenone, nitroesters and nitroaromatic explosives. *J Biol Chem* **277**: 21906–21912.

Khan H, Barna T, Harris RJ, Bruce NC, Barsukov I, Munro AW, Moody PCE, Scrutton NS (2004) Atomic resolution structures and solution behavior of enzyme-substrate complexes of *Enterobacter cloacae* PB2 pentaerythritol tetranitrate reductase. *J Biol Chem* **279**: 30563–30572.

Koder RL, Haynes CA, Rodgers ME, Rodgers DW, Miller A-F (2002) Flavin thermodynamics explain the oxygen insensitivity of enteric nitroreductases. *Biochemistry* **41**: 14197–14205.

Kubata BK, Kabututu Z, Nozaki T, Munday CJ, Fukuzumi S, Ohkubo K, Lazarus M, Lazarus M, Maruyama T, Martin SK, Duszenko M, Urade Y (2002) A key role for old yellow enzyme in the metabolism of drugs by *Trypanosome cruzi*. *J Exp Med* **196**: 1241–1251.

Li R, Bianchet MA, Talalay P, Amzel M (1995) The three-dimensional structure of NADP(H):quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proc Natl Acad Sci USA* **92**: 8846–8850.

Marcus RA, Sutin N (1985) Electron transfers in chemistry and biology. *Biochim Biophys Acta* **811**: 265–322.

Massey V, Schopfer LM (1986) Reactivity of old yellow enzyme with alpha-NADPH and other pyridine nucleotides. *J Biol Chem* **261**: 1215–1222.

Nivinskas H, Staškevičienė S, Šarlauskas J, Koder RL, Miller A-F, Čėnas N (2002) Two-electron reduction of quinones by *Enterobacter cloacae* NAD(P)H:nitroreductase: quantitative structure-activity relationships. *Arch Biochem Biophys* **403**: 249–258.

O'Brien PJ (1991) Molecular mechanisms of quinone cytotoxicity. *Chem Biol Interact* **80**: 1–41.

Tedeschi G, Chen S, Massey V (1995) DT-diaphorase. Redox potential, steady-state, and rapid reaction studies. *J Biol Chem* **270**: 1198–1204.

Wardman P (1989) Reduction potentials of one-electron couples involving free radicals in aqueous solutions. *J Phys Chem Ref Data* **18**: 1637–1755.