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# Two-electron reduction of quinones by *Enterobacter cloacae* NAD(P)H:nitroreductase: quantitative structure-activity relationships

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## Abstract

*Enterobacter cloacae* NAD(P)H:nitroreductase (NR; EC 1.6.99.7) catalyzes two-electron reduction of a series of quinoidal compounds according to a “ping-pong” scheme, with marked substrate inhibition by quinones. The steady-state catalytic constants ( $k_{\text{cat}}$ ) range from 0.1 to 1600 s<sup>-1</sup>, and bimolecular rate constants ( $k_{\text{cat}}/K_{\text{m}}$ ) range from 10<sup>3</sup> to 10<sup>8</sup> M<sup>-1</sup> s<sup>-1</sup>. Quinones, nitroaromatic compounds and competitive to NADH inhibitor dicumarol, quench the flavin mononucleotide (FMN) fluorescence of nitroreductase. The reactivity of NR with single-electron acceptors is consistent with an “outer-sphere” electron transfer model, taking into account high potential of FMN semiquinone/FMNH<sup>-</sup> couple and good solvent accessibility of FMN. However, the single-electron acceptor 1,1'-dibenzyl-4,4'-bipyridinium was far less reactive than quinones possessing similar single-electron reduction potentials ( $E_7^1$ ). For all quinoidal compounds except 2-hydroxy-1,4-naphthoquinones, there existed parabolic correlations between the log of rate constants of quinone reduction and their  $E_7^1$  or hydride-transfer potential ( $E_7^0(\text{Q}/\text{QH}^-)$ ). Based on pH dependence of rate constants, a single-step hydride transfer seems to be a more feasible quinone reduction mechanism. The reactivities of 2-hydroxy-1,4-naphthoquinones were much higher than expected from their reduction potential. Most probably, their enhanced reactivity was determined by their binding at or close to the binding site of NADH and dicumarol, whereas other quinones used the alternative, currently unidentified binding site. © 2002 Elsevier Science (USA). All rights reserved.

**Keywords:** Nitroreductase; Quinones; Electron transfer; Mechanism

Bacterial oxygen-insensitive NAD(P)H:nitroreductases (NR; EC 1.6.99.7)<sup>1</sup> contain flavin mononucleotide (FMN) in their active center and perform two-electron reduction of nitroaromatic compounds to nitroso and, subsequently, to hydroxylamine products [1–5]. Bacterial NRs are of considerable interest due to their participation in the biodegradation of explosives and other important polynitroaromatic environmental pollutants [6] and their utility in the antibody- and gene-directed enzyme prodrug

therapies [7–9]. Currently, the transfection of tumor cells by *Escherichia coli* nitroreductase represents a promising method for enhancing the low mammalian cell activity with respect to two-electron reductive activation of nitroaromatic alkylating agents, e.g., CB-1954 ((5-aziridin-1-yl)-2,4-dinitrobenzamide) [9]. However, certain aspects of the catalytic mechanism of bacterial oxygen-insensitive nitroreductases, e.g., their substrate specificity, and the general mechanism of two-electron (hydride) nitroreduction remain vaguely understood.

*Enterobacter cloacae* nitroreductase is a homodimeric FMN-containing 24.5-kDa protein, originally characterized by Bryant and DeLuca [3], and is now known to be a member of a larger family of proteins including *Salmonella typhimurium* [2] and *E. coli* nitroreductases [10] and *Vibrio fischeri* FMN reductase [11]. The crystal structure of *E. cloacae* NR is homologous to the struc-

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<sup>1</sup> Abbreviations used: NR, nitroreductase; FMN, flavin mononucleotide; Q, quinone;  $E_7^1$ , single-electron reduction potential;  $E_7^0$ , standard (two-electron reduction) potential;  $E_7^0(\text{Q}/\text{QH}^-)$ , potential for a net hydride transfer; VdWvol., van der Waals volume;  $k_{\text{cat}}$ , catalytic constant;  $k_{\text{cat}}/K_{\text{m}}$ , bimolecular rate constant;  $K_{\text{iq}}$ , quinone substrate inhibition constant;  $k_{11}$ ,  $k_{22}$ , electron self-exchange constants.

ture of *E. coli* NR [12,13]. *E. cloacae* NR reduces polynitrobenzenes, polynitrophenyl-*N*-nitramines, and nitrofurans in two successive two-electron transfers to their hydroxylamines and follows a “ping-pong” kinetic mechanism [14,15]. The activity of *E. cloacae* nitroreductase is relatively insensitive to the particular structure of its nitroaromatic substrates, but it increases with an increase in their electron-accepting potency [3,15]. However, these data did not permit characterization the mechanism of two-electron (hydride) transfer in nitroreductase reaction, since the energetics of the two-electron reduction of nitroaromatic compounds in aqueous medium, i.e., their two-electron reduction potentials, and the proton accepting properties of their single- and two-electron reduced forms are insufficiently characterized.

Quinones represent another group of oxidizing substrates of bacterial nitroreductases [16]. In addition, they are widely used as model substrates for other flavoenzymes. In this respect, the main advantage of quinones is their well-characterized pH-dependent potentials for single- and two-electron reduction [17–23]. Using quinones, a quantitative relationship between one- and two-electron transfers in the reactions of NAD(P)H-oxidizing flavoenzymes has been proposed [24, and references therein]. The reactivity of quinones with flavoproteins is also interesting in view of therapeutic action and/or toxicity of quinones and their presence among the products of environmental pollution [22,25,26, and references therein].

In the present work, we have attempted to characterize the mechanism of two-electron transfer of *E. cloacae* nitroreductase using a series of quinoidal oxidants with well-characterized single ( $E_7^1$ )- and two ( $E_7^0$ )-electron reduction potential values.

## Materials and methods

**Materials.** *E. cloacae* NR was expressed in *E. coli*, purified, and stored as previously described [27]. The enzyme concentration was determined spectrophotometrically using  $\epsilon_{454} = 14.3 \text{ mM}^{-1} \text{ cm}^{-1}$  for the bound FMN cofactor. Quinoidal compounds were synthesized according to published methods: 2,5-diaziridinyl-1,4-benzoquinone [28], 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone [29]; 2-hydroxy-3-methyl-1,4-naphthoquinone [30]; 1-hydroxy-9,10-anthraquinone [31]; 1,4-dihydroxy-9,10-anthraquinone [31]; and 9,10-anthraquinone-2-sulfonate [31]. The purity of quinones was determined using melting points, TLC, NMR, and elemental analysis. All other compounds were obtained from Sigma or Aldrich and used as received.

**Methods.** Kinetic measurements were carried out in 0.1 M Tris–Cl (pH 7.0), containing 0.5 mM desferrioxamine at 25 °C. The rates of NR-catalyzed oxidation of NADH by various quinones, riboflavin, 1,1'-dibenzyl-

4,4'-bipyridinium, and  $\text{Fe}(\text{EDTA})^-$  were determined by monitoring NADH oxidation ( $\Delta\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ) using a Hitachi-557 spectrophotometer. Corrections were introduced when necessary for the formation of reaction products absorbing at 340 nm. The reduction of ferricyanide was monitored according to a decrease of ferricyanide absorbance at 420 nm ( $\Delta\epsilon_{420} = 1.0 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The 1,4-benzoquinone-mediated reduction of cytochrome *c* was monitored in the presence of 150  $\mu\text{M}$  NADH, 50  $\mu\text{M}$  1,4-benzoquinone, 50  $\mu\text{M}$  cytochrome *c*, and 20 nM NR, according to an increase in absorbance at 550 nm ( $\Delta\epsilon_{550} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The catalytic constant ( $k_{\text{cat}}$ ) and the bimolecular rate constant ( $k_{\text{cat}}/K_{\text{m}}$ ) of quinone reduction correspond to the reciprocal intercepts and slopes of plots  $[E]/v$  vs  $1/[Q]$ , where  $[E]$  is enzyme concentration, and  $[Q]$  is concentration of quinone.  $k_{\text{cat}}$  is the number of NADH molecules oxidized by a single active center of the enzyme per second. The quinone substrate inhibition constants ( $K_{\text{iq}}$ ) were obtained from Eq. (1) which describes the “ping-pong” scheme of enzyme accompanied by the competitive inhibition of quinones versus NADH [32]:

$$v/[E] = \{k_{\text{cat}}K_{\text{iq}}[Q][\text{NADH}]\} / \{K_{\text{iq}}([Q][\text{NADH}] + K_{\text{m}(\text{Q})}[\text{NADH}] + K_{\text{m}(\text{NADH})}[Q]) + K_{\text{m}(\text{NADH})}[Q]^2\}. \quad (1)$$

Alternatively,  $K_{\text{iq}}$  were determined at several fixed NADH concentrations as the intercepts of plots  $[E]/v$  vs  $[Q]$  with the  $x$ -axis and, subsequently, were obtained by extrapolating the data to zero NADH concentration. The pH dependency of reactions of NR was examined between pH 5.8 and 8.0, using 0.1 M Tris–acetate buffer solutions.

The *E. cloacae* nitroreductase FMN fluorescence quenching experiments were performed in 0.1 M Tris–Cl, pH 7.0, at 25 °C, using a Hitachi-MPF4 spectrofluorometer. Typically, 5–6  $\mu\text{M}$  enzyme was used. The dissociation constants ( $K_{\text{d}}$ ) of NR complexes with quinones were calculated from the fluorescence intensity changes ( $\Delta I$ ) (Eq. (2)),

$$\Delta I/\Delta I_{\text{max}} = (B - (B^2 - 4[E][Q])^{0.5})/2[E], \quad (2)$$

where  $\Delta I_{\text{max}}$  is the maximal fluorescence change at fully saturating quinone concentration, and  $B = [E] + [Q] + K_{\text{d}}$  [33].

**Molecular volume calculations and statistical analysis.** Van der Waals volumes (VdWvol) of quinoidal compounds were calculated by the neuronal networks method using Virtual MSC (Japan). The multiparameter regression analysis was performed using Statistica (version 4.3, StatSoft).

## Results

During *E. cloacae* nitroreductase-catalyzed oxidation of 150  $\mu\text{M}$  NADH by 50  $\mu\text{M}$  2-methyl-1,4-naphthoqui-

none (menadione) at pH 5.8–7.0, the rapid oxidation of stoichiometric to menadione amount of NADH is followed by the second slower phase of oxidation of excess NADH (Fig. 1). Evidently, the second phase is limited by the rate of reoxidation of two-electron reduced menadione by oxygen, since its rate markedly increases with an increase in pH (Fig. 1) [24,34]. In contrast, hydroxy-substituted naphthoquinones oxidize excess NADH in a single phase (Fig. 1). This may be explained by the rapid reoxidation of reduced hydroxy-substituted naphthohydroquinones at neutral pH [34]. During NR-catalyzed oxidation of NADH by 1,4-benzoquinone at pH 7.0, the reduction of added cytochrome *c* took place with less than 14% of NADH oxidation rate. However, this rate was achieved after a lag period of 1.5–2.0 min. Since at  $\text{pH} \leq 7.0$  cytochrome *c* can be reduced only by 1,4-benzosemiquinone, but not by hydroquinone [24], this means that the single-electron flux, expressed as the ratio of cytochrome *c* reduction rate and a doubled rate of NADH oxidation [24], is less than 7%. The existence of a lag period may be explained by the disproportionation of benzoquinone and the product of its two-electron reduction, hydroquinone, with subsequent formation of benzosemiquinone, which, subsequently, reduces cytochrome *c*. The analogous pattern of the benzoquinone-mediated reduction of cytochrome *c* was observed under anaerobiosis.

In this study, a number of structurally diverse quinones were used as *E. cloacae* NR substrates (Table 1). Based on two commonly accepted mechanisms for two-electron (hydride) reduction of quinones [35,36], one may propose the following two mechanisms for the two-electron reduction of quinones by reduced anionic FMN of *E. cloacae* NR ( $\text{E-FMNH}^-$ ) [36].

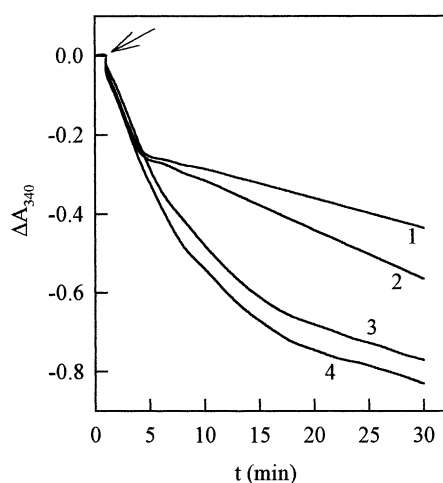
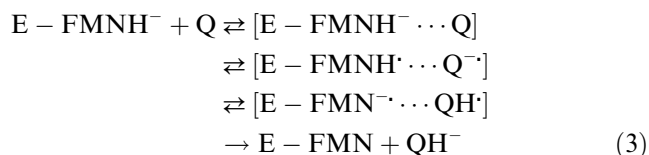
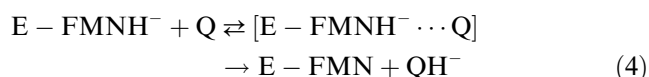


Fig. 1. Kinetics of oxidation of 150  $\mu\text{M}$  NADH by 50  $\mu\text{M}$  menadione (1–3) and 50  $\mu\text{M}$  5-hydroxy-1,4-naphthoquinone (4) in the presence of 20 nM *E. cloacae* NR at pH 5.8 (1), 7.0 (2,4), and 8.0 (3). The time of enzyme addition indicated by arrow.

First is a three-step ( $\text{e}^-$ ,  $\text{H}^+$ ,  $\text{e}^-$ ) hydride transfer with the partly rate-limiting transfer of the first electron and proton and the transient formation of ion–radical pairs or charge–transfer complexes:



For this mechanism, the reaction rate should increase with an increase in quinone single-electron reduction potentials at pH 7.0 ( $\text{E}_7^1$ ), determined by pulse radiolysis, and  $\text{pK}_a$ s for their semiquinones ( $\text{pK}_a(\text{QH}^\cdot)$  [36], which are presented in Table 1. Second is a single-step hydride transfer:

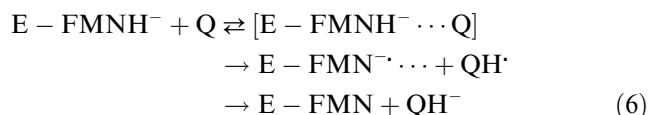


For a net hydride transfer model (Eq. (4)), we may use the approach that Carlson and Miller [35] applied for the analysis of the single-step hydride reduction of quinones at pH 7.0. The reaction rate should increase with an increase in the potential of hydride transfer (potential of quinone/anionic hydroquinone redox couple,  $\text{E}_7(\text{Q}/\text{QH}^-)$ ). The latter is equal to the standard (voltammetric two-electron reduction) potential of quinone ( $\text{E}_7^0$ ), if  $\text{pK}_a$  of corresponding hydroquinone ( $\text{pK}_a(\text{QH}_2)$ , Table 1) is equal to or below 7.0, or

$$\text{E}_7(\text{Q}/\text{QH}^-) = \text{E}_7^0 - 0.029 \text{V}(\text{pK}_a(\text{QH}_2) - 7.0), \quad (5)$$

if  $\text{pK}_a(\text{QH}_2) > 7.0$ . The values of  $\text{E}_7^1$ ,  $\text{pK}_a(\text{QH}^\cdot)$ , and  $\text{E}_7(\text{Q}/\text{QH}^-)$  of quinones are given in Table 1.

Hypothetically, there also may exist an intermediate mechanism of two-step hydride transfer:



with a rate-limiting hydrogen atom (concerted electron and proton) transfer, whose rate should also depend on  $\text{E}_7^1$  and  $\text{pK}_a(\text{QH}^\cdot)$  of quinone [36]. It is difficult to distinguish this mechanism from the rate-limiting electron transfer and the subsequent fast proton transfer [45]. It has been suggested that this mechanism may take place at pH below  $\text{pK}_a(\text{QH}^\cdot)$  [36]. However,  $\text{pK}_a(\text{QH}^\cdot)$  of the examined quinones are in the range of 2.8–5.1 (Table 1), and the studies of NADH oxidation in acidic media are problematic due to NADH instability. Therefore, we have also examined riboflavin ( $\text{pK}_a(\text{QH}^\cdot) = 8.5$  (Table 1)) as nitroreductase substrate.

Since the quinone reductase activity of homologous *E. coli* nitroreductase is supposed to be influenced by oxidant size [16], Table 1 also contains the calculated  $\text{VdWvol}$  of quinoidal compounds.

Table 1

Single-electron reduction potentials of quinones at pH 7.0 ( $E_7^1$ ),  $pK_a$  of their semiquinones ( $pK_a(QH^{\cdot-})$ ), standard (voltammetric) two-electron reduction potentials of quinones at pH 7.0 ( $E_7^0$ ),  $pK_a$  of hydroquinones ( $pK_a(QH_2)$ ), hydride transfer potentials ( $E_7(Q/QH^{\cdot-})$ ) (calculated by Eq. (5)), and van der Waals volumes (VdWvol) of quinones

No.	Compound	$E_7^1$ (V)	$pK_a(QH^{\cdot-})$	$E_7^0$ (V)	$pK_a(QH_2)$	$E_7(Q/QH^{\cdot-})$	VdWvol ( $\text{\AA}^3$ )
1	1,4-Benzoquinone	0.09	4.10	0.28	9.9	0.195	124.6
2	2-Methyl-1,4-benzoquinone	0.01	4.45	0.21	10.0	0.120	142.5
3	2,3-Dichloro-1,4-naphthoquinone	-0.035	–	0.08	–	–	206.5
4	2,5-Diaziridinyl-1,4-benzoquinone <sup>a</sup>	-0.054	5.00	–	–	–	199.2
5	2,6-Dimethyl-1,4-benzoquinone	-0.08	4.75	0.16	10.4	0.058	160.6
6	5-Hydroxy-1,4-naphthoquinone	-0.09	3.65	-0.02	8.5	-0.060	179.7
7	5,8-Dihydroxy-1,4-naphthoquinone	-0.11	2.80	-0.06	7.8	-0.084	192.3
8	9,10-Phenanthrene quinone	-0.12	–	0.02	8.8	-0.034	218.3
9	1,4-Naphthoquinone	-0.15	4.10	0.04	9.3	-0.029	168.1
10	2-Methyl-5-hydroxy-1,4-naphthoquinone	-0.16	–	-0.08	–	–	199.6
11	Trimethyl-1,4-benzoquinone	-0.17	5.00	0.11	10.8	0.00	178.5
12	2-Methyl-1,4-naphthoquinone	-0.20	4.50	-0.03	9.8	-0.114	187.2
13	2,5-Dimethyl-3,6-diaziridinyl-1,4-benzoquinone <sup>b</sup>	-0.23	–	–	–	–	237.7
14	9,10-Anthraquinone-2,6-disulfonate <sup>c</sup>	-0.25	3.00	-0.20	7.4	-0.21	314.5
15	Tetramethyl-1,4-benzoquinone	-0.26	5.10	0.04	11.2	-0.086	197.5
16	1,4-Dihydroxy-9,10-anthraquinone	-0.27	3.30	–	–	–	245.5
17	Mitomycin C <sup>d</sup>	-0.31	–	-0.13	$\geq 10.0$	$\leq -0.22$	313.9
18	Riboflavin	-0.318	8.5	-0.21	6.7	-0.21	422.7
19	1,8-Dihydroxy-9,10-anthraquinone	-0.325	3.95	–	–	–	245.5
20	Adriamycin	-0.34	2.80	-0.30	8.1	-0.34	600.5
21	9,10-Anthraquinone-2-sulfonate	-0.38	3.25	-0.23	8.65	-0.25	266.4
22	1-Hydroxy-9,10-anthraquinone	-0.385	4.6	–	–	–	231.5
23	2-Hydroxy-1,4-naphthoquinone	-0.41	4.7	-0.14	9.0	-0.20	179.7
24	2-Methyl-3-hydroxy-1,4-naphthoquinone <sup>e</sup>	-0.46	–	-0.18	8.9	-0.24	199.6

Note. The values of  $E_7^1$ ,  $pK_a(QH^{\cdot-})$ ,  $E_7^0$ , and  $pK_a(QH_2)$  are taken from [17–23,38,39], unless stated otherwise.

<sup>a</sup>  $E_7^1$  value from [40],  $pK_a$  of semiquinone from [41].

<sup>b</sup> J. Butler, personal communication.

<sup>c</sup>  $pK_a$  of hydroquinone from [42].

<sup>d</sup>  $E_7^0$  and  $pK_a$  of hydroquinone from [43].

<sup>e</sup>  $E_7^1$  value calculated assuming that 3-methyl group should decrease  $E_7^1$  of 2-hydroxy-1,4 naphthoquinone by 0.05 V; other parameters from [44].

The reduction of quinones by *E. cloacae* NR followed the “ping-pong” scheme, as evidenced by a series of parallel Lineweaver–Burk plots at fixed concentrations of NADH and varied quinone concentrations (Fig. 2A), with pronounced inhibition at high quinone concentrations. The values of  $k_{cat}$ , obtained by means of extrapolation of maximal reaction rates at varied NADH concentrations to infinite NADH concentration, are given in Table 2, together with the bimolecular steady-state reduction rate constants of quinones,  $k_{cat}/K_m$ . The value of  $k_{cat}/K_m$  of NADH ( $(5.3 \pm 0.7) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) was almost identical for several electron acceptors used (1,4-benzoquinone, 2,5-diaziridinyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone) and matched the value determined in the nitroreductase reaction [15]. The reactions of *E. cloacae* NR with single-electron acceptors ferricyanide,  $\text{Fe}(\text{EDTA})^-$  and 1,1'-dibenzyl-4,4'-bipyridinium were studied also (Table 2). The  $k_{cat}/K_m$  of ferricyanide and  $\text{Fe}(\text{EDTA})^-$  decrease about 1.6 times upon an increase of buffer solution concentration from 0.05 to 0.2 M Tris–Cl, pH 7.0.

Next, we examined the character of *E. cloacae* NR inhibition by quinones. Except for the derivatives of 2-hydroxy-1,4-naphthoquinone, the kinetic data of

quinone reduction did not follow Eq. (1), which describes the “ping-pong” scheme of the reaction accompanied by the competitive to NADH inhibition of NR by quinones. Thus, the quinone substrate inhibition constants ( $K_{iq}$ ) were determined at several fixed NADH concentrations as the intercepts of plots  $[E]/v$  vs  $[Q]$  with the  $x$ -axis (data not shown). The obtained  $K_{iq}$  values were plotted against NADH concentrations (Fig. 2B). It is evident, that 2-hydroxy-1,4-naphthoquinone acts as a competitive inhibitor to NADH, since its  $K_{iq}$  decreased with a decrease in NADH concentration, giving  $K_{iq} = 17 \mu\text{M}$  at zero NADH concentration (Fig. 2B). In addition,  $K_{iq}$  of 2-hydroxy-1,4-naphthoquinone increased in the presence of dicumarol, a competitive to NADH inhibitor of *E. cloacae* NR ( $K_i = 60 \text{ nM}$ ) (Fig. 2B). 2-Methyl-3-hydroxy-1,4-naphthoquinone acted as a competitive to NADH inhibitor also (data not shown). This shows that the derivatives of 2-hydroxy-1,4-naphthoquinone compete for a NADH binding site in the oxidized enzyme form. Surprisingly, the other quinones examined acted as noncompetitive to NADH inhibitors, since their  $K_{iq}$  did not depend on NADH concentration (Fig. 2B) and did not change in the presence of

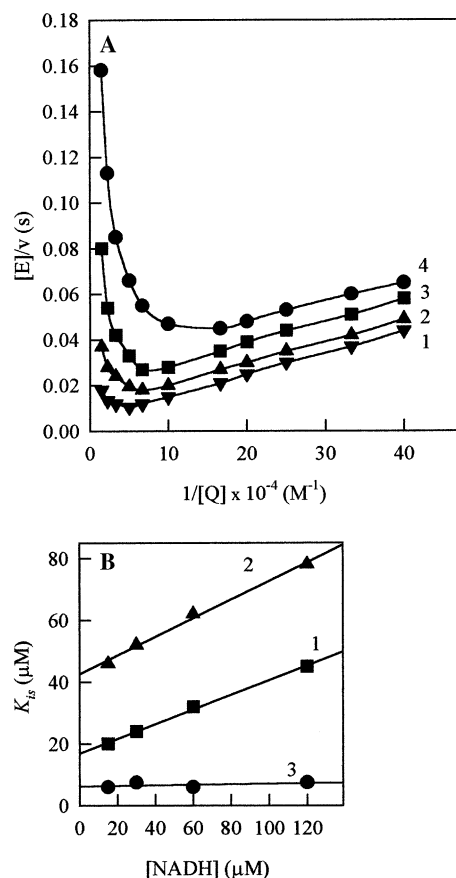


Fig. 2. (A) The dependence of the rate of enzymatic oxidation of NADH by 2-hydroxy-1,4-naphthoquinone on the concentration of substrates. Concentration of NADH, 120  $\mu M$  (1), 60  $\mu M$  (2), 30  $\mu M$  (3), and 15  $\mu M$  (4), pH 7.0. (B) The dependence of the quinone substrate inhibition constants ( $K_{is}$ ) on NADH concentration: 2-hydroxy-1,4-naphthoquinone (1), 2-hydroxy-1,4-naphthoquinone in the presence of 100 nM dicumarol (2), and 5,8-dihydroxy-1,4-naphthoquinone (3), pH 7.0.

100 nM dicumarol. The  $K_{iq}$  values of quinones examined are given in Table 2.

Previously, *p*-nitrobenzoic acid has been shown to be a competitor to NADH inhibitor of *E. cloacae* NR with  $K_{iq} = 220 \mu M$  [14]. According to our preliminary studies, *m*-dinitrobenzene was a more efficient competitive to NADH inhibitor. The  $K_{iq}$  value of *m*-dinitrobenzene decreased with a decrease in NADH concentration, reaching 36  $\mu M$  at zero NADH concentration, and increased in the presence of dicumarol (data not shown).

The increase in pH from 5.8 to 8.0 increased  $k_{cat}/K_m$  of tetramethyl-1,4-benzoquinone, 2-methyl-5-hydroxy-1,4-naphthoquinone, and riboflavin by three to four times (Fig. 3).

The FMN fluorescence intensity of *E. cloacae* NR is about 20% of the intensity of free FMN. Dicumarol quenched the FMN fluorescence by 85% (Fig. 4A). Quinones also quenched the fluorescence of FMN (Fig. 4B); however, the extent of quenching by 2-methyl-1,4-

naphthoquinone was only 20%. Binding of menadione to nitroreductase was characterized by  $K_d = 18 \pm 3 \mu M$ , close to the menadione  $K_{iq}$  in NR-catalyzed reduction (Table 2). A competitive to NADH inhibitor, 2-hydroxy-1,4-naphthoquinone, quenched the FMN fluorescence to a greater extent (Fig. 4B). However, this is attributed not to a different mode of NR inhibition, but to its strong absorbance at 460 nm, since 5-hydroxy-1,4-naphthoquinone, possessing similar absorbance characteristics, quenched the FMN fluorescence to a similar extent (Fig. 4B). *m*-Dinitrobenzene also quenched the FMN fluorescence by 16% with  $K_d = 32 \pm 4 \mu M$  (Fig. 4B).

## Discussion

Single-electron reduction of quinones by flavoenzymes dehydrogenases–electrontransferases, e.g., NADPH:cytochrome P-450 reductase (EC 1.6.2.4) and ferredoxin: NADP<sup>+</sup> reductase (EC 1.18.1.2), follows an “outer-sphere” electron-transfer mechanism, the reaction rate constants increasing with an increase in  $E_7^1$  of oxidants and being relatively insensitive to their structure [46,47]. The mechanism of quinone reduction by two-electron transferring flavoenzymes is unclear. In the most extensively studied two-electron reduction reactions of mammalian DT-diaphorase (NAD(P)H: quinone reductase, EC 1.6.99.2) [26,48,49], quinone reactivity does not correlate with their redox potential, being strongly influenced by their structure. The specificity of DT-diaphorase for the particular quinone structure is not completely clear. On the other hand, in the nonenzymatic two-electron (hydride) oxidation of 1,4-dihydropyridines, quinone reactivity increases with an increase in their reduction potential [35,36]. In addition,  $\log k_{cat}/K_m$  of nitroaromatic compounds in *E. cloacae* NR-catalyzed reduction follows parabolic (second-order) dependence on their  $E_7^1$ , reaching the limiting value at sufficiently high reduction potential [15].

In the present study, we have undertaken an initial attempt to distinguish between charge-transfer energetics and substrate structure effects in quinone reduction by *E. cloacae* nitroreductase. Except for derivatives of 2-hydroxy-1,4-naphthoquinone, which were much more reactive than one may expect from their  $E_7^1$  values, the  $\log k_{cat}$  and  $\log k_{cat}/K_m$  of compounds 1–22 (Tables 1 and 2) increased with an increase in their  $E_7^1$ , approaching the limiting values at high reduction potential (Figs. 5A and B). Interestingly, the reactivity of riboflavin, which accepts electron and proton (net hydrogen atom) during reduction at pH 7.0 and hypothetically may follow a two-step hydride transfer mechanism (Eq. (4)), did not deviate from the correlations. Our data (Fig. 2B) demonstrate that the binding site of 2-hydroxy-1,4-naphthoquinones are identical to or strongly

Table 2

Catalytic constants ( $k_{\text{cat}}$ ), bimolecular rate constants ( $k_{\text{cat}}/K_m$ ), and quinone substrate inhibition constants ( $K_{\text{iq}}$ ) of quinoidal compounds and single-electron acceptors in *Enterobacter cloacae* nitroreductase-catalyzed reactions (pH 7.0, 25 °C)

No.	Compound	$k_{\text{cat}}$ (s <sup>-1</sup> )	$k_{\text{cat}}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	$K_{\text{iq}}$ (μM)
1	1,4-Benzoquinone	1200 ± 50	(1.4 ± 0.1) × 10 <sup>8</sup>	78 <sup>a</sup>
2	2-Methyl-1,4-benzoquinone	666 ± 37	(5.0 ± 0.4) × 10 <sup>7</sup>	100 <sup>a</sup>
3	2,3-Dichloro-1,4-naphthoquinone	250 ± 18	(1.3 ± 0.1) × 10 <sup>7</sup>	64 <sup>a</sup>
4	2,5-Diaziridinyl-1,4-benzoquinone	1610 ± 80	(2.4 ± 0.2) × 10 <sup>7</sup>	148 <sup>a</sup>
5	2,6-Dimethyl-1,4-benzoquinone	704 ± 82	(1.8 ± 0.2) × 10 <sup>7</sup>	50 <sup>a</sup>
6	5-Hydroxy-1,4-naphthoquinone	77 ± 5.2	(1.2 ± 0.1) × 10 <sup>7</sup>	24 <sup>a</sup>
7	5,8-Dihydroxy-1,4-naphthoquinone	111 ± 8.7	(2.5 ± 0.1) × 10 <sup>7</sup>	6 <sup>a</sup>
8	9,10-Phenanthrene quinone	333 ± 12	(2.4 ± 0.1) × 10 <sup>7</sup>	44 <sup>a</sup>
9	1,4-Naphthoquinone	200 ± 15	(2.2 ± 0.1) × 10 <sup>7</sup>	22 <sup>a</sup>
10	2-Methyl-5-hydroxy-1,4-naphthoquinone	150 ± 10	(1.0 ± 0.1) × 10 <sup>6</sup>	100 <sup>a</sup>
11	Trimethyl-1,4-benzoquinone	400 ± 30	(1.0 ± 0.1) × 10 <sup>7</sup>	200 <sup>a</sup>
12	2-Methyl-1,4-naphthoquinone	50 ± 3.0	(3.5 ± 0.1) × 10 <sup>6</sup>	20 <sup>a</sup>
13	2,5-Dimethyl-3,6-diaziridinyl-1,4-benzoquinone	71 ± 5.0	(2.9 ± 0.1) × 10 <sup>5</sup>	≥ 400 <sup>a</sup>
14	9,10-Anthraquinone-2,6-disulfonate	0.83 ± 0.05	(1.2 ± 0.1) × 10 <sup>4</sup>	500 <sup>a</sup>
15	Tetramethyl-1,4-benzoquinone	17 ± 1.1	(3.8 ± 0.1) × 10 <sup>4</sup>	≥ 1000 <sup>a</sup>
16	1,4-Dihydroxy-9,10-anthraquinone	1.5 ± 0.1	(2.6 ± 0.2) × 10 <sup>4</sup>	N.D.
17	Mitomycin C	1.0 ± 0.1	(2.3 ± 0.2) × 10 <sup>4</sup>	N.D.
18	Riboflavin	5.3 ± 0.2	(1.0 ± 0.1) × 10 <sup>5</sup>	N.D.
19	1,8-Dihydroxy-9,10-anthraquinone	1.0 ± 0.1	(5.0 ± 0.3) × 10 <sup>4</sup>	N.D.
20	Adriamycin	≤ 0.1	≤ 10 <sup>3</sup>	N.D.
21	9,10-Anthraquinone-2-sulfonate	0.83 ± 0.1	(2.5 ± 0.1) × 10 <sup>4</sup>	120 <sup>a</sup>
22	1-Hydroxy-9,10-anthraquinone	1.5 ± 0.1	(1.2 ± 0.1) × 10 <sup>4</sup>	N.D.
23	2-Hydroxy-1,4-naphthoquinone	160 ± 9.0	(1.2 ± 0.1) × 10 <sup>7</sup>	17 <sup>b</sup>
24	2-Methyl-3-hydroxy-1,4-naphthoquinone	111 ± 10	(3.7 ± 0.2) × 10 <sup>6</sup>	12 <sup>b</sup>
25	1,1'-Dibenzyl-4,4'-bipyridinium ( $E_7^1 = -0.354$ V)	< 0.03	< 10 <sup>2</sup>	N.D.
26	Ferricyanide ( $E_7^1 = 0.41$ V)	39 ± 1.5	(5.0 ± 0.3) × 10 <sup>5</sup>	N.D.
27	Fe(EDTA) <sup>-</sup> ( $E_7^1 = 0.12$ V)	5.0 ± 0.3	(1.5 ± 0.1) × 10 <sup>4</sup>	N.D.

<sup>a</sup> Noncompetitive to NADH inhibitor.

<sup>b</sup> Competitive to NADH inhibitor.

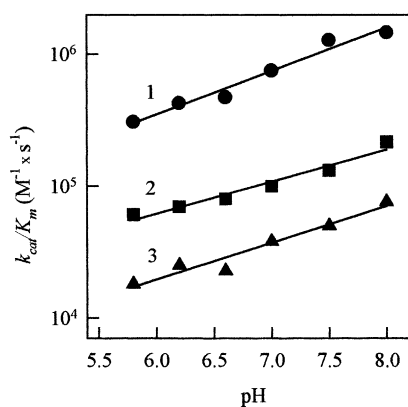


Fig. 3. pH Dependence of  $k_{\text{cat}}/K_m$  of 2-methyl-5-hydroxy-1,4-naphthoquinone (1), riboflavin (2), and tetramethyl-1,4-benzoquinone (3) in NR-catalyzed reaction.

overlap with NADH and dicumarol binding sites, whereas other quinones bind in a different way. This may explain the increased reactivity of 2-hydroxy-1,4-naphthoquinones (Figs. 5A and B). The domain of dicumarol binding of *E. cloacae* NR has not been characterized so far. However, in homologous *V. fischeri* FMN reductase, dicumarol binds between the isoalloxazine ring of FMN and the side chain of Phe-124 residue, interacting with Ser-42 and Ile-43 [50]. Since

Phe-124 and Ser-40 are conserved in the active center of *E. coli* and *E. cloacae* NR [5,12,13], one may suggest that dicumarol may bind in the analogous domain of nitroreductases [12]. The FMN fluorescence quenching by quinones and nitroaromatics (Figs. 4A and B) shows that these compounds bind close to FMN. Thus, the existence of the second binding site of quinones in *E. cloacae* NR may seem unexpected. On the other hand, the X-ray analysis of *E. coli* NR demonstrates the presence of two potential channels for substrate access to FMN, the first one close to Lys-14, Lys-74, and Phe-70 and the second one close to Phe-124 [12]. These conserved residues occupy analogous positions in the active center of *E. cloacae* NR [13], thus pointing to possible existence of two quinone access (binding) sites. The criteria of quinone preferences for each site should be elucidated in the future.

In more detailed analysis using the electron-proton-electron hydride transfer model (Eq. (1)), we considered the possible quinone reactivity increase upon an increase in  $\text{p}K_{\text{a}}(\text{QH}^{\cdot})$  of semiquinones [36] (Table 1). In addition, it has been suggested that Phe-124 residue in *E. coli* NR, which is conserved in *E. cloacae* NR also, causes sterical hindrances for the reduction of bulky riboflavin derivatives [16]. Thus, the reaction rate may decrease with an increase in van der Waals volume of substrate (Table 1).

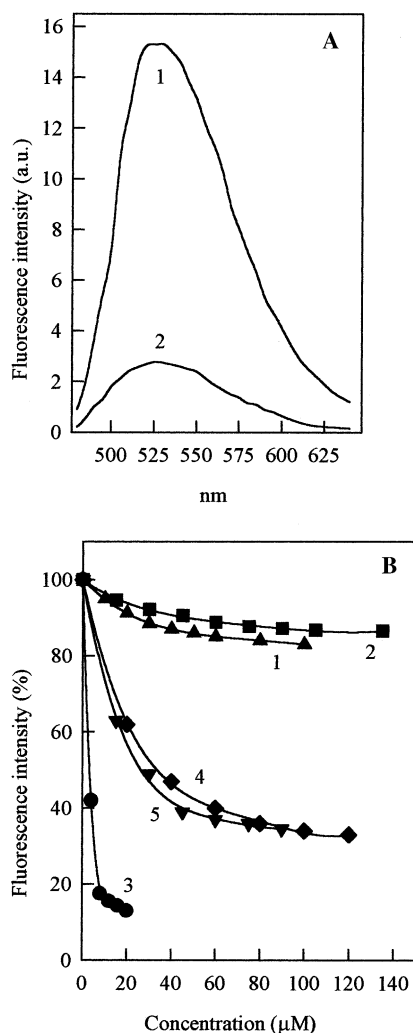


Fig. 4. (A) Fluorescence spectra of FMN of *E. cloacae* NR in the absence (1) and in the presence (2) of 12  $\mu\text{M}$  dicumarol. Enzyme concentration, 6  $\mu\text{M}$ , excitation wavelength, 460 nm. (B) The quenching of *E. cloacae* NR FMN fluorescence by menadione (1), *m*-dinitrobenzene (2), dicumarol (3), 5-hydroxy-1,4-naphthoquinone (4), and 2-hydroxy-1,4-naphthoquinone (5). Enzyme concentration, 6  $\mu\text{M}$ , emission wavelength, 530 nm, excitation wavelength, 460 nm (1–3), or 500 nm (4,5), pH 7.0.

It is possible that the nonlinear character of plots in Figs. 5A and B is caused by the limitation of  $k_{\text{cat}}/K_{\text{m}}$  by substrate diffusion, or by the change in the rate-limiting step ( $k_{\text{cat}}$ ) in the enzyme catalysis. Thus, only the rate constants of low-potential compounds 6, 7, 9, 11, 12, 14–16, 18–22 with available values of  $\text{p}K_{\text{a}}(\text{QH}^{\cdot})$  (Table 1), except 2-hydroxy-1,4-naphthoquinone derivatives, were analyzed using the linear multiparameter regression:

$$\log(\text{rate constant}) = a + bE_7^1 + c\text{VdWvol} + d\text{p}K_{\text{a}}(\text{QH}^{\cdot}). \quad (7)$$

The calculated coefficients in the Eq. (7) (Table 3) demonstrate that the reactivity of quinones indeed in-

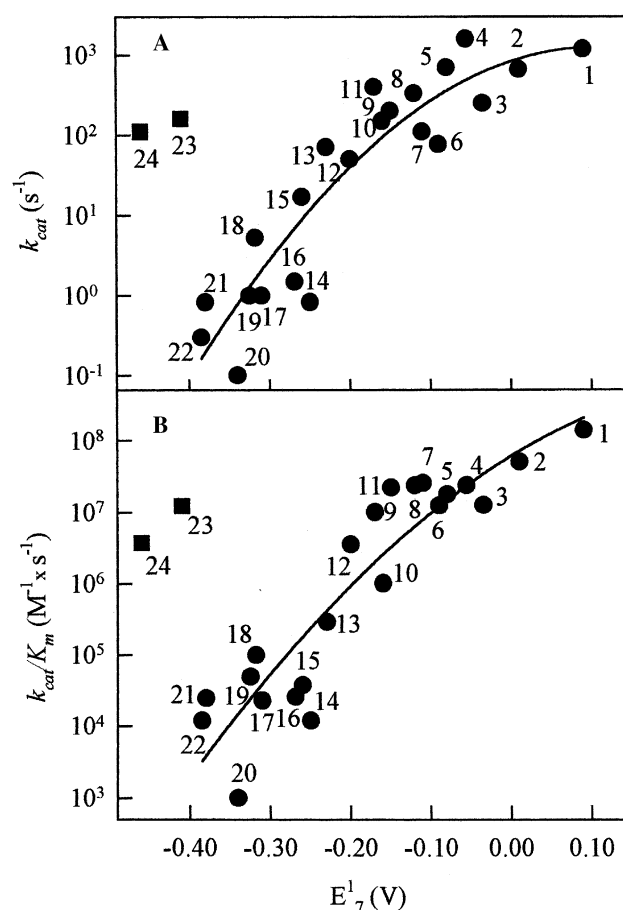


Fig. 5. The parabolic relationships between  $\log k_{\text{cat}}$  (A) or  $\log k_{\text{cat}}/K_{\text{m}}$  (B) of quinoidal oxidants in *E. cloacae* NR-catalyzed reduction and their single-electron reduction potentials ( $E_7^1$ ). The numbers of compounds, rate constants, and redox potentials are taken from Tables 1 and 2. The correlations representing the second order polynomial regression describe the reactivities of compounds 1–22.

creases with an increase in  $\text{p}K_{\text{a}}(\text{QH}^{\cdot})$  and with a decrease in  $\text{VdWvol}$ .

Alternatively, analyzing quinone reactivity in terms of the single-step hydride transfer model (Eq. (4)), we also observed an increase of  $\log k_{\text{cat}}$  and  $\log k_{\text{cat}}/K_{\text{m}}$  on  $E_7(\text{Q}/\text{QH}^{\cdot})$  for all compounds with available  $\text{p}K_{\text{a}}(\text{QH}_2)$  (Table 1), except 2-hydroxy-1,4-naphthoquinones, which showed an enhanced reactivity (Figs. 6A and B). Again, the multiparameter analysis using the linear regression (Eq. (8)) was confined to the rate constants of low-potential compounds 6–9, 11, 12, 14, 15, 17, 18, 20, and 21 (Table 1):

$$\log(\text{rate constant}) = a + bE_7(\text{Q}/\text{QH}^{\cdot}) + c\text{VdWvol}. \quad (8)$$

Interestingly, in this case we failed to observe the well-expressed increase in the reactivity of quinones with a decrease in their  $\text{VdWvol}$  (Table 3) and thus did not obtain equivocal support for the role of Phe-124 in the steric control of reaction.

Table 3

The results of multiparameter analysis of quinone reduction rate constants ( $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$ ) according to Eqs. (7) and (8)

Rate constant	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>r</i> <sup>2</sup>
$k_{\text{cat}}^a$	1.9844 ± 0.3857	8.2355 ± 1.2437	−0.0025 ± 0.0010	0.4074 ± 0.0689	0.9276
$k_{\text{cat}}/K_m^a$	8.2965 ± 0.6813	11.3064 ± 2.1966	−0.0036 ± 0.0017	0.1980 ± 0.1216	0.8670
$k_{\text{cat}}^b$	2.3137 ± 0.3694	13.3827 ± 2.7207	0.0028 ± 0.0022	–	0.8670
$k_{\text{cat}}/K_m^b$	7.5105 ± 0.5977	13.3041 ± 4.4016	−0.0006 ± 0.0036	–	0.7978

Note. Coefficients *b*, *c* and *d* represent the parameter weights, characterizing the influence of reduction potential ( $E_7^1$  or  $E_7(\text{Q}/\text{QH}^-)$ ), van der Waals volume of quinones (VdWvol), and  $\text{p}K_a$  of their semiquinones ( $\text{p}K_a(\text{QH}^-)$ ) on the corresponding rate constants.

<sup>a</sup>  $E_7^1$ , VdWvol, and  $\text{p}K_a(\text{QH}^-)$  as variables (Eq. (7)).

<sup>b</sup>  $E_7(\text{Q}/\text{QH}^-)$  and VdWvol as variables (Eq. (8)).

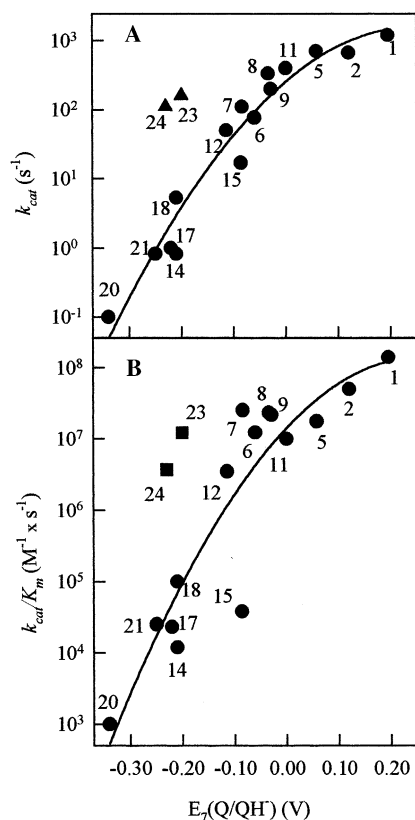


Fig. 6. The relationships between  $\log k_{\text{cat}}$  (A) or  $\log k_{\text{cat}}/K_m$  (B) of quinoidal oxidants in *E. cloacae* NR-catalyzed reduction and their hydride transfer potentials ( $E_7(\text{Q}/\text{QH}^-)$ ). The numbers of compounds, rate constants, and redox potentials are taken from Tables 1 and 2. The correlations representing the second order polynomial regression describe the reactivities of compounds except 2-hydroxy-1,4-naphthoquinone derivatives (compounds 23 and 24).

Although the correlations (Eqs. (7) and (8)) represent significant simplification, they show that the reactivity of quinones in *E. cloacae* NR-catalyzed reduction may be equally well described in terms of three-step (Eq. (1)) and single-step (Eq. (2)) hydride transfer mechanisms. Since the initial single-electron transfer step is involved in a three-step hydride transfer mechanism, it is important to analyze the reactivity of NR with single-electron acceptors.

According to the model of “outer-sphere electron transfer” [51], the rate constant of electron transfer be-

tween reagents ( $k_{12}$ ) depends on electron self-exchange constants of redox protein ( $k_{11}$ ), electron acceptor ( $k_{22}$ ), and equilibrium constant of reaction ( $K$ ) ( $\log K = n\Delta E_1^7(\text{V})/0.059$ ,  $n$ , the number of electrons transferred):

$$k_{12} = (k_{11}k_{22}K \cdot f)^{1/2} \quad (9)$$

and

$$\log f = (\log K)^2/4 \log(k_{11}k_{22}/Z^2), \quad (10)$$

where  $Z$  is a frequency factor ( $10^{11} \text{ M}^{-1} \text{ s}^{-1}$ ). The  $k_{11}$  of protein may be obtained as follows [52]:

$$\log k_{11} = (\log k_{12} - 0.5 \log K + \log Z) - \log k_{22} - [(\log Z - \log k_{12})^2 + \log K(\log Z - \log k_{12})]^{1/2}. \quad (11)$$

The  $E_7^0$  of *E. cloacae* NR is  $-0.19 \text{ V}$ , with an upper limit of  $-0.29 \text{ V}$  for FMN/FMN semiquinone couple and a lower limit of  $-0.09 \text{ V}$  for FMN semiquinone/FMNH<sup>-</sup> couple [37]. Thus, single-electron acceptors may oxidize FMN semiquinone more easily than FMNH<sup>-</sup>, and the transfer of the first electron may be rate limiting. The analogous mechanism is characteristic of the oxidation of reduced DT-diaphorase by ferricyanide, where the oxidation of FAD<sup>-•</sup> is much faster than the oxidation of reduced FAD, since the redox potential of FAD/FAD<sup>-•</sup> couple ( $-0.20 \text{ V}$ ) is more negative than the potential of FAD<sup>-•</sup>/reduced FAD ( $-0.118 \text{ V}$ ) [48]. The results of the calculations, using  $-0.09 \text{ V}$  as a lower limit for the FMN semiquinone/FMNH<sup>-</sup> couple of *E. cloacae* NR and  $k_{\text{cat}}/K_m$  values of single-electron acceptors as  $k_{12}$  (Table 2), are given in Table 4. The difference in  $k_{11}$  values obtained using various oxidants is usually attributed to the decreased ability of inorganic complexes to penetrate into the protein globule and, consequently, to the increased distance of the electron transfer [51]. It is important to note that  $k_{11}$  values of NR in reactions with inorganic complexes are even higher than  $k_{11}$  of NADPH:cytochrome P-450 reductase, which performs an obligatory single-electron transfer [46] (Table 4). High self-exchange constants of cytochrome P-450 reductase are related to good solvent accessibility of the pyrimidine side of isoalloxazine ring of FMN [53]. According to the X-ray data, the O2,N3,O4 part of pyrimidine ring of FMN of *E. coli* and *E. cloacae* NR is also

Table 4

Electron self-exchange rate constants ( $k_{22}$ ) of single-electron accepting oxidants, and electron self-exchange rate constants ( $k_{11}$ ) of *E. cloacae* NR and NADPH:cytochrome P-450 reductase (P-450R) calculated by Eq. (11)

Oxidant	$k_{22}$ ( $M^{-1} s^{-1}$ )	$k_{11}$ ( $M^{-1} s^{-1}$ )	
		<i>E. cloacae</i> NR	P-450 R
1,1'-Dibenzyl-4,4'-bipyridinium	$1.0 \times 10^8$	> 4.0	$1.0 \times 10^4$
Ferricyanide	$4.6 \times 10^5$	> $2.0 \times 10^{-2}$	$6.0 \times 10^{-3}$
Fe(EDTA) <sup>-</sup>	$6.9 \times 10^4$	> 1.30	$4.0 \times 10^{-2}$

Note. The values of  $k_{22}$  of oxidants and  $k_{11}$  of P-450R taken from [46].

close to the surface of protein globule [12,13]. Thus, the reactivity of *E. cloacae* NR with single-electron acceptors is not restricted, being the result of superposition of two opposite factors, namely, good accessibility of FMN to oxidant and high potential of FMN semiquinone/FMNH<sup>-</sup> couple. However, the single-electron accepting 1,1'-dibenzyl-4,4'-bipyridinium is much less reactive than two-electron accepting quinones with similar  $E_7^1$  values (Fig. 5B, Tables 1 and 2). It shows that NR strongly prefers two-electron acceptors.

Typically, the difference between  $E_7^1$  and  $E_7(Q/QH^-)$  of examined quinones is about -50 to -100 mV (Table 2). In addition, the difference between potential of FMNH<sup>-</sup>/FMN semi-quinone couple and  $E_7^0$  of *E. cloacae* NR is 100 mV. It makes the single-step hydride transfer by 150–200 mV more exothermic than the initial single-electron transfer. One may argue that the stabilization of ion-radical pairs (Eq. (1)) may decrease the free energy of activation of the single-electron transfer [36] and make it more favorable. However, some additional conclusions may be obtained from the pH dependence of  $k_{cat}/K_m$  of oxidants (Fig. 3). The  $k_{cat}/K_m$  values of riboflavin and several quinones exhibit the same pH dependence (Fig. 3), although the potential of the single-electron reduction of riboflavin should increase with a decrease in pH, whereas the  $E^1$  values of quinones do not change between pH 5.8 and 8.0 (Table 1). Although we are not aware of the pH profile of *E. cloacae* NR FMN semiquinone, the data of Fig. 3 seem to be inconsistent with a sequential e<sup>-</sup>, H<sup>+</sup>, e<sup>-</sup>-transfer (Eq. (1)), since in this case the reactivity of riboflavin should relatively increase over the reactivity of quinones at lower pH. Thus, the scheme of single-step hydride transfer (Eq. (2)) seems to be more feasible.

In the single-electron reduction of quinones and nitroaromatics by flavoenzymes dehydrogenases-electrontransferases, quinones are more reactive than nitroaromatics in view of their higher electron self-exchange rate constants ( $k_{22}$ , Eq. (9)) [46,47]. The comparison of reactivities of nitroaromatics and quinones in *E. cloacae* NR-catalyzed reactions is problematic, since we are not aware of the potentials of two-electron re-

duction of nitroaromatics. The data of our work and the previous observations [14] show that nitroaromatics are the competitors to NADH inhibitors, binding at or close to the NADH and dicumarol binding sites, thus sharing the properties of 2-hydroxy-1,4-naphthoquinone derivatives (Fig. 2B). However, other quinones bind in a different way. Using  $E_7^1$  as the correlation parameter and the previously obtained [15]  $k_{cat}/K_m$  for some nitroaromatic compounds (*p*-dinitrobenzene,  $E_7^1 = -0.257$  V,  $k_{cat}/K_m = 3.1 \times 10^6 M^{-1} s^{-1}$ ; *p*-nitrobenzaldehyde,  $E_7^1 = -0.315$  V,  $k_{cat}/K_m = 8.0 \times 10^5 M^{-1} s^{-1}$ ; *p*-nitroacetophenone,  $E_7^1 = -0.355$  V,  $k_{cat}/K_m = 8.0 \times 10^5 M^{-1} s^{-1}$ ; and *p*-nitrobenzoic acid,  $E_7^1 = -0.425$  V,  $k_{cat}/K_m = 4.0 \times 10^4 M^{-1} s^{-1}$ ), one may conclude that nitroaromatic compounds are less reactive than 2-hydroxy-1,4-naphthoquinones, but more reactive than other quinones with the same  $E_7^1$  values (Fig. 5B).

Previously, we have observed kinetic isotope effects on  $k_{cat}/K_m$  of quinones during their two-electron reduction by *Clostridium chuyveri* diaphorase [54], and mixed single- and two-electron reduction by lipoamide dehydrogenase [55], using 4-*S*-deuterated NADH as substrate. It shows that proton or hydride is directly transferred from N-5 position of reduced flavin to quinone. Kinetic isotope effect studies, which are currently under way, will result in a more rigorous scheme of two-electron reduction of quinones and nitroaromatic compounds by *E. cloacae* NR and related bacterial oxygen-insensitive nitroreductases.

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