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ABSTRACT

Thermotoga maritima peroxiredoxin–nitroreductase hybrid enzyme (Prx–NR) consists of a FMN-containing nitroreductase (NR) domain fused to a peroxiredoxin (Prx) domain. These domains seem to function independently as no electron transfer occurs between them. The reduction of quinones and nitroaromatics by NR proceeded in a two-electron manner, and follows a ‘ping-pong’ scheme with sometimes pronounced inhibition by quinone substrate. The comparison of steady- and presteady-state kinetic data shows that in most cases, the oxidative half-reaction may be rate-limiting in the catalytic cycle of NR. The enzyme was inhibited by dicumarol, a classical inhibitor of oxygen-insensitive nitroreductases. The reduction of quinones and nitroaromatic compounds by Prx–NR was characterized by the linear dependence of their reactivity ($\log k_{\text{cat}}/K_m$) on their single-electron reduction potentials E_7^{\ominus} , while the reactivity of quinones markedly exceeded the one with nitroaromatics. It shows that NR lacks the specificity for the particular structure of these oxidants, except their single-electron accepting potency and the rate of electron self-exchange. It points to the possibility of a single-electron transfer step in a net two-electron reduction of quinones and nitroaromatics by *T. maritima* Prx–NR, and to a significant diversity of the structures of flavoenzymes which may perform the two-electron reduction of quinones and nitroaromatics.

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Introduction

Bacterial oxygen-insensitive NAD(P)H:nitroreductases (NRs) contain flavin mononucleotide (FMN)¹ in their active center, and perform a net two-electron reduction of nitroaromatic compounds to nitroso, and, subsequently, to hydroxylamine products ([1–11], and references therein). There are two groups of NRs with low sequence homology, which are similar with *Escherichia coli* NADPH-dependent major nitroreductase-A [3], e.g., *Vibrio harveyi* FMN-reductase [4], and *Salmonella typhimurium* nitroreductase [5], or with *E. coli* NAD(P)H-dependent minor nitroreductase-B [6], e.g.,

Enterobacter cloacae nitroreductase [2,7–9], *Vibrio fischeri* FMN-reductase [10], and *Thermus thermophilus* NADH-oxidase [11]. The physiological role(s) of NRs are unclear except their possible antioxidant functions, because they are induced by oxidative stress and other environmental hazards [12]. On the other hand, bacterial NRs are of considerable interest due to their participation in the biodegradation of explosives and other polynitroaromatic environmental pollutants ([12,13], and references therein), and their possible utility in the antibody-, gene-, or virus- directed prodrug therapies ([14,15], and references therein).

It is generally accepted that a net two-electron (hydride) reduction of nitroaromatics by NRs is linked to the instability of their FMN semiquinone state, which makes the single-electron transfer thermodynamically unfavorable [8,10]. However, the other aspects of the catalytic mechanisms of bacterial oxygen-insensitive NRs, e.g., their substrate specificity, and the general mechanism of two-electron nitroreduction remain vaguely understood. Except for the more detailed examination of *E. cloacae* NR [7,16], most kinetic studies of other NRs were confined to a limited number of oxidants with sometimes poorly characterized reduction thermodynamics ([3,6,17], and references therein). The few available crystal structures of *E. cloacae* NR and *E. coli* NR-B with bound nitroaromatic compounds and other

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¹ Abbreviations used: Ar-NO₂, nitroaromatic compound; BCP, bacterioferritin comigratory protein; CB-1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; DNBF, 4,6-dinitrobenzofuroxan; DZQ, diaziridinyl-1,4-benzoquinone; E_7^{\ominus} , single-electron reduction potential at pH 7.0; FMN, flavin mononucleotide; Grx, glutaredoxin; k_{cat} , catalytic constant; k_{cat}/K_m , second-order rate reaction rate constant; K_{q} , quinone substrate inhibition constant; MeDZQ, 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone; NQO1, NAD(P)H:quinone oxidoreductase; NR, nitroreductase; Prx, peroxiredoxin; Prx-NR, peroxiredoxin–nitroreductase hybrid enzyme; Q, quinone; SN-23862, 5-(bis(2,2'-chloroethyl-amino))-2,4-dinitrobenzamide; TNC, 1,3,6,8-tetranitrocarbazole; TNT, 2,4,6-trinitrotoluene; VdWvol, van der Waals volume.

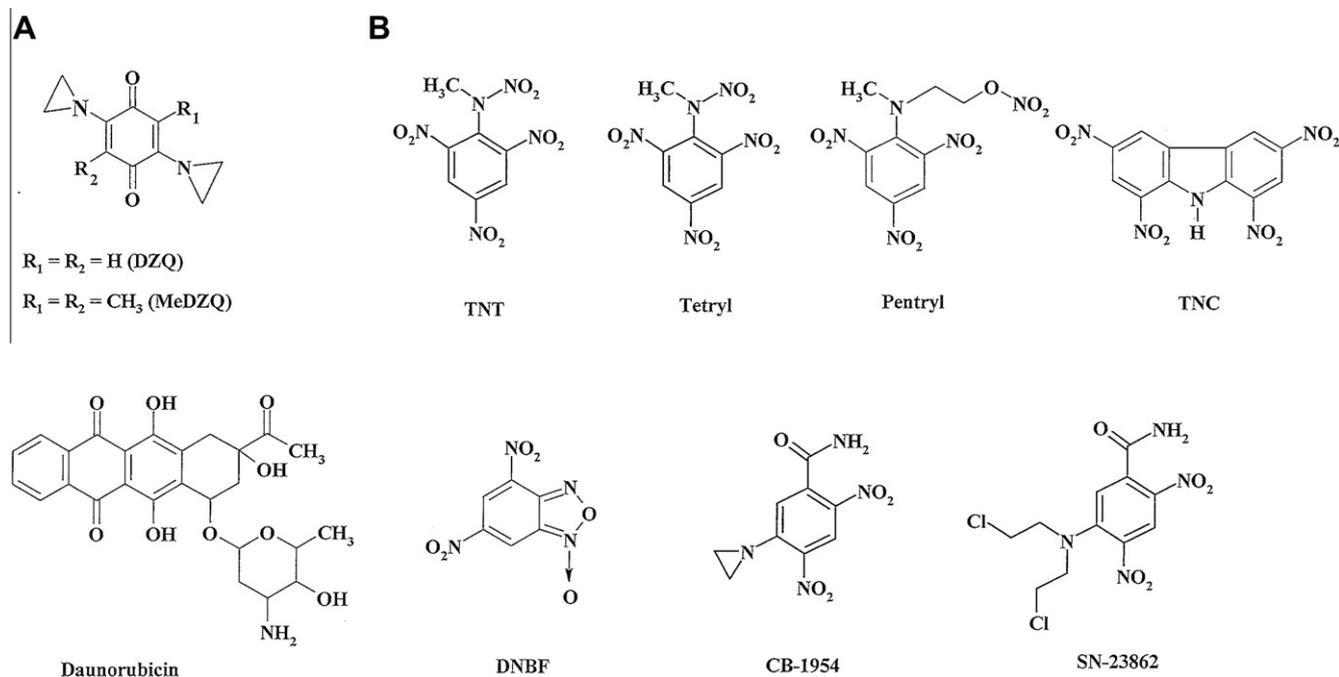


Fig. 1. The formulae of nontrivial quinones and nitroaromatic compounds used in this study.

aromatic ligands point to multiple and sometimes unproductive modes of binding [9,18–20]. A more thorough examination of the catalysis of NRs, including studies of their new representatives would be thus very informative in this respect.

Recently, an NR-related sequence has been found in the thermophilic bacterium *Thermotoga maritima* as a polypeptide of 321 amino acids (Genbank accession number NP_228196). It is constituted of a peroxidoredoxin (Prx) domain of the BCP (bacterioferritin comigratory protein)-type at the N-terminus (~140 amino acids) that contains a catalytic cysteine which is regenerated externally by the glutaredoxin (Grx) system, and an FMN-containing NR domain at the C-terminus (~170 amino acids), which contains an NAD(P)H-binding site [21]. The extra 10 amino acids constitute a linker between the two domains. Previous physicochemical and structural characterization of its Prx domain [21] and of the holoenzyme [22] have shown that the Prx and NR domains function independently without the transfer of the redox equivalents between the domains, i.e., NAD(P)H does not provide the electrons for the reduction of Prx-linked oxidants, and the reduction of NR-linked oxidants does not involve the Prx domain [21].

In this study, we analyze the mechanisms of reactions of Prx–NR with two classes of biologically important xenobiotics, quinones and aromatic nitrocompounds. Apart from the series of model compounds, the studies involved a number of therapeutically/toxicologically important substances such as antitumour agents DZQ, MeDZQ, daunorubicin, CB-1954, and SN-23862, and the environmental contaminants, such as the explosives TNT, tetryl, pentryl, TNC, and DNBF (Fig. 1).

Materials and methods

Enzymes and chemicals

The recombinant *T. maritima* Prx–NR was obtained as previously described [22]. Its concentration was determined according to the absorbance of FMN ($\epsilon_{462} = 11 \text{ mM}^{-1} \text{ cm}^{-1}$). *E. cloacae* NR was a generous gift of Dr. Ronald L. Koder (City College, CUNY, NY, USA). Model quinones and nitroaromatic compounds (the derivatives of

1,4-benzoquinone, 1,4-naphthoquinone, 9,10-phenanthrene quinone, 9,10-anthraquinone, and nitrobenzenes), NADH, NADPH, dicumarol, cytochrome c, superoxide dismutase (SOD), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma–Aldrich, Applichem, and Fluka. Daunorubicin was obtained from Minmedprom (Russia). Diaziridinyl-1,4-benzoquinone (DZQ) and 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone (MeDZQ) were synthesized as described [23]. 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB-1954) and 5-(bis(2,2'-chloroethyl-amino))-2,4-dinitrobenzamide (SN-23862) synthesized according to the established methods [24], were the generous gift of Dr. Vanda Miškinienė (Institute of Biochemistry, Vilnius). 2,4,6-Trinitrotoluene (TNT), 2,4,6-trinitrophenyl-N-methylnitramine (tetryl), 2,4,6-trinitrophenyl-N-nitroaminoethylnitrate (pentryl), 1,3,6,8-tetranitrocarbazole (TNC), and 4,6-dinitrobenzofuroxan (DNBF) were synthesized as described previously [7]. The purities of synthesized compounds were characterized by their melting points, TLC, ^1H NMR, UV, and IR spectroscopy and elemental analysis. The formulae of nontrivial quinoidal and nitroaromatic compounds are given in Fig. 1.

Steady-state kinetic studies

The steady-state kinetic measurements were performed spectrophotometrically using the Hitachi-557 spectrophotometer in 0.1 M Tris–HCl (pH 7.0), containing 1 mM EDTA, at 25 °C. The initial rates of enzymatic NADH-mediated quinone- or nitroreduction were determined by monitoring the oxidation of NADH ($\Delta\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$), after the correction for the NADH oxidase activity of Prx–NR, 0.03 s^{-1} , and for the nonenzymatic oxidation of NADH by high potential 1,4-benzoquinone and 2-methyl-1,4-benzoquinone, or for the spectral changes at 340 nm, corresponding to two(four)-electron reduction of nitrobenzenes [7]. The steady-state parameters of reactions, the catalytic constants (k_{cat}) and the bimolecular rate constants ($k_{\text{cat}}/K_{\text{m}}$) of the oxidants at fixed concentration of NADH correspond to the reciprocal intercepts and slopes of Lineweaver–Burk plots, $[E]/v$ vs. $1/[Q]$, where v is the reaction rate, $[E]$ is the enzyme concentration, and $[Q]$ is the

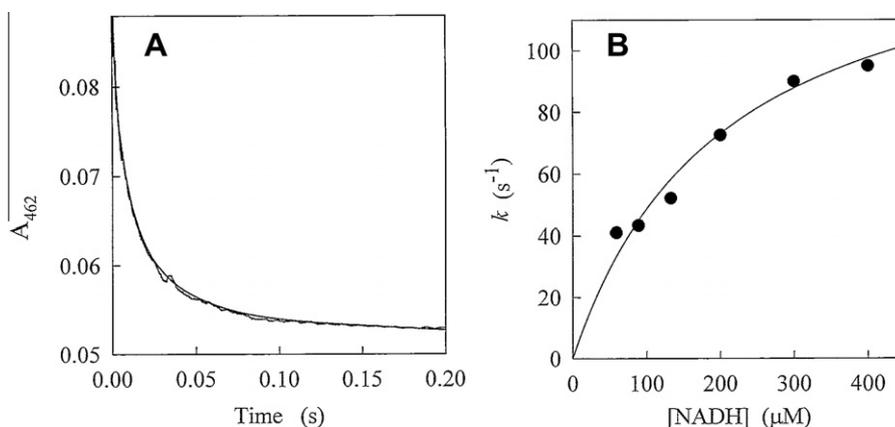


Fig. 2. (A) The kinetics of reduction of Prx-NR (5 μM after mixing) by 300 μM NADH, monitored at 462 nm. (B) The dependence of obtained pseudofirst-order reduction rate constant (k) on NADH concentration.

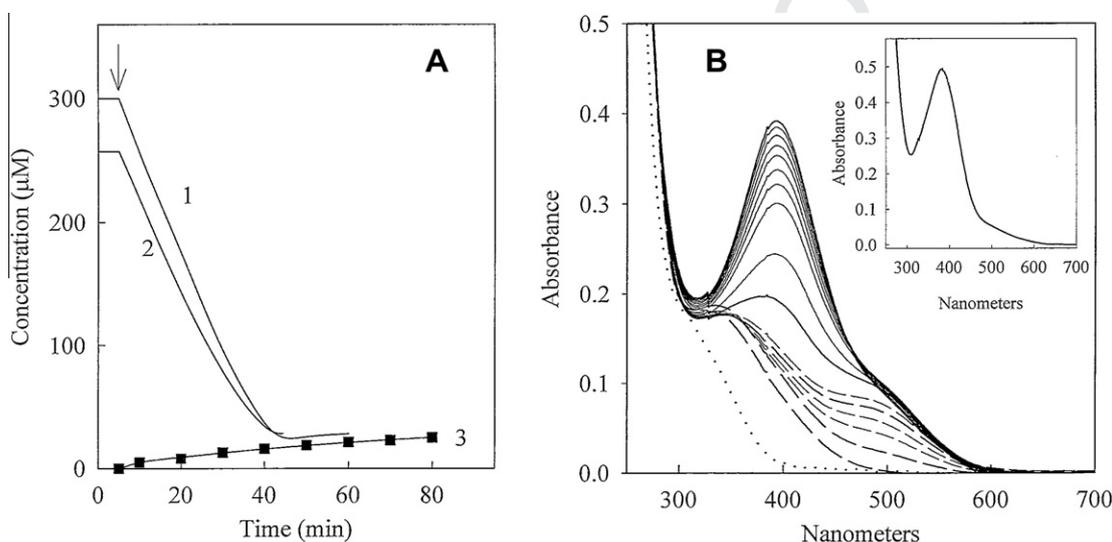


Fig. 3. (A) The oxidation of 300 μM NADH by 50 μM tetryl in the presence of 40 nM Prx-NR: oxidation of NADH, monitored spectrophotometrically at 340 nm (1), consumption of oxygen, monitored polarographically (2), and the formation of nitrite (3); (B) the kinetics of formation of products of reduction of tetryl (50 μM) by 40 nM Prx-NR in the presence of NADPH regeneration system: the dashed lines were recorded in each 10 min, the solid lines were recorded in each 20 min. The insert shows the spectrum of the reduction product(s) of 50 μM tetryl by 40 nM *E. cloacae* nitroreductase after 2 h in the presence of NADPH regeneration system.

concentration of quinone or nitroaromatic oxidant. k_{cat} represents a number of molecules of NADH oxidized by a single active center of the enzyme per second. These parameters were obtained by the fitting of kinetic data to the parabolic expression using Statistica (version 4.30, StatSoft, 1993), or SigmaPlot 2000 (version 6.10, SPSS Inc.). For the kinetics with a pronounced oxidizing quinone substrate (Q) inhibition, the data were fit to Eq. (1), where K_{iq} is the apparent constant of quinone substrate inhibition at fixed concentration of NADH:

$$\frac{v}{[E]} = \frac{k_{cat}}{1 + K_m/[Q] + [Q]/K_{iq}} \quad (1)$$

and further linearly extrapolated to $[NADH] = 0$. In separate experiments of nitroreduction, the NADPH regeneration system (10 U/ml glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, and 15 μM NADP⁺) has been used. In some experiments, the quinone/nitroaromatic-mediated reduction of cytochrome *c* (50 μM) was monitored spectrophotometrically following an increase in absorbance at 550 nm ($\Delta\epsilon_{550} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction rate was corrected for a direct reduction of 50 μM cytochrome *c* by Prx-NR, 0.2 s^{-1} . The oxygen uptake was measured using the Clark-

type electrode (Digital model 10, Rank Brothers Ltd.). The formation of nitrite was monitored spectrophotometrically, measuring an increase in the absorbance at 540 nm in the presence of sulfanilamide, naphthylethylene diamine dihydrochloride, and 4-fold diluted reaction mixture as described [7]. The solutions of NaNO₂ (10–100 μM) were used for the calibration.

Presteady-state kinetic measurements

The presteady-state kinetic measurement of reduction of Prx-NR by excess NADH was carried out by a stopped-flow SX.17 MV spectrophotometer (Applied Photophysics), using 0.1 M Tris-HCl containing 1 mM EDTA at pH 7.0 and 25 °C. The kinetics of absorbance decrease at 462 nm was analyzed according to the single-exponent fit using the Applied Photophysics software.

Protein sequence alignment

Protein sequence alignment of nitroreductase domain of Prx-NR and group A and B nitroreductases and related enzymes was

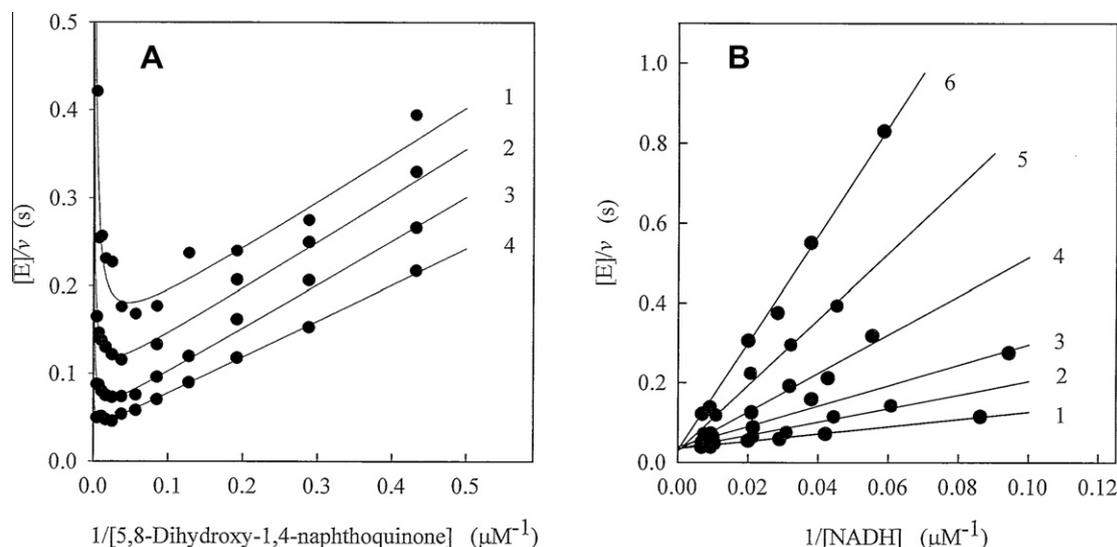


Fig. 4. (A) The rates of Prx–NR-catalyzed oxidation of NADH by 5,8-dihydroxy-1,4-naphthoquinone. The concentration of NADH, 10 μM (1), 30 μM (2), 50 μM (3), and 150 μM (4); (B) inhibition of the Prx–NR-catalyzed oxidation of NADH by 5,8-dihydroxy-1,4-naphthoquinone (30 μM) by dicumarol. The concentration of dicumarol, 0 μM (1), 0.5 μM (2), 1.0 μM (3), 2.5 μM (4), 5.0 μM (5), and 10 μM (6).

performed using Clustal Omega software (www.ebi.ac.uk/Tools/msa/clustalo/).

Molecular volume calculations of oxidants

Quantum mechanical calculations of the Van der Waals volumes (VdWvol) of compounds were performed using PC Spartan '04 package (Wavefunction, Inc. 1991–2003).

Results

Kinetic studies of the FMN reduction in Prx–NR

It has been shown previously that both NADH and NADPH reduce the FMN moiety of Prx–NR, leading to the disappearance of the absorbance of FMN in Prx–NR at 460 nm [22]. This is not observed with DTT, which can reduce the Prx redox moiety [22]. Because of the low NADH oxidase activity of Prx–NR, 0.03 s^{-1} , which may just marginally affect the data of the measurements, the subsequent presteady-state reduction experiments were performed under the aerobic conditions. In the presence of an excess NADH, the FMN absorbance decrease at 460 nm followed the pseudofirst order kinetics (Fig. 2A). The dependence of the pseudofirst order rate constant (k) on the concentration of NADH (Fig. 2B) yielded the maximal reduction rate constant, $146 \pm 24 \text{ s}^{-1}$, and the apparent second-order reduction rate constant, $7.3 \pm 1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Although NADPH displayed a similar capacity to reduce FMN, its activity towards Prx–NR was not investigated in the present paper, except its use in the NADPH regeneration system.

Two-electron reduction of quinones and nitroaromatic compounds by Prx–NR

Next, we attempted to find whether the reduction of quinones and nitroaromatics by Prx–NR occurs in a single- or two-electron manner. The percentage of the single-electron flux in the reduction of quinones by NAD(P)H-oxidizing flavoenzymes may be determined using the 1,4-benzoquinone-mediated reduction of added cytochrome *c*, and is equal to the ratio between the cytochrome *c* reduction rate and a doubled rate of NAD(P)H oxidation [25]. This

is based on the fact that at pH <7.2 the rate of reduction of cytochrome *c* by 1,4-hydroquinone is negligible, whereas 1,4-benzo-semiquinone, formed during single-electron reduction, reduces cytochrome *c* at a high rate ($k \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) [25]. During Prx–NR-catalyzed reduction of 50 μM 1,4-benzoquinone by 150 μM NADH, the rate of reduction of added cytochrome *c* (50 μM) after the correction for the direct enzymatic reduction of cytochrome *c*, 0.2 s^{-1} , was equal to 12% of NADH oxidation rate. It shows that the percentage of single-electron flux makes up 6%, which is consistent with the two-electron reduction mechanism. During the enzymatic oxidation of 150 μM NADH by 50 μM 2-methyl-1,4-naphthoquinone (menadione) at pH 5.8, menadione oxidized a close to stoichiometric amount of NADH in a single phase, which was followed by a slower subsequent oxidation of excess NADH. This is consistent with a two-electron reduction of menadione, because 2-methyl-1,4-naphthohydroquinone autooxidizes slowly at the acidic pH, and is unable to support efficiently the enzymatic oxidation of excess NAD(P)H [26].

Using NADPH regeneration system, *p*-dinitrobenzene was reduced by Prx–NR in two(four)-electron manner into *p*-hydroxylaminonitrobenzene which possesses the characteristic absorbance at 340–350 nm (data not shown, the reference spectrum is given in Ref. [27]). During the enzymatic oxidation of NADH by *p*-dinitrobenzene, the reduction of added 50 μM cytochrome *c* at 15–20% of NADH oxidation rate takes place after the correction for the direct reduction of cytochrome *c* by Prx–NR. This reaction was not inhibited by 100 U/ml superoxide dismutase. This is in line with the previously demonstrated direct reduction of cytochrome *c* by hydroxylamine derivatives of polynitrobenzenes [7,28], and rules out the formation of nitroaromatic free radical intermediates which would be reoxidized by oxygen with the formation of superoxide. In addition, we found that using 500 μM DTT which can reduce Prx moiety of Prx–NR [22], we failed to detect a measurable reduction of *p*-dinitrobenzene (500 μM) by Prx–NR. Thus, these data additionally point to the absence of exchange in redox equivalents between NR and Prx moieties of Prx–NR, which could have contributed to the catalytically important reduction of NR-linked oxidants. The reduction of another nitroaromatic compound, tetryl (Fig. 1) by Prx–NR is accompanied by the oxidation of significant excess NADH, uptake of O_2 lower than stoichiometric to NADH, and formation of nitrite (Fig. 3A). This reaction was accompanied

Table 1

Single-electron reduction potentials of quinones and nitroaromatic compounds at pH 7.0 (E_7^1), their van der Waals volumes (VdWvol), and their catalytic constants (k_{cat}) and bimolecular rate constants (k_{cat}/K_m) in *Thermotoga maritime* Prx–NR-catalyzed reactions at pH 7.0 and 25 °C in the presence of 150 μM NADH unless otherwise indicated.

No.	Compound	$E_7^1(\text{V})^a$	VdWvol (\AA^3)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
<i>Quinones</i>					
1	1,4-Benzoquinone	0.09	124	70 ± 10	$(1.0 \pm 0.4) \times 10^7$
2	2-Methyl-1,4-benzoquinone	0.01	142	27 ± 4	$(1.7 \pm 0.1) \times 10^6$
3	2,3-Dichloro-1,4-naphthoquinone	-0.035	187	34 ± 1	$(6.9 \pm 0.8) \times 10^6$
4	2,5-Diaziridiny-1,4-benzoquinone (DZQ)	-0.054	199	n.d.	$(1.7 \pm 0.1) \times 10^6$
5	2,3-Dimethyl-1,4-benzoquinone	-0.07	160	30 ± 3	$(1.1 \pm 2.9) \times 10^7$
6	2,6-Dimethyl-1,4-benzoquinone	-0.08	160	56 ± 4	$(5.6 \pm 1.4) \times 10^6$
7	5-Hydroxy-1,4-naphthoquinone	-0.09	180	102 ± 19^b	$(2.7 \pm 0.7) \times 10^7$
8	5,8-Dihydroxy-1,4-naphthoquinone	-0.11	192	28 ± 2^b	$(2.5 \pm 0.2) \times 10^6$
9	9,10-Phenanthrenequinone	-0.12	218	111 ± 14^b	$(2.9 \pm 0.6) \times 10^6$
10	1,4-Naphthoquinone	-0.15	168	99 ± 9^b	$(1.8 \pm 0.2) \times 10^6$
11	2-Methyl-5-hydroxy-1,4-naphthoquinone	-0.16	200	54 ± 4	$(8.6 \pm 0.8) \times 10^5$
12	2-Methyl-1,4-naphthoquinone	-0.20	187	n.d.	$(1.2 \pm 0.2) \times 10^5$
13	2,5-Dimethyl-3,6-diaziridiny-1,4-benzoquinone (MeDZQ)	-0.23	238	n.d.	$(4.7 \pm 0.4) \times 10^3$
14	Tetramethyl-1,4-benzoquinone	-0.26	197	n.d.	$(4.6 \pm 0.5) \times 10^3$
15	9,10-Anthraquinone-2,6-disulphonate	-0.25	266	n.d.	$(3.4 \pm 0.3) \times 10^3$
16	1,4-Dihydroxy-9,10-anthraquinone	-0.30	245	n.d.	$(3.4 \pm 0.5) \times 10^2$
17	1,8-Dihydroxy-9,10-anthraquinone	-0.33	245	n.d.	$(3.8 \pm 0.3) \times 10^2$
18	Daunorubicin	-0.34	600	n.d.	$(6.8 \pm 0.5) \times 10^3$
19	2-Hydroxy-1,4-naphthoquinone	-0.41	180	n.d.	$(6.5 \pm 0.4) \times 10^3$
20	2-Hydroxy-3-methyl-1,4-naphthoquinone	-0.46	200	n.d.	$(8.1 \pm 0.8) \times 10^2$
<i>Aromatic nitrocompounds</i>					
21	1,3,6,8-Tetranitrocarbazole	-0.12	270	10 ± 1.5	$(2.2 \pm 0.6) \times 10^5$
22	Pentryl	-0.14	266	15 ± 2.0	$(5.8 \pm 1.4) \times 10^5$
23	Tetryl	-0.16	218	n.d.	$(5.6 \pm 0.6) \times 10^4$
24	2,4,6-Trinitrotoluene	-0.25	182	n.d.	$(4.8 \pm 0.1) \times 10^3$
25	1,4-Dinitrobenzene	-0.26	143	n.d.	$(2.2 \pm 0.2) \times 10^3$
26	4,6-Dinitrobenzofuroxane	-0.26	167	n.d.	$(4.2 \pm 0.4) \times 10^3$
27	1,2-Dinitrobenzene	-0.29	142	n.d.	260 ± 40
28	4-Nitrobenzaldehyde	-0.32	142	n.d.	430 ± 40
29	1,3-Dinitrobenzene	-0.35	143	n.d.	80 ± 15
30	CB-1954	-0.38	217	n.d.	100 ± 12
31	SN-23862	-0.43	266	n.d.	70 ± 15
32	4-Nitrobenzyl alcohol	-0.48	147	n.d.	42 ± 10
33	Nitrobenzene	-0.49	123	n.d.	11 ± 2.0

^a E_7^1 values taken from Refs. [31–34].

^b k_{cat} determined by extrapolation to infinite concentrations of NADH and quinone substrate according to a 'ping-pong' scheme.

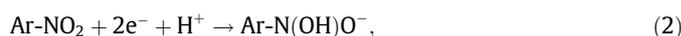
257 by the reduction of added cytochrome *c* at 18–25% NADH oxidation
 258 rate, which also was not inhibited by 100 U/ml superoxide dismu-
 259 tase. The spectral changes of tetryl during the reduction by Prx–NR
 260 using the NADPH regeneration system are similar to those taking
 261 place during *E. cloacae* NR-catalyzed reaction [7], with a final for-
 262 mation of product(s) absorbing at 380–385 nm (Fig. 3B). Thus,
 263 our findings are in line with a net two(four)-electron of tetryl by
 264 Prx–NR, followed by the further reduction, and the subsequent
 265 *N*-denitration of its (di)hydroxylamine derivatives [7]. In this case,
 266 the nonstoichiometric uptake of O_2 accompanying the oxidation of
 267 excess NADH by tetryl may be attributed to the oxidation of its
 268 dihydroxylamine derivatives by O_2 , which is characteristic for
 269 dihydroxylamine derivatives of other polynitroaromatic com-
 270 pounds such as TNT [29,30].

271 Steady-state kinetic studies and substrate specificity of quinone- and 272 nitroreductase reactions of Prx–NR

273 At fixed concentrations of NADH and varied concentrations of
 274 5,8-dihydroxy-1,4-naphthoquinone (naphthazarine), the steady-
 275 state kinetics of Prx–NR is characterized by a 'ping-pong' scheme
 276 with a pronounced quinone-substrate inhibition (Fig. 4A). The inhi-
 277 bition constant for naphthazarin, K_{iq} , decreased with a decrease in
 278 NADH concentration, thus showing that naphthazarin is a competi-
 279 tive inhibitor with respect to NADH that binds to the oxidized en-
 280 zyme form. The K_{iq} for naphthazarin obtained by extrapolation to
 281 $[\text{NADH}] = 0$, was equal to $95 \pm 18 \mu\text{M}$. The k_{cat} of this reaction
 282 determined by the extrapolation of the maximal reaction rate to

the infinite concentration of NADH in the plot $[E]/V_{\text{max}}$ vs. $1/$
 [NADH], was equal to $28 \pm 2.0 \text{ s}^{-1}$. The k_{cat}/K_m for NADH obtained
 using 5,8-dihydroxy-, 5-hydroxy-1,4-naphthoquinone, or 9,10-
 phenanthrenequinone as electron acceptors was equal to
 $7.2 \pm 1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, i.e., close to the apparent second-order
 reduction rate constant obtained in the presteady-state studies.
 We also found that Prx–NR was inhibited by dicumarol, a classical
 inhibitor of oxygen-insensitive NRs [6,7,10] (Fig. 4B), which acted
 as a competitive inhibition with respect to NADH with
 $K_i = 0.70 \pm 0.03 \mu\text{M}$, thus showing that it competes for the NADH
 binding site in the oxidized enzyme form.

In order to characterize the oxidizing substrate specificity for
 Prx–NR, the main problem is to distinguish the roles of their elec-
 tron(hydride)-accepting potency and structural peculiarities. For
 this reason, we studied the reduction of a series of quinones and
 nitroaromatic compounds with varying electron/hydride accepting
 potency (single-electron reduction potential, E_7^1), and steric param-
 eters (Van der Waals volume, VdWvol) (Table 1). The use of E_7^1 in
 the analysis of two-electron(hydride) transfer is justified, because
 there exists a parallel between the thermodynamics of electron
 and hydride transfer in quinone reduction [10,31]. The thermody-
 namics of two-electron reduction of nitroaromatic compounds is
 less understood, because their electrochemical reduction in aque-
 ous media is irreversible [35]. However, the reduction of nitroaro-
 matic compounds (Ar-NO_2) into their nitroso derivatives (Ar-NO)
 proceeds with the rate-limiting hydride transfer:



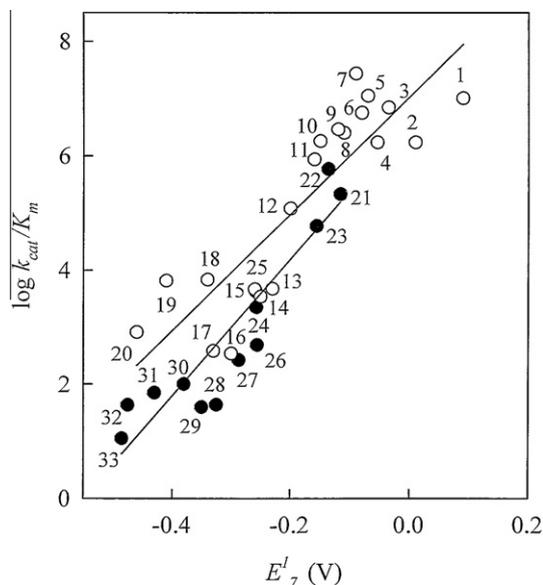


Fig. 5. The relationship between the $\log k_{\text{cat}}/K_m$ of quinones (blank circles) and nitroaromatic compounds (solid circles) in Prx–NR-catalyzed reduction, and their single-electron reduction potentials at pH 7.0 (E_7^1). The numbers of quinone and nitroaromatic compounds, their redox potentials, and their reduction rate constants are given in Table 1.

with the subsequent fast protonation and dehydration of Ar-N(OH) O^- [7,32]. The heats of formation of Ar-N(OH) O^- obtained by the quantum mechanical calculations show a good correlation with the heats of formation of nitroaromatic anion-radicals (Ar-NO $_2^-$), and also with the values of their E_7^1 [7]. For these reasons, E_7^1 was used in the description of the reactivity of nitroaromatics as well. As it is shown in Fig. 5, the $\log k_{\text{cat}}/K_m$ of reduction of quinones exhibited a linear but rather scattered positive dependence on their E_7^1 values ($r^2 = 0.873$, $F(1,18) = 57.39$). The similar trend was observed in the reduction of nitroaromatics ($r^2 = 0.872$, $F(1,11) = 74.61$). One may note that the reactivity of nitroaromatic compounds is by one-two orders of magnitude lower than that of quinones possessing the same value of E_7^1 (Fig. 5). An introduction of VdWvol of quinones or nitroaromatics (Table 1) as the second variable in the multiparameter regression did not improve the correlations.

Discussion

In this work, we characterized quinone- and nitroreductase reactions of a hybrid peroxiredoxin–nitroreductase protein (Prx–NR) of anaerobic thermophilic bacterium *T. maritima*. Prx–NR is a relatively unique protein, being present only in some thermotogales. Not entirely in line with our initial expectations, the flavo-protein domain does not serve as a peroxiredoxin reductase in this case, since no electrons are exchanged between the NR and Prx domain [22,36].

The kinetic characterization of Prx–NR (Figs. 2–5) is important in view of the vaguely understood mechanism(s) of the two-electron reduction of quinones and nitroaromatic compounds by different nitroreductases. As a thermophilic enzyme, Prx–NR is also interesting in the context of the biodegradation of nitroaromatic and other redox active environmental pollutants, because the use of thermophilic microorganisms in the bioremediation may contribute to the increased operational longevity of the systems [37,38].

To some extent, Prx–NR shares the properties of group B nitroreductases, because it uses both NADH and NADPH as the reducing substrates. However, the sequence analysis of its NR domain (res-

idues 142–321, GenBank accession number NP_228196) revealed a modest homology, 20–24%, with the sequences of *E. coli* NR-B (NC_000913.2), and *E. cloacae* NR (M6803). More important, the residues analogous to Phe-124, Phe-70, Ser-40, Lys-14, and Lys-74 which participate in the binding of nitroaromatic compounds or the inhibitor dicumarol in *E. coli* NR-B or *E. cloacae* NR [6,9,18,19], are absent in Prx–NR (data not shown). On the other hand, the comparison of Prx–NR sequence with that of *E. coli* NR-A (Protein Data Bank Entry 1BKJ) also shows a modest homology, 18%, but also points to a possible conservation of several residues participating in the binding of the isoalloxazine ring of FMN, Arg-15 (Arg-154 in Prx–NR), Gly-130,131 (Gly-235 and Arg-236 residues in Prx–NR), and also Tyr-128 (substituted into aromatic Phe-260 in Prx–NR) [39] (data not shown). Thus, it is possible that Prx–NR may share some properties of group A nitroreductases as well. In addition, the absence of conserved analogues for Phe-124 and Ser-40 which participate in the binding of dicumarol in *E. coli* NR-B and homologous NADPH:FMN oxidoreductase of *V. fischeri* [10,18,19], may be responsible for the relatively weak binding of dicumarol by Prx–NR, $K_i = 0.7 \mu\text{M}$ (Fig. 4B), which is less efficient than in the case of *E. cloacae* NR ($K_i = 0.06 \mu\text{M}$ [7]), or mammalian NAD(P)H: quinone oxidoreductase (NQO1, $K_i = 0.01 \mu\text{M}$ [40]).

Considering the mechanism of catalysis of Prx–NR, it follows a ‘ping-pong’ scheme (Fig. 4A) like in other oxygen-insensitive NRs of *E. coli* and *E. cloacae* with quinone substrate inhibition, thus showing that NAD(P)H and electron acceptors bind at the same site in the oxidized and reduced enzyme forms, respectively [3,6]. The maximal rate of reduction of FMN of Prx–NR by NADH, $146 \pm 24 \text{ s}^{-1}$ (Fig. 2B) is lower than that of *E. coli* NR-B, $>500 \text{ s}^{-1}$ [17], and *E. cloacae* NR, $>1000 \text{ s}^{-1}$ [7]. The comparison of presteady-state and steady-state kinetic data of Prx–NR (Fig. 2B,4A, Table 1) shows that the oxidative half-reaction is rate-limiting in the catalysis, with the exception of 5-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone, and 9,10-phenanthrene quinone, where the reductive half-reaction may be partly rate-limiting. Similarly, the oxidative half-reaction is a rate-limiting step in the reduction of CB-1954, menadione, and nitrofurazone by *E. coli* NR-B [17], and, probably, in the reduction of the low-potential oxidants by *E. cloacae* NR [7,16]. Thus, in this aspect, Prx–NR of *T. maritima* is similar to the above B group nitroreductases.

The most interesting problem in our case is the elucidation of the oxidizing substrate specificity for Prx–NR in the context of other oxygen-insensitive NRs. In the reactions of *E. coli* NR-A and NR-B, the reactivity of the oxidants increased upon increase in their reduction potential, in order 4-nitrobenzoic acid and CB-1954 ($E_7^1 \approx -0.40 \text{ V}$) < nitrofurazone and menadione ($E_7^1 = -0.25 \text{ V} - -0.20 \text{ V}$) < 1,4-benzoquinone ($E_7^1 = 0.09 \text{ V}$) [3,6,17], although this dependence has not been thoroughly analyzed. On the other hand, *E. cloacae* NR was characterized by a parabolic dependence of $\log k_{\text{cat}}/K_m$ of nitroaromatic compounds on their E_7^1 [7]. The reactivity of quinones towards *E. cloacae* NR also displayed a parabolic dependence on their E_7^1 , although their reactivity was generally by one order of magnitude lower than that of nitroaromatic compounds with the same reduction potential [16]. It shows that *E. cloacae* NR exhibited a certain preference towards nitroaromatic compounds in comparison to quinones. This enzyme was also characterized by a certain substrate specificity, because it discriminated the compounds with large WdWvol such as daunorubicin, and, possibly, 9,10-anthraquinones, and also displayed an enhanced reactivity towards 2-hydroxy-1,4-naphthoquinones [16]. However, the data of Fig. 5 show that none of these features are characteristic for Prx–NR, which shows little sensitivity to the structure of the oxidants. Besides, the enhanced reactivity of quinones in comparison to nitroaromatic compounds is similar to the regularities in the reactions of single-electron transferring flavoenzymes dehydrogenases-

electrontransferases, such as NADPH: cytochrome P-450 reductase and ferredoxin:NADP⁺ reductase [41,42]. In these cases, the enhanced reactivity of quinones is attributed to the high electron self-exchange rate constant of quinone/semiquinone redox couple, $\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [43], which significantly exceeds that of $\text{ArNO}_2/\text{ArNO}_2^-$ redox couple, $\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$ [44]. Thus, it may point to a multistep (e^-, H^+, e^-) mechanism in the two-electron reduction of quinones and nitroaromatic compounds by Prx–NR with a partly limiting step of first electron transfer. In addition, in terms of k_{cat}/K_m , the reactivity of quinone- and nitroaromatic oxidants towards Prx–NR is by one or two orders of magnitude lower than towards *E. coli* NR-B and *E. cloacae* NR (Table 1, Fig. 5, [7,16,17]). Here we may also consider some decline in the driving force for the reaction, because the standard potential for FMN of Prx–NR is equal to -0.185 V at pH 8.0 [22], which corresponds to $E_1^1 = -0.125$ to -0.155 V at pH 7.0. This is more positive than the E_7^0 value for *E. cloacae* NR, -0.190 V [8], and therefore it may decrease the reactivity of quinones and nitroaromatic compounds towards Prx–NR. On the other hand, taken together with the relatively inefficient inhibition of Prx–NR by dicumarol (Fig. 4B), our data may also point to a relatively inefficient and structure-unspecific binding of quinones and nitroaromatic compounds in the active center of NR domain of Prx–NR, i.e., to the flexibility of its active center. In this case, the thermodynamics of hydride(electron) transfer, and, possibly, the internal electron self-exchange in the separate series of the oxidants may play a major role in the determination of their reactivity. To some extent, this is characteristic for the active center of *E. coli* NR-B, where nitrofurazone was found to bind in the unproductive conformation [20], and the binding of CB-1954 occurred at two distant domains [18,19]. Taken together, our data testify in favor of the considerable but yet insufficiently studied diversity among the possible structural motifs of flavoenzymes prerequisite for the two(four)-electron reduction of nitroaromatic compounds. We also suggest that our data may provide some information linking the mechanisms of single- and two-electron reduction of quinones by flavoenzymes, thus complementing our previous studies [31,45]. The latter points to the stability of the flavin semiquinone state as to the most important factor deciding the single- or two-electron reduction of quinones, and also to a minor role of the surroundings of the isoalloxazine ring, which may be putatively responsible for the appropriate binding and activation of these oxidants [45]. One may note that no detectable formation of FMN semiquinone at 380 or 600 nm has been observed during the potentiometric titration of FMN of Prx–NR [22], which points to its instability like in *E. cloacae* NR and *V. fischeri* FMN reductase [8,10]. Thus, concerning the further studies of these physiologically important reactions, our next aims are to characterize the role of the interface between the NR and Prx domains in the binding of the oxidizing substrates and inhibitors for NR, and its impact on their reduction mechanism. The preliminary studies of the isolated NR domain of Prx–NR [22] show that it reduces quinones in two-electron manner, although with a decreased by 1.5–2.5 times reactivity.

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