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Two-electron reduction of nitroaromatic compounds by *Thermotoga maritima* hybrid peroxiredoxin–nitroreductase enzyme

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Explosives such as 2,4,6-trinitrotoluene (TNT) and related polynitroaromatics being toxic environmental pollutants, numerous efforts are directed towards their biodegradation. In this work, we studied the NADPH-dependent two-electron reduction of a number of nitroaromatic compounds by a peroxiredoxin–nitroreductase hybrid enzyme from *Thermotoga maritima* (Prx–NR). We have found that the peroxiredoxin and nitroreductase domains of Prx–NR function independently. The activity of Prx–NR towards nitroaromatics is not influenced by their particular structure and is characterized by a linear log k_{cat}/K_m dependence on their single-electron reduction potentials (E_7^1). The reduction of polynitroaromatic explosives *N*-nitramines such as tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) and pentryl (2,4,6-trinitrophenyl-*N*-nitroaminoethylnitrate) was accompanied by the formation of nitrite, which implies their reductive *N*-denitration, while *p*-dinitrobenzene is reduced to *p*-hydroxylaminonitrobenzene. Taken together, these data indicate that nitroreductase reactions of Prx–NR share common features with those of other bacterial oxygen-insensitive nitroreductases, e. g., *Enterobacter cloacae* nitroreductase. However, the activity of Prx–NR is 10–100 times lower than that of *E. cloacae* NR.

Key words: 2,4,6-trinitrotoluene, explosives, nitroreductases, biodegradation, bioreductive activation

Abbreviations: TNT, 2,4,6-trinitrotoluene; TNC, 1,3,6,8-tetranitrocarbazole; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; SN23862, 5-[bis(2,2'-chloroethylamino)]-2,4-dinitrobenzamide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Prx–NR, peroxiredoxin–nitroreductase hybrid enzyme; NR, nitroreductase; NQO1, NAD(P)H : quinone oxidoreductase; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant in enzymatic steady-state reactions; E_7^1, single-electron reduction potential at pH 7.0.

INTRODUCTION

Polynitroaromatic explosives such as 2,4,6-trinitrotoluene (TNT) and related compounds are toxic environmental pollutants causing methemoglobinemia, cataract, liver and kidney diseases, and an increased cancer incidence ([1] and references cited therein). These toxic effects are mainly attributed to the prooxidant action of nitroaromatics, i. e. their flavoenzyme-catalyzed single-electron reduction to anion-radicals, and subsequent redox-cycling leading to the formation of reactive oxygen species (ROS, i. e. $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$) ([1], and references cited therein). For this reason, numerous efforts are directed towards the bioremediation of explosive residues in soil, groundwater, and industrial wastes ([2] and References cited therein). Typically, the first step of biodegradation of explosives by microorganisms or plants is their two(four)-electron reduction to corresponding hydroxylamine derivatives by oxygen-insensitive NAD(P)H : nitroreductases [3].

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These enzymes contain flavinmononucleotide (FMN) in their active site and are in a destabilized FMN semiquinone state [4]. Another potential biomedical application of oxygen-insensitive nitroreductases is their use in various combined antitumour therapies of bioreductively activated nitroaromatic prodrugs, where they may perform the two-electron reduction of aziridinyl- or di(chloroethylamino)-substituted nitrobenzenes faster than the corresponding mammalian enzymes [5].

Recently, peroxiredoxin–nitroreductase (Prx–NR) natural fusion enzyme from the thermophile *Thermotoga maritima* has been purified and partly characterized [6]. The mechanism and structure–activity relationships of this enzyme deserve some interest, because the use of thermophiles in biodegradation systems typically increases their operational stability [7]. In this work, we examined the reduction of a series of nitroaromatic compounds including several explosives and antitumour agents by *T. maritima* Prx–NR.

EXPERIMENTAL

Recombinant Prx–NR (m. w. 37.2 kD) was purified as described previously [6]. Enzyme concentration was determined according to the absorbance of FMN at 460 nm (ε_{460} = 11 $mM^{-1}\,cm^{-1}$). Explosives TNT, tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine), pentryl (2,4,6-trinitrophenyl-*N*-nitroaminoethylnitrate), 1,3,6,8-tetranitrocarbazole (TNC), and 4,6-dinitrobenzofuroxan (DNBF) were synthesized as described elsewhere [8, 9]. The antitumour agents 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) and 5-[bis(2,2'-chloroethylamino)]-2,4-dinitrobenzamide (SN23862), synthesized according to the established methods [5, 10], were the generous gift of Dr. Vanda Miškinienė. The formulae of nontrivial nitroaromatic compounds are given in Fig. 1. Other nitroaromatic compounds, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and other chemicals were obtained from Sigma.

Kinetic experiments were performed in 0.1 M K-phosphate (pH 7.0) containing 1 mM EDTA at 25 °C. Reaction rates were monitored spectrophotometrically according to a decrease in NADPH absorbance ($\Delta\varepsilon_{340}$ = 6.2 $mM^{-1}\,cm^{-1}$), using a Hitachi-557 spectrophotometer. Steady-state kinetic parameters were obtained using a saturating NADH concentration, 150 μM, and varying concentrations of nitroaromatic compounds. Catalytic constants (k_{cat}) and bimolecular rate constants of the reduction of nitroaromatics (k_{cat}/K_m) correspond to reciprocal intercepts and slopes in the Lineweaver–Burk coordinates, respectively. k_{cat} is the number of NADPH molecules oxidized by a single enzyme molecule per second. Kinetic parameters were obtained by the Quasi-Newton or Simplex-Quasi-Newton nonlinear estimation methods using Statistica (version 4.30, StatSoft, 1993). Reaction rates were corrected for an intrinsic NADPH : oxidase activity of Prx–NR, 0.03 s^{-1} and for the formation of 340 nm-absorbing products of reduction of nitroaromatic compounds [11]. In separate cases, the NADPH regeneration system (10 U/ml glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, and 15 μM $NADP^+$) was used. The reduction of 5.5'-dithio-

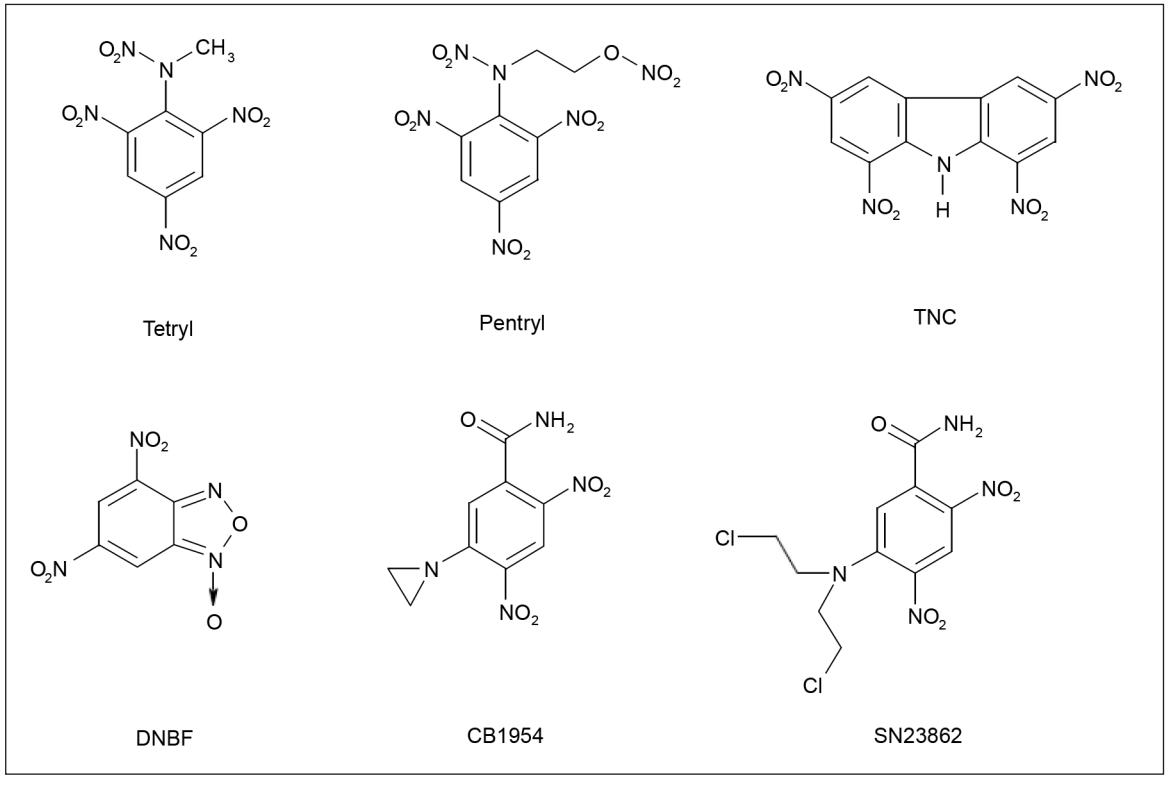
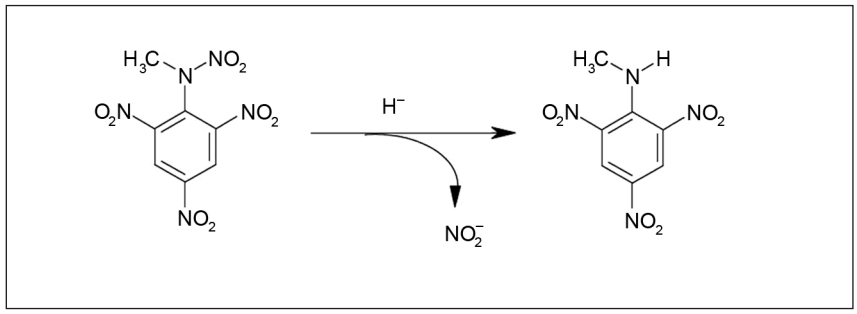


Fig. 1. The structure of nontrivial nitroaromatic explosive compounds



bis-(2-nitrobenzoic acid) (DTNB) was followed according to the absorbance rise at 412 nm ($\Delta\varepsilon_{412}$ = 27.2 $mM^{-1}\,cm^{-1}$). The formation of nitrite in the reaction was monitored spectrophotometrically according to the formation of a diazo compound [12]. Oxygen consumption during the reaction was monitored using a Digital model 10 oxygen electrode (Rank Brothers Ltd.).

RESULTS AND DISCUSSION

First, we have found that Prx and NR domains of *T. maritima* Prx-NR function independently, i. e. that the exchange of redox equivalents between these domains does not occur at a catalytically competent rate. In the presence of 200 μM NADPH, which reduces the NR domain, the reduction of 2 mM DTNB, the latter being reduced by catalytic –SH groups of a Prx domain, takes place at the rate <0.01 s^{-1}. Thus, in the further studies, the possible reduction of nitroaromatic compounds by the Prx domain was neglected.

Second, we found that Prx–NR reduced nitroaromatic compounds in the two(four)-electron way. In the presence of NADPH regeneration system, *p*-dinitrobenzene was reduced into *p*-hydroxylaminonitrobenzene which possesses the characteristic absorbance at 340–350 nm (Fig. 2). The reaction was not accompanied by an increased oxygen consumption which would indicate the formation of a nitroanion-radical and its redox cycling. During the reduction of tetryl, we observed an initial accumulation of a product of its *N*-denitration, *N*-methylpicramide, which possesses a characteristic 2 : 1 absorbance ratio at 340 nm and 430 nm, respectively [13] (Fig. 3). In the absence of NADPH regeneration system, this reaction step is accompanied by nitrite formation with a stoichiometric (1 : 1) ratio between the nitrite formed and the NADPH oxidized. Subsequently, we observed the formation of unidentified products absorbing at 400 nm, which evidently were the products of *N*-methylpicramide nitroreduction (Fig. 3). These data imply the occurrence of a reductive *N*-denitration of tetryl, proceeding as a single-step hydride transfer (Scheme). The formation of nitrite accompanied by the appearance of 400 nm-absorbing product(s) is also characteristic of the reduction of tetryl by *Enterobacter cloacae* nitroreductase [11]. However, in

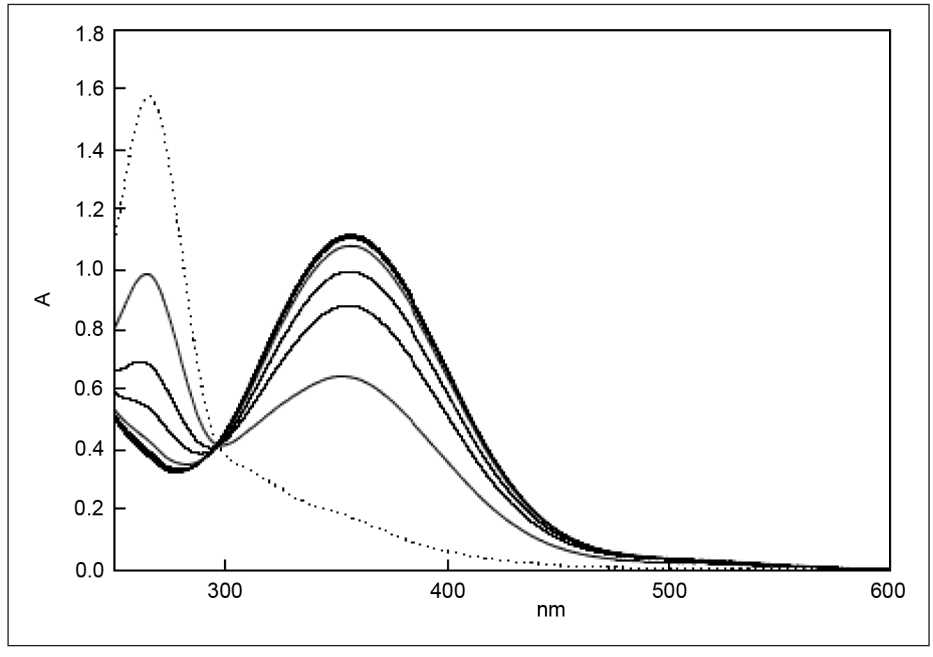


Fig. 2. Reduction of 100 μM *p*-dinitrobenzene by 40 nM Prx–NR using the NADPH regeneration system. The dotted curve represents the absorbance spectra of the reaction mixture before Prx–NR addition. The subsequent spectra were recorded each 5 min

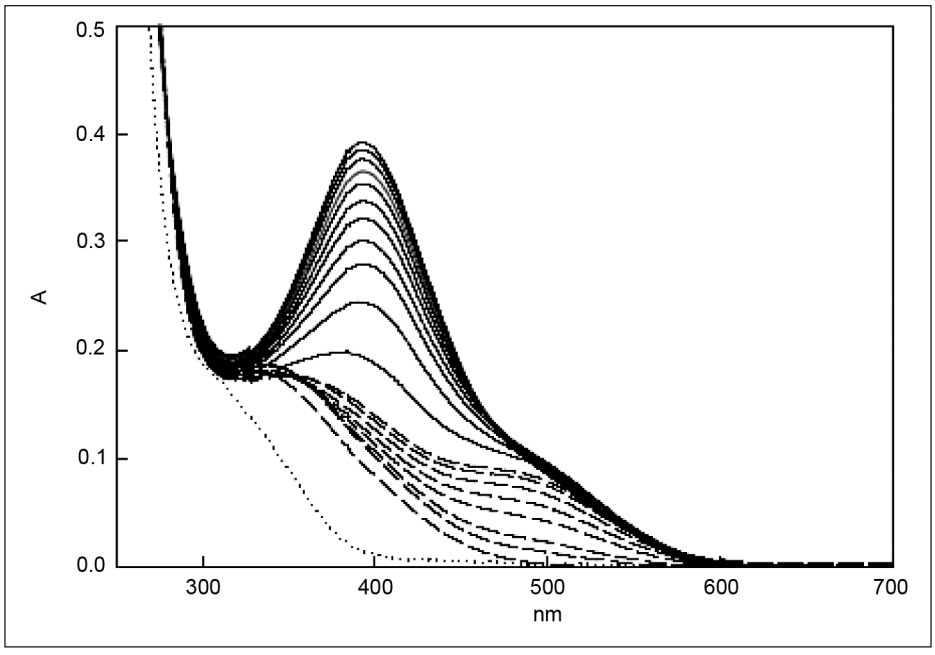


Fig. 3. Reduction of 50 μM tetryl by 40 nM Prx–NR using the NADPH regeneration system. The dotted curve represents the absorbance spectra of the reaction mixture before Prx–NR addition. The dashed curves reflecting the initial formation of *N*-methylpicramide were recorded each 10 min. The solid curves reflecting the further course of the reaction were recorded each 20 min

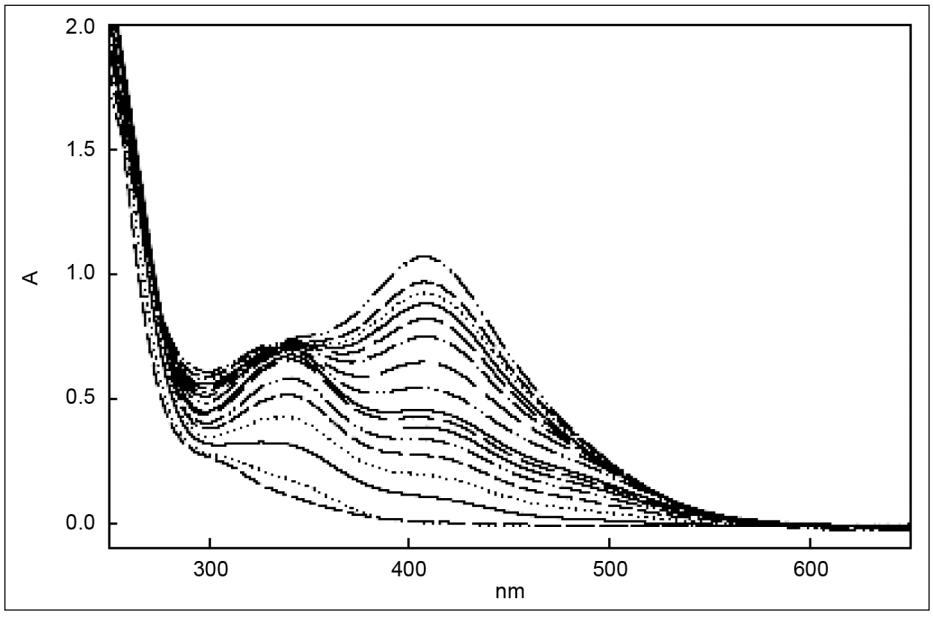


Fig. 4. Reduction of 100 μM pentryl by 40 nM Prx–NR using the NADPH regeneration system. The lower curve represents the absorbance spectra of the reaction mixture before Prx–NR addition. The subsequent spectra showing the initial absorbance increase at 340 nm and 430 nm, and the further course of reaction were recorded each 10 min

this case we did not observe the transient formation of *N*-methylpicramide [11]. This means a different nitroreductase specificity of *E. cloacae* NR and *T. maritima* Prx–NR, i. e. the possible preference of *E. cloacae* NR to aromatic nitrogroups and the preference of Prx–NR to the *N*-nitramine group (Scheme). Similar absorbance spectra of reduction products with a transient accumulation of 340 nm and 430 nm-absorbing species in the presence of a NADPH regeneration system (Fig. 4) and 1 : 1 nitrite / NADPH stoichiometry were also characteristic of the Prx–NR-catalyzed reduction of pentryl, another polynitroaromatic *N*-nitramine explosive. The reduction of tetryl and pentryl by Prx–NR was not accompanied by an increased oxygen consumption, either.

Table. **Kinetic parameters of reduction of nitroaromatic compounds by Prx–NR at fixed concentration of NADPH, 150 μM. The single-electron reduction potentials of nitroaromatics are taken from Refs. [14, 15]**

No.	Oxidant	E_7^1 (V)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
1.	1,3,6,8-Tetranitrocarbazole	–0.12	10 ± 1.5	$(2.2 \pm 0.6) \times 10^5$
2.	Pentryl	–0.14	15 ± 2.0	$(5.8 \pm 1.4) \times 10^5$
3.	Tetryl	–0.16	–	$(5.6 \pm 0.6) \times 10^4$
4.	2,4,6-Trinitrotoluene	–0.25	–	$(4.8 \pm 0.1) \times 10^3$
5.	1,4-Dinitrobenzene	–0.26	–	$(2.2 \pm 0.2) \times 10^3$
6.	4,6-Dinitrobenzofuroxane	–0.26	–	$(4.2 \pm 0.4) \times 10^3$
7.	1,2-Dinitrobenzene	–0.29	–	260 ± 40
8.	4-Nitrobenzaldehyde	–0.32	–	43 ± 4.0
9.	1,3-Dinitrobenzene	–0.35	–	80 ± 15
10.	CB1954	–0.38	–	100 ± 12
11.	SN23862	–0.43	–	70 ± 15
12.	4-Nitrobenzyl alcohol	–0.48	–	42 ± 2.0
13.	Nitrobenzene	–0.49	–	11 ± 2.0

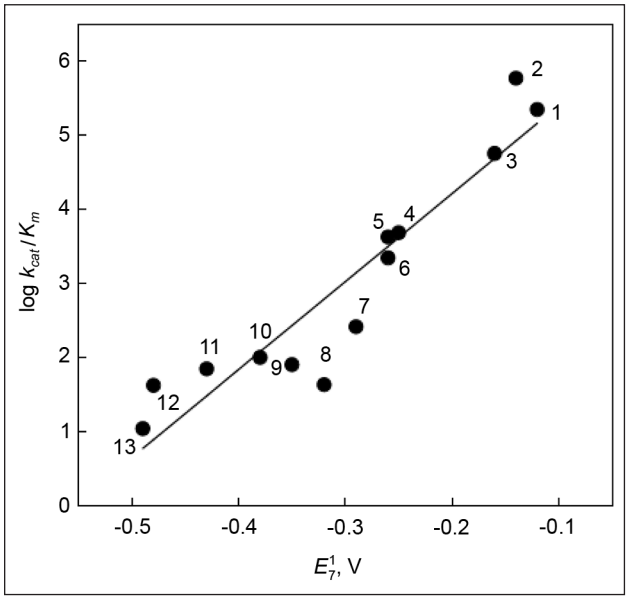


Fig. 5. Dependence of the reactivity (log k_{cat} / K_m) of nitroaromatic compounds on their single-electron reduction potentials E_7^1. The numbers of the compounds are taken from Table

Further, we examined the substrate specificity of Prx–NR, using a series of nitroaromatic compounds with a wide range of single-electron reduction potentials, E_7^1, which reflect the energetics of a nitroaromatic compound / nitroanion radical redox couple [14, 15]. This parameter may be also used in the analysis of two-electron reduction of nitroaromatic compounds by oxygen-insensitive nitroreductases [11], because there is an evident parallelism between the energetics of single-electron and two-electron (hydride) reduction of nitroaromatics. Our data show the presence of a linear relationship between the reactivity (log k_{cat} / K_m) (Table 1) and E_7^1 of nitroaromatic compounds (Fig. 5). This means that there is no substrate preference of Prx–NR for their particular structure, e. g., single, or two, or three aromatic rings, and that the reaction rate is mainly determined by the energetics of nitroreduction. It also shows that there are no sterical hindrances to access the active site FMM by bulky polyciclic nitroaromatic oxidants. These regularities of Prx–NR are analogous to those observed in nitroreductase reactions of *E. claocae* NR [11]. However, the reactivity of Prx–NR in terms of k_{cat} / K_m is 10–100 times lower than that of *E. cloacae* NR [11], including its low reduction rates of antitumour bioreductive agents CB1954 and SN23862 (Table, Fig. 5), which are similar to that of mammalian NAD(P)H : quinone oxidoreductase (NQO1) [10].

CONCLUSIONS

In this work, for the first time, we have characterized the nitroreductase reactions of the *T. maritima* peroxiredoxin–nitroreductase fusion enzyme, which potentially may be important in the future development of *T. maritima*-based systems of explosive biodegradation, owing to the thermophilic nature of this species. Overall, the substrate specificity and reaction mechanism of *T. maritima* NR are similar to those of the best described oxygen-insensitive nitroreductase, *E. cloacae* NR, although there are some differences in their pathways of the reduction of polynitroaromatic *N*-nitramines. However, the reactivity of Prx–NR is 10–100 times lower than that of *E. cloacae* NR, which restricts the application of Prx–NR in nitroaromatic contaminant bioremediation systems and in combined antitumour therapies.

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DVIELEKTRONINĖ NITROAROMATINIŲ JUNGINIŲ REDUKCIJA *THERMOTOGA MARITIMA* PEROKSIREDOKSINO–NITROREDUKTAZĖS HIBRIDINIU FERMENTU

S a n t r a u k a

Kadangi 2,4,6-trinitrotoluenas ir analogiški nitroaromatiniai sprogmenys – toksiški aplinkos teršalai, jų biodegradacijos mechanizmų tyrimai yra pakankamai svarbūs. Šiame kontekste ištyrėme NADPH-priklausomą nitroaromatinių junginių dvielektroninę redukciją *Thermotoga maritima* peroksiredoksino–nitroreduktazės (Prx–NR) hibridiniu fermentu. Nustatėme, kad Prx–NR nitroreduktazinis ir peroksiredoksino domenai funkcionuoja nepriklausomai. Nitroaromatinių junginių reaktingumas (log k_{cat}/K_m) Prx–NR atžvilgiu yra nespecifinis, ten stebima tiesinė jo priklausomybė nuo junginių vienelektroninės redukcijos potencialo (E_7^1). Polinitroaromatinių *N*-nitramininių sprogmenų tetrilo (2,4,6-trinitrofenil-*N*-metilnitramino) ir pentrilo (2,4,6-trinitrofenil-*N*-nitroaminoetilnitrato) redukcija buvo lydima nitrito susidarymo. Tai rodo, kad vyksta jų redukcinė *N*-denitracija. Alternatyviai, *p*-dinitrobenzenas yra redukuojamas į *p*-hydroksilaminobenzeną. Apibendrinant galima teigti, kad Prx–NR nitroaromatinių junginių redukcijai būdingi panašūs bruožai kaip ir kitoms bakterijų kilmės deguoniui nejautrioms nitroreduktazėms, pvz., *Enterobacter cloacae* nitroreduktazei, tačiau Prx–NR reaktingumas yra 10–100 kartų mažesnis nei *E. cloacae* NR.