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Biochimica et Biophysica Acta 1366 (1998) 275–283

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Nitroreductase reactions of *Arabidopsis thaliana* thioredoxin reductase

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Received 30 March 1998; revised 28 May 1998; accepted 10 June 1998

Abstract

Arabidopsis thaliana NADPH:thioredoxin reductase (TR, EC 1.6.4.5) catalyzed redox cycling of aromatic nitrocompounds, including the explosives 2,4,6-trinitrotoluene and tetryl, and the herbicide 3,5-dinitro-*o*-cresol. The yield of nitro anion radicals was equal to 70–90%. Redox cycling of tetryl was accompanied by formation of *N*-methylpicramide. Bimolecular rate constants of nitroaromatic reduction (k_{cat}/K_m) and reaction catalytic constants (k_{cat}) increased upon an increase in oxidant single-electron reduction potential (E_7^1). Using compounds with an unknown E_7^1 value, the reactivity of TR increased parallelly to the increase in reactivity of ferredoxin:$NADP^+$ reductase of *Anabaena* PCC 7119 (EC 1.18.1.2). This indicated that the main factor determining reactivity of nitroaromatics towards TR was their energetics of single-electron reduction. Incubation of reduced TR in the presence of tetryl or 2,4-dinitrochlorobenzene resulted in a loss of thioredoxin reductase activity, most probably due to modification of reduced catalytic disulfide, whereas nitroreductase reaction rates were unchanged. This means that on the analogy of quinone reduction by TR (D. Bironaitė, Ž. Anusevičius, J.-P. Jacquot, N. Čėnas, Biochim. Biophys. Acta 1383 (1998) 82–92), FAD and not catalytic disulfide of TR was responsible for the reduction of nitroaromatics. Tetryl, 2,4,6-trinitrotoluene and thioredoxin increased the FAD fluorescence intensity of TR. This finding suggests that nitroaromatics may bind close to the thioredoxin-binding site at the catalytic disulfide domain of TR, and induce a conformational change of enzymes (S.B. Mulrooney, C.H. Williams Jr., Protein Sci. 6 (1997) 2188–2195). Our data indicate that certain nitroaromatic herbicides, explosives and other classes of xenobiotics may interfere with the reduction of thioredoxin by plant TR, and confer prooxidant properties to this antioxidant enzyme. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Thioredoxin; Thioredoxin reductase; Nitroaromatics; Redox cycling

1. Introduction

Thioredoxin (TRX), a disulfide-reducing redox protein, and flavoenzyme NADPH:thioredoxin reductase (TR, EC 1.6.4.5) form a ubiquitous redox system present in both prokaryotic and eukaryotic organisms [1]. TRX participates in cell proliferation [1,2], and performs antioxidant functions, scavenging activated oxygen species [3] and regenerating proteins inactivated by oxidative stress [4]. In plants, various thioredoxin types participate in light-mediated enzyme regulation in oxygenic photosynthesis and germination [5,6].

The best characterized NADPH:thioredoxin re-

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); E_7^1, single-electron reduction potential at pH 7.0; FNR, ferredoxin:$NADP^+$ reductase; k_{cat}, catalytic constant; k_{cat}/K_m, bimolecular rate constant; TNT, 2,4,6-trinitrotoluene; TR, NADPH:-thioredoxin reductase; TRX, thioredoxin

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PII: S0005-2728(98)00128-5

ductase from *Escherichia coli* is a homodimer and contains one FAD and one redox active disulfide per 33 kDa subunit [7]. The catalysis of *E. coli* TR shares analogous steps with other NAD(P)H:disulfide reductases, e.g., lipoamide dehydrogenase, glutathione and trypanothione reductases, and involves the transfer of redox equivalents from NADPH to FAD, from reduced FAD to catalytic disulfide of TR, and then from the newly formed enzyme dithiol to the disulfide of TRX [7]. However, in contrast with other disulfide reductases, TR does not form a FAD-thiolate charge-transfer complex in two-electron reduced state [7]. In catalysis, TR cycles between two- and four-electron reduced forms, $FADH_2/S_2$ and $FADH_2/(SH)_2$, whereas other disulfide reductases cycle between fully oxidized and two-electron reduced forms, FAD/S_2 and $FAD/(SH)_2$ [8,9]. According to X-ray data, NADP(H) and disulfide substrate of glutathione and trypanothione reductases bind at the vicinity of the FAD and catalytic disulfide, respectively, their binding sites being spatially separated by the isoalloxazine ring of FAD [10], whereas in TR, NADP(H) binds close to catalytic disulfide, and the access of NADP(H) to FAD (or access of TRX to catalytic disulfide) requires the rotation of the NADPH-binding domain with respect to the FAD domain [11]. The mechanism of mammalian TR, which is structurally different from bacterial enzyme being a dimer of two 58 kDa subunits [1,12], is similar to glutathione reductase [13].

Nitroaromatic and nitroheterocyclic compounds (nitrobenzenes, nitrofurans and nitroimidazoles) are used as antibacterial, antiprotozoal and anticancer agents [14–16]. Their therapeutic and/or cytotoxic action is most often related to single- or two-electron reduction, catalyzed by low-potential NAD(P)H-oxidizing flavoenzymes [16]. Another important problem related to a reductive metabolism of the nitro group is the biodegradation of nitroaromatic environment pollutants (dyes, pesticides, explosives) by plants, bacteria and fungi [17]. The mechanisms of interaction of nitroaromatics with flavoenzyme NAD(P)H:disulfide reductases deserve some interest. Although these enzymes catalyze obligatory two-electron (hydride) transfer between their pyridine nucleotide and disulfide substrates [7], they reduce nitroaromatics in a single-electron way, thus initiating their redox cycling [18–22]. In these cases, the physiological antioxidant functions of the above enzymes are converted into prooxidant ones. Besides, certain nitrofurans inhibit glutathione and trypanothione reductases [18–20,22]. These phenomena could be of some toxicological importance, e.g., the trypanocidal action of nitroaromatics may result from their interaction with trypanothione reductase or trypanosome lipoamide dehydrogenase [18,20,23], or the cytotoxic effects of 2,4-dinitrochlorobenzene may be related to its interaction with mammalian thioredoxin reductase [24].

The aim of this paper is to study the interaction of nitroaromatic and nitroheterocyclic compounds with NADPH:thioredoxin reductase from *Arabidopsis thaliana*, a plant-type cytosolic enzyme possessing 45% sequence homology and marked structural analogy to *E. coli* TR [25,26]. Recent steady-state kinetics studies also point to an analogous mechanism of catalysis of *A. thaliana* and *E. coli* thioredoxin reductases [27].

2. Materials and methods

A. thaliana thioredoxin reductase and thioredoxin were prepared as described previously [25]. Concentrations of TR and TRX were determined spectrophotometrically, using $\varepsilon_{454} = 11.3\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{280} = 10.9\ mM^{-1}\ cm^{-1}$ [25,28], respectively. Ferredoxin:$NADP^+$ reductase (FNR, EC 1.18.1.2) from *Anabaena* PCC 7119, prepared as described previously [29], was a generous gift from Dr. M. Martinez-Julvez and Prof. C. Gomez-Moreno (Zaragoza University, Spain). The concentration of FNR was determined using $\varepsilon_{459} = 9.4\ mM^{-1}\ cm^{-1}$. NADPH, $NADP^+$, cytochrome *c*, superoxide dismutase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glucose 6-phosphate and reduced glutathione were obtained from Sigma, while catalase and glucose-6-phosphate dehydrogenase were obtained from Fermentas (Vilnius, Lithuania). 2,4,6-Trinitrotoluene (TNT) was obtained by nitration of 4-nitrotoluene [30], and 4-amino-2,6-dinitrotoluene by reduction of TNT by H_2S [31]. 3,5-Dinitro-*o*-cresol was synthesized by nitration of *o*-cresol [32]. *N*-Methylpicramide (2,4,6-trinitrophenyl-*N*-methylamine) was obtained by the reaction of 2,4,6-trinitrochlorobenzene with methylamine [33], tetryl (2,4,6-trinitrophenyl-*N*-

methylnitroamine) was obtained by nitration of *N*-methylpicramide [34]. Chinifur (2-(5′-nitro(furo-2′-yl)ethene-1-yl)-4-(*N*,*N*-diethylamino)-1-methylbut-1-ylaminocarbonyl-4-quinoline) was a generous gift from Dr. N.M. Sukhova (Institute of Organic Synthesis, Riga, Latvia). The structural formula of chinifur was presented in our previous work [35]. Other nitrocompounds used in this study were obtained from Reakhim (Russia).

All experiments were performed in 0.1 M K-phosphate buffer solution (pH 7.0) containing 1 mM EDTA, at 25°C. Reaction rates were monitored spectrophotometrically, using a Hitachi-557 spectrophotometer. The NADPH:thioredoxin reductase reaction of TR was monitored with the TRX-mediated reduction of DTNB (300 μM) ($\Delta\varepsilon_{412} = 13.6$ mM^{-1} cm^{-1}) [36]. Typically, 40 μM NADPH were used. The NADPH:nitroreductase reaction of TR and ferredoxin:$NADP^+$ reductase was monitored following the rate of NADPH oxidation ($\Delta\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}). Nitroradical-mediated reduction of cytochrome *c* (50 μM) during the NADPH:nitroreductase reaction of TR was monitored following the increase in absorbance at 550 nm ($\Delta\varepsilon = 20$ mM^{-1} cm^{-1}). The kinetic parameters of reactions, the catalytic constant (k_{cat}), and the bimolecular rate constant (k_{cat}/K_m) correspond to the reciprocal intercepts and slopes of the Lineweaver-Burke plots, [E]/*V* vs. 1/[S], where *V* is reaction rate, [E] and [S] are enzyme and substrate concentrations, respectively. k_{cat} corresponds to molecules of NADPH oxidized by enzyme active site per second. Since 1 mol NADPH reduces 2 moles DTNB [36], the rates of DTNB reduction were divided by 2. The activity of ferredoxin:$NADP^+$ reductase determined using 1 mM ferricyanide as acceptor [29] (concentration of NADPH, 200 μM) was equal to 100 s^{-1}.

The fluorescence spectra of FAD of TR (470–670 nm) were recorded using a Hitachi MPF-4 spectrofluorimeter (excitation wavelength 455 nm, enzyme concentration 3 μM). Fluorescence excitation spectra (330–500 nm) were recorded at 518 nm emission wavelength.

The oxygen consumption in the course of the enzymatic reaction was monitored using a Clark electrode, at 25°C.

The inactivation of reduced TR by nitrocompounds was studied as follows. The enzyme was incubated in the presence of nitrocompound, NADPH (40 μM), 2 mM glucose 6-phosphate, 5 μg/ml glucose-6-phosphate dehydrogenase at 25°C, for 5–80 min. After incubation, an aliquot of enzyme was introduced into a spectrophotometer cell (factor of dilution 100), and the rate of DTNB reduction was recorded in the presence of 40 μM NADPH and 2 μM TRX.

3. Results

3.1. Nitroreductase reactions of thioredoxin reductase

Using TRX as electron acceptor, the kinetic parameters of *A. thaliana* TR were close to those determined in our previous work [27]: k_{cat} of the reaction was equal to 7.3 s^{-1}, K_m NADPH was equal to 1.8 μM, and K_m TRX was equal to 1.1 μM.

Thioredoxin reductase catalyzed the oxidation of NADPH at the expense of nitroaromatics. At a fixed concentration of nitroaromatics and varied NADPH (5–50 μM) reaction rates did not change, pointing to micromolar K_m for NADPH. Kinetic parameters of the nitroreductase reaction at saturated NADPH concentration (40 μM) are presented in Table 1, together with values of nitroaromatic single-electron reduction potentials (E_7^1). One should note that the log of k_{cat}/K_m and k_{cat} of the nitroreductase reaction increased upon the increase in E_7^1 of nitrocompounds (Fig. 1). Since E_7^1 values for a number of nitrocompounds examined are not available, we have carried out parallel studies on their reactivity towards ferredoxin:$NADP^+$ reductase. It is well established that this enzyme catalyzes single-electron reduction of nitrocompounds to their anion radicals, and that a linear relationship exists between log k_{cat}/K_m and E_7^1 of nitroaromatics in FNR-catalyzed reactions [35,38]. The values of k_{cat}/K_m of nitrocompounds in their reactions with *Anabaena* FNR are presented in Table 1. The data of Fig. 2 indicate that, in general, the reactivity of nitroaromatics towards TR increases upon the increase in their reactivity towards FNR.

We have found that TR reduced nitroaromatics in a single-electron way, initiating their redox cycling. During nitroreductase reaction, we have observed the reduction of added cytochrome *c*, that was partly inhibited by 30 μg/ml superoxide dismutase (50–

70%). This points to the reoxidation of nitro anion radicals formed by oxygen with formation of $O_2^{-\cdot}$ and participation of nitro anion radicals and superoxide in the reduction of cytochrome *c* [38]. The yield of free radicals expressed as the ratio between the rate of cytochrome *c* reduction and the double rate of NADPH enzymatic oxidation was equal to 80–90% (nitrofurantoin, *o*-dinitrobenzene, TNT) and to 70% (tetryl). In separate assays, we have found that TR catalyzed the oxidation of excess NADPH over the amount of nitroaromatic compound, and that the reaction was accompanied by oxygen consumption. The subsequent addition of catalase caused reappearance of oxygen, thus pointing to the formation of H_2O_2 as the final reaction product. Next, we have examined the effect of TRX on the rate of nitroreductase reaction of TR, monitored according to NADPH oxidation. In the presence of NADPH, TR, TRX and nitrocompound, the resulting reaction rate was a sum of separately determined nitrocompound and TRX-dependent reaction rates. Besides, the accelerating effect of thioredoxin was observed only at the beginning of the reaction, until the stoichiometric amount of NADPH to added TRX (2–4 μM) was consumed.

Monitoring absorption spectra of several nitrocompounds (*o*-dinitrobenzene, 2,4,6-trinitrotoluene)

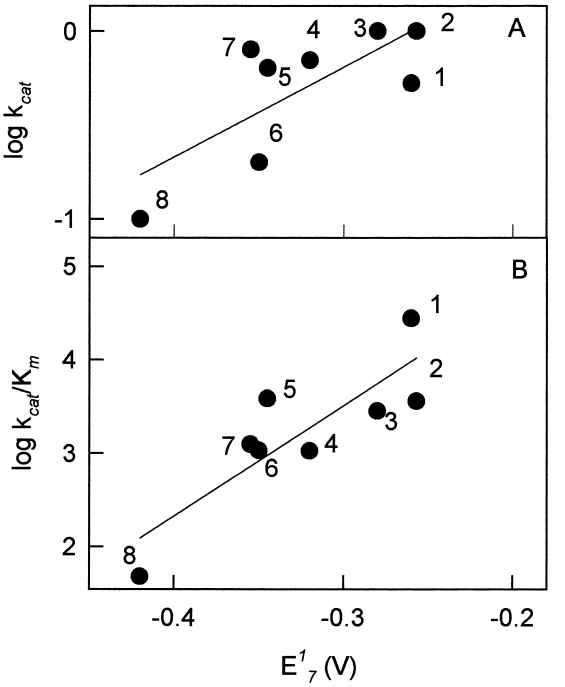


Fig. 1. The dependence of *A. thaliana* TR nitroreductase reaction catalytic constants (A) and bimolecular reaction rate constants (B) on single-electron reduction potentials of nitroaromatics. The numbers of nitrocompounds are taken from Table 1.

Table 1
Kinetic parameters of the nitroreductase reaction of *A. thaliana* thioredoxin reductase (TR), bimolecular rate constants of nitroaromatic reduction by *Anabaena* PCC 7119 ferredoxin:NADP$^+$ reductase (FNR), and single-electron reduction potentials of nitroaromatics (E_7^1), pH 7.0

No.	Compound	E_7^1 (V)[a]	k_{cat} (s^{-1}) (TR)	k_{cat}/K_m (M^{-1} s^{-1})	
				TR	FNR
1	Nitrofurantoin	−0.255	0.53	2.7×10^4	1.2×10^{4}[b]
2	*p*-Dinitrobenzene	−0.257	1.0	3.6×10^3	3.0×10^4
3	*o*-Dinitrobenzene	−0.287	1.0	2.8×10^3	2.2×10^3
4	4-Nitrobenzaldehyde	−0.315	0.7	1.06×10^3	8.3×10^{3}[b]
5	*m*-Dinitrobenzene	−0.345	0.64	3.8×10^3	2.5×10^3
6	3,5-Dinitrobenzoic acid	−0.350	0.2	1.6×10^3	1.1×10^3
7	4-Nitroacetophenone	−0.355	0.8	1.25×10^3	1.0×10^{3}[b]
8	4-Nitrobenzoic acid	−0.425	0.1	4.8×10^1	2.5×10^{2}[b]
9	2,4,6-Trinitrotoluene	–	1.8	6.2×10^3	5.3×10^3
10	Tetryl	–	1.47	5.7×10^3	9.0×10^4
11	2,4-Dinitrochlorobenzene	–	0.44	1.5×10^3	2.3×10^4
12	Chinifur	–	0.4	2.0×10^4	6.0×10^{4}[b]
13	4-Amino-2,6-dinitrotoluene	–	–	3.3×10^2	1.0×10^3
14	3,5-Dinitro-*o*-cresol	–	–	2.0×10^2	1.0×10^3

[a]Taken from [37].
[b]Taken from [35].

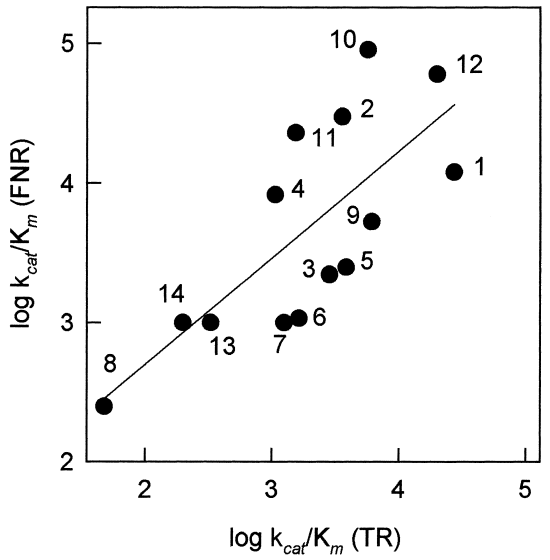
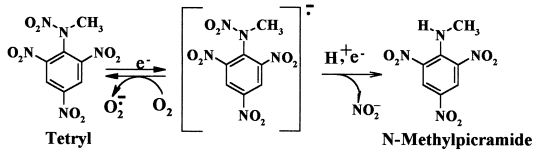


Fig. 2. The relationship between bimolecular rate constants in nitroreductase reactions of *A. thaliana* thioredoxin reductase (TR) and *Anabaena* ferredoxin:$NADP^+$ reductase (FNR). The numbers of compounds are taken from Table 1.

in the presence of TR, micromolar amounts of NADPH and NADPH regeneration system, glucose 6-phosphate and glucose-6-phosphate dehydrogenase, we did not observe their reduction by TR under aerobic conditions. In contrast, redox cycling of tetryl by TR under aerobic conditions was accompanied by spectral changes (Fig. 3) consistent with the formation of *N*-methylpicramide as a reaction product. Evidently, TR reduced tetryl according to a mechanism characteristic for ferrredoxin:$NADP^+$ reductase [39], where the redox cycling of tetryl was accompanied by parallel elimination of nitrite and formation of *N*-methylpicramide (Eq. 1). In accordance with this, the spectra of the reaction products obtained after incubation of tetryl with *Anabaena* FNR and NADPH-regeneration system were analogous to those presented in Fig. 3 (data not shown).



(1)

3.2. Inactivation of TR by nitroaromatics

It is known that 2,4-dinitrochlorobenzene inactivated reduced human thioredoxin reductase, modifying reduced active site thiol [24]. We have found that incubation of reduced *A. thaliana* TR in the presence of 2,4-dinitrochlorobenzene resulted in a loss of TRX-reductase activity (Fig. 4A). By extrapolation to infinite 2,4-dinitrochlorobenzene concentration (Fig. 4B), a maximal value of the pseudo-first order inactivation rate constant (k_i) was obtained, being equal to 0.04 min^{-1}. The bimolecular inactivation rate constant was equal to 0.28 mM^{-1} min^{-1} (4.66 M^{-1} s^{-1}). Among other compounds investigated, tetryl inactivated reduced TR even faster than 2,4-dinitrochlorobenzene (Fig. 4A,B), with a maximal rate of inactivation of 0.2 min^{-1}, and a bimolecular rate constant of 2.5 mM^{-1} min^{-1} (41.7 M^{-1} s^{-1}). It is known that tetryl undergoes ring substitution reactions with various nucleophiles [33], thus inactivation of TR may probably involve a modification of

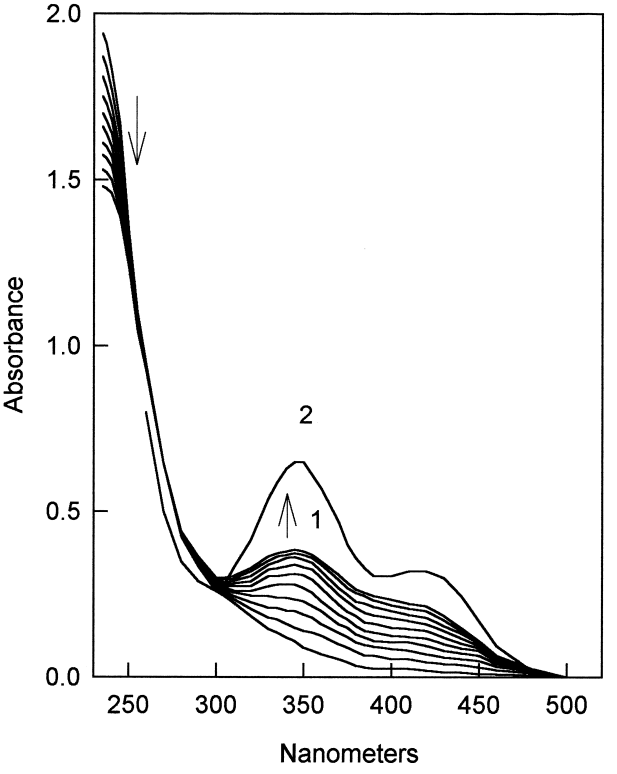


Fig. 3. The spectral changes of 50 μM tetryl during its aerobic reduction by 70 nm thioredoxin reductase, 10 μM NADPH, 5 mM glucose 6-phosphate, and 30 μg/ml glucose-6-phosphate dehydrogenase (1). The spectra were recorded every 40 min. For comparison, the spectrum of 50 μM *N*-methylpicramide is presented (2).

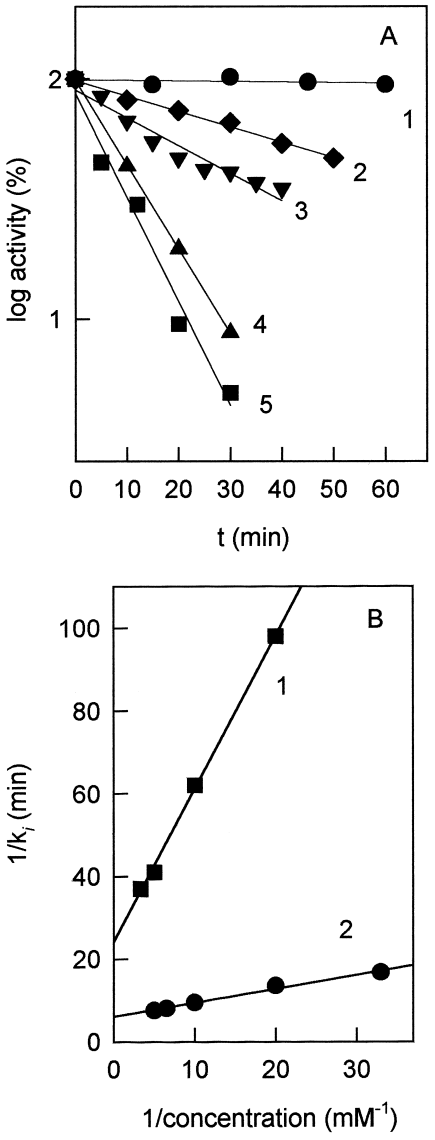


Fig. 4. Inactivation of reduced thioredoxin reductase by tetryl and 2,4-dinitrochlorobenzene. (A) Kinetics of inactivation of reduced TR. Additions: none (1), 100 μM 2,4-dinitrochlorobenzene (2), 200 μM 2,4-dinitrochlorobenzene (3), 100 μM tetryl (4), and 200 μM tetryl (5). (B) The dependence of pseudo-first order inactivation rate constant on the concentration of 2,4-dinitrochlorobenzene (1) and tetryl (2).

reduced active site thiol by tetryl. In a control experiment, tetryl reacted with reduced glutathione with $k = 0.63$ M^{-1} s^{-1}, yielding a product with increased absorbance at 320–360 nm (data not shown).

The incubation of oxidized TR with 200 μM tetryl or 2,4-dinitrochlorobenzene for 1–1.5 h did not change enzyme TRX-reductase activity. The inactivation of reduced enzyme by tetryl neither affected its nitroreductase activity using tetryl or TNT as electron acceptor, nor changed low intrinsic NADPH-oxidase activity of TR. This indicates that tetryl may interact with several sites on TR. The alkylation of the active site thiol by tetryl is a relatively slow process (Fig. 4), and appears to be a separate event from nitroreductase activity of TR using tetryl as electron acceptor. Besides, during 5–10 min registration of reaction kinetics, we did not observe a time-dependent increase in nitroreductase activity of TR.

3.3. The binding of nitroaromatics to TR

As observed for *E. coli* TR [36,40], FAD of *A. thaliana* enzyme was fluorescent with $\lambda_{max} = 518$ nm, possessing approx. 25% of the fluorescence intensity of free FAD. A possibility exists that the binding of nitroaromatics to TR may quench the FAD fluorescence. However, we have found that tetryl and 2,4,6-trinitrotoluene (60–500 μM) *increased* the fluorescence intensity of FAD (Fig. 5A). This increase in fluorescence intensity was accompanied by a proportional increase in intensity of fluorescence excitation spectra (data not shown). On the other hand, *o*-, *m*- and *p*-dinitrobenzenes (1 mM) or 2,4-dinitrochlorobenzene (0.5 mM) did not change the fluorescence intensity. The data linearization in double-reciprocal coordinates, 1/compound concentration, 1/relative increase in intensity (Fig. 5C), yielded binding constants and maximal increases in fluorescence intensity. They were equal to 220 μM and 50% (tetryl), and 180 μM and 35% (TNT).

Further, we have found that the addition of excess thioredoxin (5–8 μM) to 3 μM thioredoxin reductase solution also increased the fluorescence intensity by maximal level, approx. 18% (Fig. 5B). In the presence of 5 μM TRX, addition of tetryl further increased FAD fluorescence (Fig. 5B). The finding that thioredoxin and certain nitroaromatics influence the FAD fluorescence in a similar way points to the possibility that these compounds may bind at close sites in the enzyme catalytic disulfide domain [11,26]. Due to low solubility of tetryl (approx. 400 μM) and relatively small fluorescence changes, we were unable to detect whether the presence of TRX decreased the affinity of tetryl to TR. However, monitoring the TRX-mediated reduction of DTNB in the presence

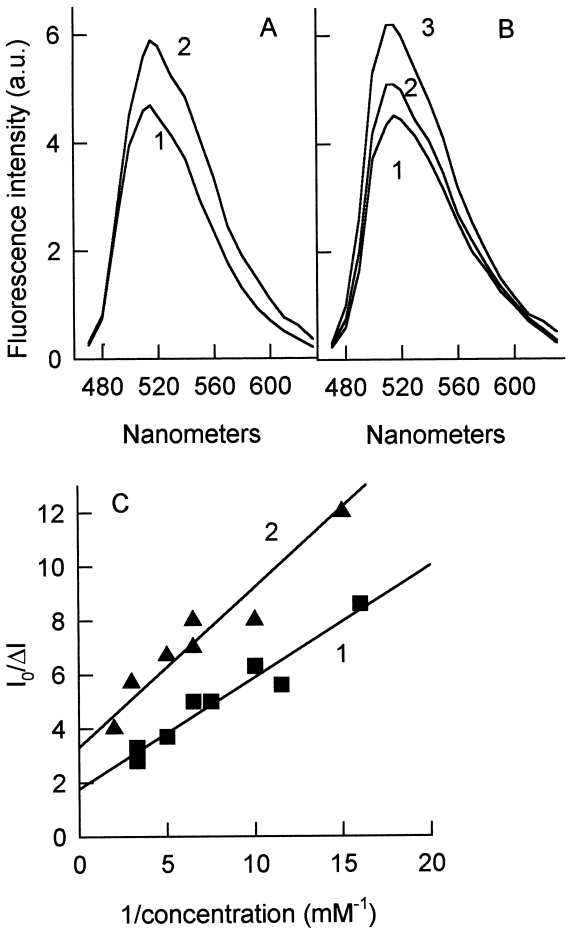


Fig. 5. The influence of nitroaromatics on the FAD fluorescence intensity of thioredoxin reductase. (A) Fluorescence of 3 μM TR, no additions (1), the same after addition of 300 μM tetryl (2). (B) Fluorescence of 3 μM TR, no additions (1), subsequent additions of 5 μM TRX (2) and 300 μM tetryl (3). (C) The dependence of a relative increase in fluorescence intensity on the concentration of tetryl (1) and 2,4,6-trinitrotoluene (2).

of tetryl and saturating NADPH concentration (40 μM), we have found that tetryl acted as non-competitive inhibitor to TRX ($K_i = 150$ μM). One may argue that this effect can be attributed to an interception of electron flux from TR to TRX by tetryl. However, the observed enzyme turnover rate in NADPH oxidation by 300 μM tetryl (0.8 s^{-1}) is much lower than the difference between enzyme maximal turnover rates in TRX-mediated DTNB reduction, 7.3 s^{-1} in the absence of tetryl, and 2 s^{-1} in the presence of 300 μM tetryl. This clearly points out that the binding of tetryl to TR slows down the rate of enzymatic reduction of TRX.

4. Discussion

The data of the present work demonstrate that *A. thaliana* NADPH:thioredoxin reductase reduces nitroaromatics in a single-electron way, and initiates their redox cycling. The reactivity of nitroaromatic compounds increased upon the increase in their single-electron reduction potential, E_7^1 (Fig. 1A,B). Besides, the reactivity of nitrocompounds investigated, including the compounds with unavailable values of E_7^1 (Table 1), increased upon increase in their reactivity towards ferredoxin:$NADP^+$ reductase (Fig. 2), a typical representative of flavoenzyme dehydrogenases-electron transferases, which reduce nitroaromatics in a single-electron way [38]. The linear relationship between logs of k_{cat}/K_m of nitroaromatics and their E_7^1 values for FNR-catalyzed reactions was established [35,38], indicating that the reaction followed a model of an 'outer-sphere' single-electron transfer [41], where the differences in structure of oxidants played a minor role, and the main factor determining the reactivity was the energetics of single-electron transfer. Thus, the same factor appears to be the most important in the nitroreductase reaction of TR. The analogous reaction rate vs. redox potential dependence was observed for the quinone reductase reaction of *A. thaliana* TR [27], pointing to a similar mechanism of quinone- and nitroreductase reactions of this enzyme. The data of our previous paper demonstrate that quinones are reduced by the FAD cofactor of *A. thaliana* TR without involvement of reduced catalytic disulfide [27]. Reduction of nitroaromatics by TR should also share this pathway, since the TRX-reductase reaction of TR was abolished by nitroaromatics that alkylate reduced catalytic disulfide of TR (Fig. 4), whereas the nitroreductase activity of TR remained unchanged. It is interesting to note that the rate of modification of *A. thaliana* TR by 2,4-dinitrochlorobenzene ($k = 4.66$ M^{-1} s^{-1}, Fig. 4B) is an intermediate between the rates of modification of human placenta thioredoxin reductase ($k = 200$ M^{-1} s^{-1} [24]) and reduced glutathione ($k = 0.03$ M^{-1} s^{-1} [24]). The reaction of tetryl with *A. thaliana* TR ($k = 41.7$ M^{-1} s^{-1}, Fig. 4B) was also much faster than the reaction with glutathione ($k = 0.63$ M^{-1} s^{-1}).

Another interesting point is that the maximal rate of nitroreductase reaction of *A. thaliana* TR reached

25% of physiological disulfide substrate reduction rate (Table 1). This ratio is much higher than that for other flavoenzyme NAD(P)H:disulfide reductases, e.g., 5% for *Trypanosoma congolense* trypanothione reductase [22], 1.5% for pig heart lipoamide dehydrogenase [21], and $<0.1\%$ for yeast glutathione reductase [20]. The latter phenomenon was most probably caused by the participation of fully reduced FAD in the nitroreductase reaction of TR. On the analogy of the quinone reductase reaction of TR, we suppose that in the nitroreductase reaction, TR shuttles between fully oxidized (FAD/S_2) and two-electron reduced ($FADH_2/S_2$) states [27], whereas in glutathione and trypanothione reductase-catalyzed reactions nitrocompounds and quinones oxidize the FAD-reduced disulfide charge-transfer complex of two-electron reduced enzyme, where the electron density on FAD is low [7,42].

The data on the nitroreductase reaction of *A. thaliana* TR (Table 1, Figs. 1–3) provide evidence that redox active nitroaromatic pollutants, e.g. the explosives tetryl and TNT, and the herbicide 3,5-dinitro-*o*-cresol, may confer prooxidant properties to this antioxidant enzyme. It is possible that the single-electron reactions of thioredoxin reductase with these nitroaromatics, together with observed inactivation of TR by tetryl (Fig. 4), could be partly responsible for their damaging action to plant cells. On the other hand, thioredoxin reductase may also participate in tetryl biotransformation to less cytotoxic *N*-methylpicramide (Fig. 3), and play a certain role in its degradation by plants or microorganisms [39].

The finding that certain nitroaromatics increased the FAD fluorescence intensity of TR instead of quenching it (Fig. 5) was unexpected; however, it enabled us to reach certain conclusions about the mode of binding of nitroaromatics to TR. Since X-ray analysis revealed a similarity between structures of *E. coli* and *A. thaliana* TR [11,26], we have used the approach of Williams et al. [40] assuming that *E. coli* TR exists as equilibrium of two conformations, namely, non-fluorescent FO (catalytic disulfide adjacent to FAD) and fluorescent FR (NAD(P)H-binding site adjacent to flavin). One may suppose that the binding of nitroaromatics or thioredoxin at the proximity of catalytic disulfide of TR may cause conformational change of *A. thaliana* TR, increasing the content of the fluorescent TR conformational state (Fig. 5A,B). The fact that tetryl inhibits the reduction of TRX by TR also favors the assumption that tetryl may bind close to the thioredoxin-binding site.

In conclusion, the data of the present work reveal the following modes of interaction of *A. thaliana* thioredoxin reductase with nitroaromatics: (i) redox cycling of nitroaromatics arising from their single-electron reduction via FAD cofactor of enzyme; (ii) the loss of thioredoxin reductase activity under the action of nitroaromatics that can alkylate reduced catalytic disulfide of TR; (iii) the inhibition of TRX-reductase activity of TR, caused by the interception of electron flux for TRX reduction by nitroaromatics, and/or by binding of certain nitroaromatics at the vicinity of the TRX-binding site. The latter mode of interaction deserves more thorough investigation, in view of the ubiquitous role of the TRX/TR system in organisms and the possibility of construction of TRX-binding site specific inhibitors of TR. This approach would be an extension of numerous studies on possible biomedical and toxicological importance of the inhibitors of analogous enzymes, trypanothione reductase and glutathione reductase [18–23].

Acknowledgements

This work was supported in part by EC grant No. ERCIC15CT961004 and NATO Linkage grant HTECH. LG 972012. We thank Prof. C. Gomez-Moreno and Dr. M. Martinez-Julvez for their generous gift of ferredoxin:$NADP^+$ reductase.

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