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The comparative study of redox properties of recombinant human cytosolic and mitochondrial NADPH:thioredoxin reductases

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We compared the redox properties of recombinant cytosolic (TrxR1) and mitochondrial (TrxR2) isoforms of human flavosulfoselenoenzyme NADPH:thioredoxin reductase. The standard redox potentials of isoenzymes (E^0_7), determined according to the redox equilibrium with the $NADP^+$/NADPH couple, were equal to –0.295 V (TrxR1) and –0.270 V (TrxR2). The more positive value of E^0_7 of TrxR2 may be attributed to the presence of His-125 at the vicinity of catalytic disulfide and selenylsulfide, instead of Tyr-116 in TrxR1. The reactivity of several quinones and nitroaromatic compounds towards TrxR1 and TrxR2 increased with their single-electron reduction potential (E^1_7). For the first time, we studied the TrxR1-catalysed reduction of a series of aromatic *N*-oxides which were reduced in a mixed single- and two-electron way. Their reactivity was close to that of quinones and nitroaromatics with the similar values of E^1_7.

Keywords: thioredoxin reductase, redox potential, xenobiotics, bioreductive activation

INTRODUCTION

NAD(P)H:thioredoxin reductases (TrxRs) are important antioxidant enzymes in both prokaryotic and eukaryotic organisms. Mammalian TrxRs (2 × 58 kD) contain FAD, catalytic disulfide, and a third cofactor, selenylsulfide in their active center, the latter being located at the C-end of the protein in the conserves sequence Gly–Cys–SeCys–Gly. The physiological substrates of TrxRs are 10–12 kD disulfide proteins thioredoxins (Trxs), which perform antioxidant and other numerous physiological functions ([1, 2] and references therein) (Scheme). During catalysis, the enzyme cycles between two- and four-electron reduced states, where the main electron density is located on dithiolate and reduced selenylsulfide. The rate-limiting catalysis step of mammalian TrxR is the two-electron transfer between dithiolate and selenylsulfide ([3–5] and references therein). Subsequently, reduced selenylsulfide reduces Trx or artificial oxidant, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), whose maximal reduction rate is close to that of Trx reduction (Scheme). Among other important oxidants of TrxR are quinones (toxins juglone and toxoflavin, anticancer aziridinyl-benzoquinones) [6, 7] and nitroaromatic compounds (antibacterial nitrofurans, polynitrobenzene environmental pollutants) [8]. Their reduction proceeds most frequently in a mixed single- and two-electron way via reduced FAD cofactor, and in certain cases, with the involvement of reduced selenosulfide [6–8] (Scheme). Because human TrxRs are overexpressed in numerous cancer lines [9, 10], these reactions

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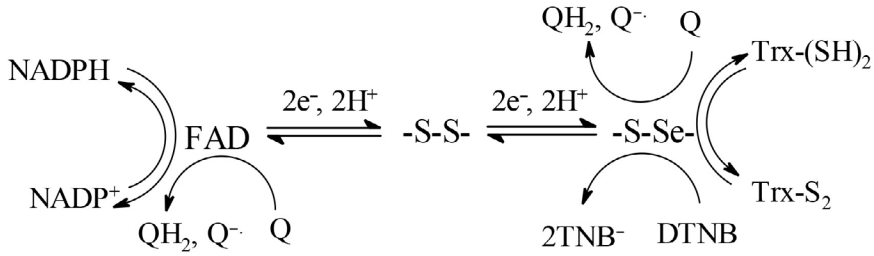

Scheme. A simplified TrxR catalysis scheme, where Q, QH_2 and $Q^{-}\cdot$ denote quinone, hydroquinone and semiquinone, and TNB^{-} is 2-thio-5-nitrobenzoic acid

leading to the formation of free radicals and to the conversion of antioxidant TrxRs into prooxidant ones may be of certain cytotoxic/therapeutic importance ([7] and references therein).

Two major isoforms of mammalian TrxR are reported, a cytosolic (TrxR1) and a mitochondrial (TrxR2) one, sharing 52–56% sequence identity, and being characterised by different pathways of antioxidant action and roles in redox signalling ([11, 12] and references therein). One may note, however, that in spite of extensive studies of both isoforms of TrxR from various sources, a limited information is available from the direct side-by-side comparison of their redox properties under identical conditions [13]. In this study, we characterised the standard redox potentials (E^0_7) of recombinant human TrxR1 and TrxR2, and compared their reactivity with nonphysiological electron acceptors, quinones and nitroaromatic compounds.

MATERIALS AND METHODS

Recombinant human TrxR1 and TrxR2, prepared as described [13], was a generous gift of Dr. Qing Cheng and Prof. Elias S. J. Arner (Karolinska Institutet, Stockholm, Sweden). The enzymes were pure as judged by Coomassie-stained SDS-PAGE. The enzyme concentration was determined from the absorbance of FAD, $\epsilon_{463} = 11.3\ \text{mM}^{-1}\ \text{cm}^{-1}$. *Chlamydomonas reinhardtii* thioredoxin (Trx), a generous gift of Prof. Nicolas Rouhier (Universite de Lorraine, Nancy), was prepared as described [14], and its concentration was determined using $\epsilon_{280} = 10.9\ \text{mM}^{-1}\ \text{cm}^{-1}$. NADPH, $NADP^+$, cytochrome *c*, superoxide dismutase, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), quinones and nitroaromatic compounds were obtained from Sigma-Aldrich and were used without additional purification. 3-Amino-1,2,4-benzotriazine-1,4-dioxide (tirapazamine) and its 7-methyl-, 7-chloro- and 7-trifluoromethyl-derivatives, synthesised as described [15], were a generous gift of Dr. Jonas Šarlauskas (Institute of Biochemistry, Vilnius). Their purity was checked by melting point, elemental analysis, and IR and NMR spectra.

Kinetic measurements were carried out spectrophotometrically, using a PerkinElmer Lambda 25 spectrophotometer in a 0.1 M K-phosphate buffer (pH 7.0), containing 1 mM EDTA, at 25°C. The rate of 0.067–1.0 mM DTNB reduction by TrxRs in the presence of 6.7–50 μM NADPH was monitored following the increase in absorbance at 412 nm ($\Delta\epsilon_{412} = 27.2\ \text{mM}^{-1}\ \text{cm}^{-1}$), considering that one molecule of DTNB (and NADPH) produces two 2-thio-5-nitrobenzoate anions. In the reverse reaction of TrxRs, the reduction of $NADP^+$ (0.063–1.0 mM) was monitored at 340 nm in the presence of Trx (3.0–30 μM) and 8 mM DTT as a reducing agent. This activity was corrected for the background reaction (reduction of $NADP^+$ by TrxR in the presence of 8 mM DTT but in the absence of Trx), which was 0.2 and 0.08 mol $NADP^+$/s × mol subunit for TrxR1 and TrxR2, respectively. The kinetic parameters of reactions, i.e. the catalytic constant (k_{cat}) and the bimolecular rate constant (k_{cat}/K_m), correspond to the reciprocal intercepts and slopes of the Lineweaver–Burk plots, $[E]/v$ versus 1/[S], where v is the reaction rate, [*E*] and [S] are the enzyme and substrate concentrations, respectively. They were obtained by fitting the kinetic data to the parabolic expression using SigmaPlot 2000 (version 11.0). The k_{cat} corresponds to the molecules of NADPH oxidised (or $NADP^+$ reduced) at saturating substrate concentration by the enzyme active site assuming one active site per subunit. Using quinones, nitroaromatic compounds and tirapazamine derivatives as oxidants,

the NADPH-acceptor reductase activity of TrxR was monitored following the rate of NADPH oxidation ($\Delta\epsilon_{340}$ = 6.2 mM^{-1} cm^{-1}) in the presence of 100 μM NADPH. In separate experiments, 50 μM cytochrome *c* was added into the reaction mixture, and its acceptor mediated reduction was monitored at 550 nm ($\Delta\epsilon_{550}$ = 20 mM^{-1} cm^{-1}). The reaction rate was corrected for the background (direct) reduction of cytochrome *c* by TrxR1, 0.15 mol of cytochrome *c* reduced per s per mol enzyme subunit.

RESULTS AND DISCUSSION

Examining the reactions of human TrxR1 and TrxR2, we followed the approach used in our previous studies of rat TrxR1 [6, 8]. The NADPH-dependent reduction of DTNB by human TrxR1 followed the 'ping-pong' mechanism, which is evident from a series of parallel Lineweaver–Burk plots obtained at varied concentrations of NADPH and fixed concentrations of DTNB (Fig. 1a). Analogous dependences were characteristic for TrxR2 (data not shown). The kinetic parameters of both enzymes, k_{cat}, the enzyme turnover rate at saturating concentrations of both substrates, k_{cat}/K_m, the apparent second-order rate constants of both substrates, are given in Table 1. Like in the previous study [13], the activity of TrxR1 is by several times higher than that of TrxR2. As in the case of rat TrxR1 [6], the reverse reaction of TrxRs, the reduction of $NADP^+$ using *C. reinhardtii* thioredoxin as the reducing substrate, also followed the 'ping-pong' mechanism (Fig. 1b). The kinetic parameters of reaction are given in Table 1. One may note that the observed 'ping-pong' patterns (Fig. 1a, b) in this case reflect the 'hybrid ping-pong' mechanism, where the reducing and oxidizing substrates react with separate redox centers, and do not affect each other's binding [16].

The standard redox potential of TrxRs (E^0_7) can be calculated according to the Haldane relationship, where the equilibrium constant (*K*) of

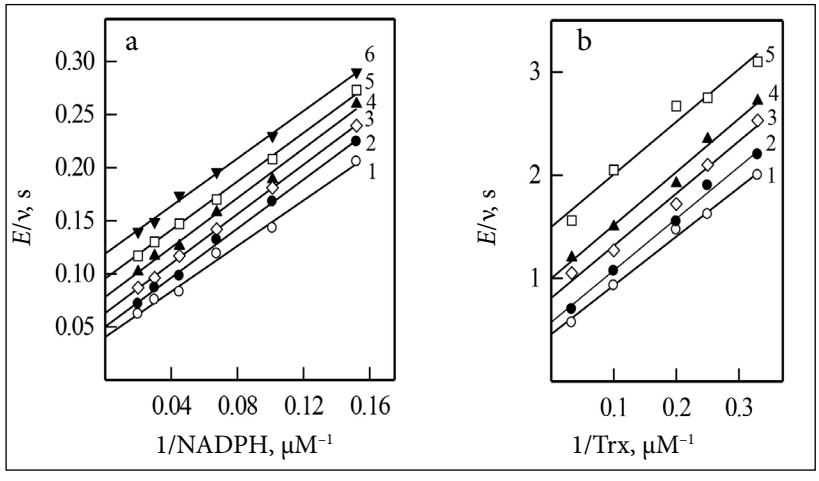

Fig. 1. Kinetics of the forward reaction of TrxR1 (A) and the reverse reaction of TrxR2 (B), pH 7.0, 25°C. (a) Concentrations of DTNB: 500 μM (1), 333 μM (2), 222 μM (3), 149 μM (4), 99 μM (5) and 67 μM (6). (b) Concentrations of $NADP^+$: 1000 μM (1), 500 μM (2), 250 μM (3), 125 μM (4) and 62.5 μM (5)

Table 1. **The kinetic parameters of recombinant thioredoxin reductases in reactions with DTNB or reduced *Cl. reinhardtii* thioredoxin at pH 7.0 and 25°C**

Enzyme	Reductant	Oxidant	k_{cat}, s^{-1}	k_{cat}/K_m, M^{-1}s^{-1}	
				Reductant	Oxidant
TrxR1	NADPH	DTNB	31.2±1.7	9.30±0.50 × 10^5	1.72±0.11 × 10^5
	Trx-$(SH)_2$	$NADP^+$	9.5±0.6	4.56±0.32 × 10^6	1.33±0.06 × 10^5
TrxR2	NADPH	DTNB	7.7±0.4	7.05±0.33 × 10^5	1.05±0.08 × 10^5
	Trx-$(SH)_2$	$NADP^+$	2.5±0.1	2.10±0.15 × 10^5	1.39±0.05 × 10^4

enzyme reaction with the $NADP^+$/NADPH couple (E^0_7 = –0.320 V) is equal to the ratio of k_{cat}/K_m for NADPH and $NADP^+$, respectively. According to the Nernst equation, the difference between the standard potentials of reactants, ΔE^0, is equal to 0.0295 V × log *K*. Using the data of Table 1, this gives the *K* values of 6.99±0.46 and 50.7±4.2, and E^0_7 values of –0.295±0.002 V and –0.270±0.002 V for TrxR1 and TrxR2, respectively. This shows that the E^0_7 of human TrxR1 is equal to the previously determined redox potential of rat TrxR1 [6]. The more positive redox potential of TrxR2, i.e. the stronger stabilization of its four-electron reduced form, can be explained by the different environments of catalytic disulfide and selenylsulfide dyad in the isoenzymes. The X-ray analysis of mouse TrxR2 shows that this dyad is flanked by basic His-143 and His-497 residues, which may stabilise the reduced state by formation of H-bonds [17]. These residues, His-125 and His-488, are conserved in human TrxR2, whereas rat and human TrxR1 contain His-472, but a second histidine is substituted by Tyr-116 ([11, 12] and references therein). This substitution may weaken the stabilization of the reduced state of TrxR1.

Because TrxR reversibly reacts with the $NADP^+$/NADPH couple, it is interesting to assess the possible relationship between the obtained E^0_7 values of TrxRs and [$NADP^+$]/[NADPH] ratio in cytosol and mitochondria. It is commonly accepted that the cytosolic [$NADP^+$]/[NADPH] ratio is below 0.01 [18], whereas mitochondrial values have been reported to range from 0.07 [19] to 0.22–0.72 [20–22]. Thus, higher E^0_7 may enable TrxR2 to exert its antioxidant functions at higher [$NADP^+$]/[NADPH] ratios, which becomes more important in the case of intramitochondrial oxidative stress when the [$NADP^+$]/[NADPH] ratio may reach 5.0 [19].

Another important problem in the functioning of TrxR is its reactions with non-physiological redox agents, including drugs and environmental contaminants, which may be partly responsible for their oxidative stress-type cytotoxicity [6–8]. The rate constants of the reduction of nonphysiological oxidants by TrxR1 and TrxR2 are given in Table 2. One may note that the reactivity of human TrxR1 is similar to that previously determined of rat TrxR1 [6]. The reduction of phenanthrene quinone, juglone and tetryl by TrxR1 was accompanied by the reduction of added cytochrome *c* at 150–190% NADPH oxidation rate, which was inhibited by 20–30% upon the addition of 30 μg/ml uperoxide dismutase (data not shown). This indicates the formation of free radicals of these compounds, which enter the equilibrium with the oxygen/superoxide redox pair. In contrast, *p*-dinitrobenzene negligibly stimulated the reduction of cytochrome c by TrxR1 which shows that it is reduced in a two-electron way. Thus, the reduction mechanisms of the above compounds by

Table 2. **The kinetic parameters of recombinant thioredoxin reductases in reactions with nonphysiological electron acceptors at pH 7.0, 25°C and [NADPH] = 100 μM. The E17 values are taken from [23] (compounds 1–7) and [24] (compounds 8–11)**

No.	Oxidant	E^1_7, V	TrxR1		TrxR2	
			k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}s^{-1}$
1.	2,3-Dichloro-1,4-naphthoquinone	–0.035	14.0±0.7	$5.7±1.0 \times 10^6$	2.4±0.2	$8.8±1.0 \times 10^5$
2.	2-Hydroxy-1,4-naphthoquinone (juglone)	–0.090	10.3±1.3	$1.5±0.2 \times 10^6$	2.8±0.2	$2.7±0.2 \times 10^5$
3.	9,10-Phenanthrene quinone	–0.120	21.6±1.8	$8.0±0.7 \times 10^6$	8.0±0.9	$4.8±0.4 \times 10^5$
4.	2,4,6-Trinitrophenyl-*N*-methylnitramine (tetryl)	–0.191	2.9±0.2	$4.5±0.3 \times 10^4$	0.7±0.1	$4.0±0.5 \times 10^3$
5.	2-Methyl-1,4-naphthoquinone	–0.200	13.6±1.2	$4.9±0.3 \times 10^4$	11.0±0.7	$6.2±0.5 \times 10^3$
6.	*p*-Dinitrobenzene	–0.255	1.1±0.1	$2.0±0.2 \times 10^3$	0.4±0.1	$3.0±0.3 \times 10^2$
7.	5-Nitrothiophene-2-aldoxime	–0.288	0.4±0.07	$1.4±0.2 \times 10^3$		
8.	7-Trifluoromethyl-tirapazamine	–0.345	0.1±0.02	$1.1±0.2 \times 10^3$		
9.	7-Chloro-tirapazamine	–0.400	≥0.06	$7.3±0.7 \times 10^2$		
10.	3-Amino-1,2,4-benzotriazine-1,4-dioxide (tirapazamine)	–0.455	≥0.04	$1.1±0.2 \times 10^2$		
11.	7-Methyl-tirapazamine	–0.474	0.3±0.04	$2.7±0.4 \times 10^2$		

human TrxR1 are the same as in rat TrxR1-catalysed reactions [6, 8]. Representatives of the previously untested group of compounds, tirapazamine derivatives, also stimulated the reduction of added cytochrome c. However, due to their low activity, the rate of cytochrome c reduction increased only from 0.15 s^{-1} in control experiments to 0.18–0.25 s^{-1} at 100–200 μM compound concentration. The partial inhibition of the reaction rate increase by superoxide dismutase shows that the reduction of tirapazamine derivatives proceeds at least partly in a single-electron way. It has previously been found that the log k_{cat}/K_m of quinones and nitroaromatic compounds in rat TrxR1-catalysed reactions follow a common linear dependence on their E^1_7, although the data are strongly dispersed [6, 8]. We observe a similar trend in human TrxR1-catalyzed reactions, including the reactivity of new group of oxidants, tirapazamine derivatives (Fig. 2). Due to the low reactivity, the reactions of tirapazamine derivatives with TrxR2 have not been studied. However, in the case of the tested compounds (Table 2), the specificity of TrxR1 and TrxR2 for them almost does not differ, because there exists a linear interdependence between their log k_{cat}/K_m (r^2 = 0.9793). Thus, based on the data obtained in this work, one may not expect that TrxR1 and TrxR2 can exert different effects on the cytotoxicity of these classes of xenobiotics.

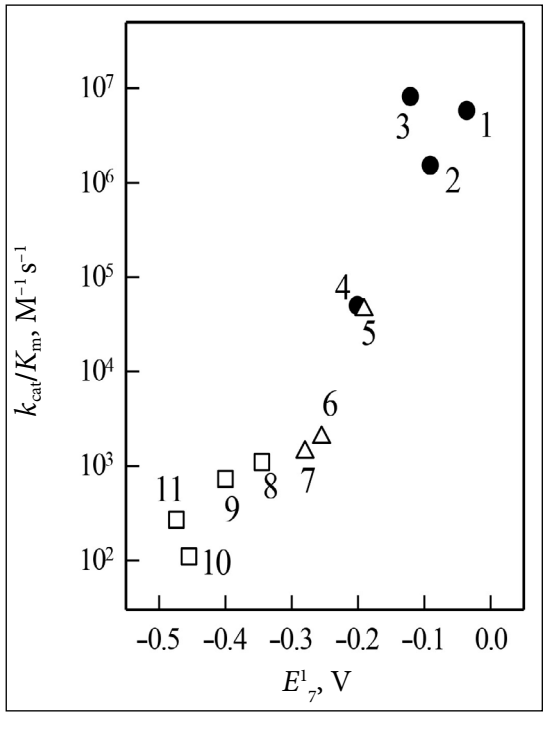

Fig. 2. The dependence of k_{cat}/K_m of nonphysiological electron acceptors on their E^1_7 in TrxR1-catalysed reactions. The numbers of compounds correspond to those in Table 2

CONCLUSIONS

The more negative value of E^0_7 of human TrxR1 as compared to TrxR2 may be caused by His116Tyr substitution. The higher redox potential of TrxR2 allows enzyme to operate at higher [$NADP^+$]/[NADPH] ratios. The reactivity of human TrxR1 towards quinoidal and nitroaromatic electron aceptors is similar to that previously determined for rat TrxR1. Human TrxR1 and TrxR2 do not possess different specificity towards the above groups of oxidants.

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PALYGINAMASIS REKOMBINANTINIŲ ŽMOGAUS CITOZOLIO IR MITOCHONDRIJŲ NADPH:TIOREDOKSINO REDUKTAZIŲ REDOKSO SAVYBIŲ TYRIMAS

S a n t r a u k a

Buvo ištirtos rekombinantinių žmogaus flavosulfoseleno fermento NADPH:tioredoksino reduktazės citozolio (TrxR1) ir mitochondrijų (TrxR2) izoformų redokso savybės. Izofermentų standartiniai redokso potencialai (E^0_7), nustatyti pagal redokso pusiausvyrą su $NADP^+$/NADPH pora, buvo lygūs –0,295 V (TrxR1) ir –0,270 V (TrxR2). Teigiamesnis TrxR2 E^0_7 gali būti susietas su His-125, esančio greta katalitinio disulfido ir seleno sulfido, buvimu TrxR2 aktyviajame centre vietoje Tyr-116, esančio TrxR1 aktyviajame centre. Keleto chinonų ir nitroaromatinių junginių reaktingumas TrxR1 ir TrxR2 atžvilgiu didėjo, didėjant jų vienelektroninės redukcijos potencialui (E^1_7). Pirmą kartą buvo ištirta serijos aromatinių *N*-oksidų redukcija TrxR1, kurie buvo redukuoti mišriu vien- ir dvielektroniniu būdu. Jų reaktingumas buvo artimas chinonų ir nitroaromatinių junginių, turinčių panašius E^1_7, reaktingumui.