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Characterization of *Deinococcus radiophilus* Thioredoxin Reductase Active with Both NADH and NADPH

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Thioredoxin reductase (TrxR, EC 1.6.4.5) of *Deinococcus radiophilus* was purified by steps of sonication, ammonium sulfate fractionation, 2'5' ADP Sepharose 4B affinity chromatography, and Sephadex G-100 gel filtration. The purified TrxR, which was active with both NADPH and NADH, gave a 368 U/mg protein of specific activity with 478-fold purification and 18% recovery from the cell-free extract. An isoelectric point of the purified enzymes was ca. 4.5. The molecular weights of the purified TrxR estimated by PAGE and gel filtration were about 63.1 and 72.2 kDa, respectively. The molecular mass of a TrxR subunit is 37 kDa. This suggests that TrxR definitely belongs to low molecular weight TrxR (L-TrxR). The Km and Vmax of TrxR for NADPH are 12.5 μM and 25 μM/min, whereas those for NADH are 30.2 μM and 192 μM/min. The Km and Vmax for 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB, a substituted substrate for thioredoxin) are 463 μM and 756 μM/min, respectively. The presence of FAD in TrxR was confirmed with the absorbance peaks at 385 and 460 nm. The purified TrxR was quite stable from pH 3 to 9, and was thermo-stable up to 70°C. TrxR activity was drastically reduced (ca. 70%) by Cu^{2+}, Zn^{2+}, Hg^{2+}, and Cd^{2+}, but moderately reduced (ca. 50%) by Ag^{+}. A significant inhibition of TrxR by N-ethylmaleimide suggests an occurrence of cysteine at its active sites. Amino acid sequences at the N-terminus of purified TrxR are H_2N-Ser-Glu-Gln-Ala-Gln-Met-Tyr-Asp-Val-Ile-Ile-Val-Gly-Gly-Gly-Pro-Ala-Gly-Leu-Thr-Ala-COOH. These sequences show high similarity with TrxRs reported in *Archaea*, such as *Methanosarcina mazei*, *Archaeoglobus fulgidus* etc.

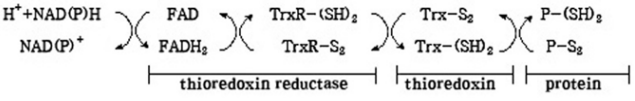
***Keywords*:** Thioredoxin reductase (TrxR) active with both NADPH and NADH, *D. radiophilus*, 37 kDa subunit, L-TrxR

The genus *Deinococcus*, which has been placed into an independent Deinococcus/Thermus phylum according to phylogeny based on 16S ribosomal RNA sequences (Madigan *et al.*, 2000), is one of prokaryotes showing peculiar features in its structure and physiology (Murray, 1986). Most unusual property of *Deinococcus* spp. is their extraordinary resistance against UV and ionizing radiations (i.e. x-ray, γ-ray), oxidative stress, and desiccation (Battista, 1997; Yun and Lee, 2000).

The toxic reactive oxygen species (ROSs) generated by UV and ionizing radiation, along with some anticancer drugs and cigarette smoking, are involved in a number of degenerative diseases including aging. However, cellular damage due to ROSs can be reduced by not only ROS scavenging enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GR), thioredoxin reductase (TrxR), and glucose 6-phosphate dehydrogenase (G6PDHase), but also by low molecular antioxitants (i.e. glutathione, V_E, V_C) and various repairing enzymes of damaged cellular components. One would assume that the extreme resistance of *Deinococcus* against UV and oxidative stress attributes to their ROS scavenging systems. Thioredoxin reductase (TrxR, EC 1.6.4.5), which catalyzes the reduction of oxidized thioredoxin using either NADH or NADPH as the donor of reducing power, is essential for recycling of thioredoxin. Thioredoxin performs various functions in cells, such as regulation of cell growth, ascorbate recycling, synthesis of deoxyribonucleotides and protecting cells from oxidative stress (Halliwell and Gutteridge, 1999; Steinling *et al*., 1999; Arnér and Holmgren, 2000; Mustacich and Powis, 2000; Norberg and Arnér, 2001).

Thioredoxin, the physiological substrate for thioredoxin reductase (TrxR), is a small protein (10-12 kDa) that has the H_2N-Trp-Cys-Gly-Pro-Cys-COOH at its active site.

Thioredoxin occurs in either an oxidized or a reduced form. Reduced thioredoxin prevents oxidation of various proteins by donating hydrogen atoms from two of the cysteine residues at its active site. Oxidized thioredoxin is reduced by thioredoxin reductase (TrxR) using NAD(P)H as an electron donor. TrxR, ubiquitously presented in prokaryotic and eukaryotic organisms, belongs to the family of pyridine nucleotide-disulfide oxidoreductases, which includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase. Enzymes of this family are typically homodimeric proteins with 1 mol of FAD prosthetic group per subunit and have conserved sites for FAD and NAD(P)H binding (Williams *et al*., 2000). Thus, TrxR is an enzyme playing a pivotal role in maintaining redox potential homeostasis of a cell by the catalytic reactions as follows.



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Interestingly, TrxRs found in prokaryotes and some eukaryotic cells are quite different from those in mammalian cells in respect of their molecular structures and substrate specificity etc. Low molecular weight TrxR (L-TrxR, 35-37 kDa subunit) is distributed in Archaea, Bacteria, Fungi, and some eukaryotes including plant (i.e. *Arabidopsis thaliana*) and intracellular parasites, i.e. *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Giardia duodenalis* (Ellis *et al*., 1994; Bruchhaus and Tannich, 1995; Brown *et al*., 1996; Dai *et al*., 1996), whereas high molecular weight TrxR (H-TrxR, 55-56 kDa subunit), which is highly similar to glutathione reductase (GR), is found in mammalian cells. Furthermore, the redox active center of H-TrxR has a motif CXXXXC that is located in the FAD binding domain, while the redox active center of L-TrxR has a motif CXXC that is located in NADPH binding domain (Williams *et al*., 2000; Hirt *et al*., 2002). H-TrxR exhibits broad substrate specificity and reacts with nondisulfide molecules such as alloxan, menadione, and Cu^{2+}; this is different from the specific substrate requirement of L-TrxR (Holmgren, 1989; Arnér and Holmgren, 2000). Following pioneering work on the thioredoxin system in *E. coli* (Thelander, 1967; Williams *et al*., 1967), extensive studies have been made upon mammalian thioredoxin system. Although genetic information on the thioredoxin system for Gram-positive coccus is easily accessible, TrxRs of Gram-positive coccus have rarely been studied. *D. radiophilus* possesses two iso-forms of TrxR; one active with both NADPH and NADH and the other specific to only NADPH. Activity of TrxR that utilises both NADPH and NADH as cofactors is exceedingly higher than that of NADPH specific TrxR (Seo and Lee, 2006). The dual specificity of the major TrxR is very peculiar since most of the TrxRs so far studied are known to be NADPH specific (Luthman and Holmgren, 1982; Brown *et al*., 1996; Watanabe *et al*., 1999; Kanzok *et al*., 2001). Since the TrxR exhibiting activity with both NADPH and NADH is a major TrxR in *D. radiophilus*, it is worthwhile investigating this enzyme to gain some insight into the thioredoxin system and furthermore into the resistance mechanism against UV and oxygen stress. Here, we report characteristics of the *D. radiophilus* TrxR that exhibits activity with both NADPH and NADH cofactors.

Materials and Methods

Bacterial culture and reagents

D. radiophilus ATCC 27603 (American Type Culture Collection, USA) was cultured in a TYGM medium [1% (w/v) tryptone, 0.5% yeast extract, 0.2% glucose, and 0.2% methionine] at 30°C for 3 days with shaking incubation at 150 rpm. Tryptone and yeast extract were obtained from Difco Lab. (USA). 2'5' ADP Sepharose™ 4B was purchased from Amersham Phamarcia Biotech (AB SE-75184, Sweden). Sephadex G-100, coomassie brilliant blue R-250, ammonium sulfate, ß-nicotineamide adenine dinucleotides (i.e. NAD^+, $NADP^+$, NADH, and NADPH), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 2,6-dichloroindo-phenol (DCIP), and materials for polyacrylamide gel electrophoresis and isoelectric focusing were purchased from Sigma Chem. (USA).

Preparation of cell-free extracts

The cells harvested by centrifugation (SUPRA 22K, Hanil, Korea) at 4,500 rpm for 20 min were washed three times with 50 mM potassium phosphate buffer (pH 7.0) and suspended in the same buffer. The cell-free extracts were obtained from sonicated-preparations of the cells at 4°C for 60 min (20 sec pulse on and 40 sec pulse off) with a sonic dismembrator (Fisher sonic dismembrator, Fisher Scientific Co., USA) and then centrifugation at 12,000 rpm for 20 min (Yun and Lee, 2004).

Determination of protein concentration and TrxR activity

Activity of TrxR was measured by monitoring for a 5 min interval, the change of absorbance at 412 nm occurring as a result of the reduction of DTNB to TNB (ε_{412}=13.6 $mM^{-1}cm^{-1}$) (Holmgren, 1977). Thioredoxin, the natural substrate of TrxR, is very expensive and difficult to obtain, so the activity of TrxR is usually assayed using an alternative substrate, DTNB; the substitution of thioredoxin by DTNB has been proved to be sufficiently specific (Holmgren, 1977; Luthman and Holmgren, 1982; Arnér *et al*., 1995). One mililiter of standard assay mixture consists of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 5 mM DTNB, 0.2 mM NADPH, and a suitable amount of enzyme. One unit of TrxR was defined as the amount of enzyme that is able to yield 1 μmol of TNB from DTNB per min at room temperature. Protein concentration was determined either by the Bradford method (1976), using BSA as a standard or measurement of absorbance at 280 nm.

Polyacrylamide gel electrophoresis and TrxR activity staining on gel

Proteins were electrophoretically resolved on 8.0% polyacrylamide gel with 25 mM Tris-glycine buffer (pH 8.8). The proteins on the gel were stained by 0.1% coomassie brilliant blue R-250 for 10 min and destained with 10% methanol and 10% acetic acid over night (Gersten, 1996). The TrxR-bands on the gel were then subjected to activity staining in the dark, by incubation in an 80 mM Tris-HCl buffer (pH 7.5) containing 2.0 mM DTNB, and either 2.0 mM NADH or 1.5 mM NADPH for about 20 min. Additionally gels was incubated for a further 20-30 min in the same staining solution but containing 40 μM DCIP and 1.0 mM MTT (Ye *et al*., 1997). A final confirmation of TrxR activity on a gel was made with thioredoxin, a product of recombinant *E. coli* (Sigma, USA) in place of DTNB; as it is the authentic substrate of TrxR.

Purification of TrxR

Dried ammonium sulfate was added to the cell-free sonic extract to reach 45% saturation and the mixture was gently stirred for 2-3 h on ice. After removal of the precipitate by centrifugation at 12,000 rpm for 15 min at 4°C, ammonium sulfate was further added to the supernatant to reach 95% saturation. Then, the mixture was kept on ice for 30 min with constant stirring. The resulting protein precipitate collected by centrifugation was then suspended in 50 mM potassium phosphate buffer (pH 7.0) and dialyzed against 5 L of the same phosphate buffer overnight at 4°C. The dialyzed enzyme preparation was subjected to 2'5' ADP Sepharose 4B affinity chromatography. After sufficient washing of the column with the same phosphate buffer to remove the unbound proteins, proteins bound to 2'5' ADP Sepharose 4B were eluted with a 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 M NaCl. Fractions with TrxR activity were pooled and concentrated by ultrafiltration (Amicon, USA) and subjected to a Sephadex G-100 column which was pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The protein was collected (1 ml/fraction) with elution rate, 0.5 ml/min. After resolution

of proteins in the fractions containing TrxR activity on 8.0% polyacryamide gel, fractions exhibiting a single protein band of TrxR were pooled and concentrated using the Amicon membrane filter. This electrophoretically homogeneous preparation was used to characterize properties of deinococcal TrxR.

Characterization of TrxR

Determination of molecular weight of TrxR

Molecular weight of purified TrxR was determined by native polyacrylamide gel electrophoresis (Gersten, 1996) and by Superose HR12 pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0) (Bollag and Edelstein, 1996). The molecular weight of the denatured enzyme was measured by 0.1% SDS-12% polyacrylamide gel electrophoresis with a number of size marker proteins.

Isoelectic focusing of the purified TrxR

Isoelectric point (pI) of the purified TrxR was determined by isoelectric focusing on 4% polyacryamide gel containing 2.5% ampholytes of which the pH span was 3 to 10. TrxR and protein markers on gel were visualized by coomassie staining and by TrxR activity staining (Ye *et al*., 1997). Amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.2), myoglobin (pI 6.8, 7.2), and lentil lectin (pI 8.2, 8.6, 8.8) (Sigma) were used as standard markers.

Kinetics of TrxR

Substrate affinity (K_m) and V_{max} as kinetic parameters for NADPH, NADH, and DTNB were determined with a Lineweaver-Burk plot. The K_m and V_{max} for NADPH and NADH were determined at a saturating concentration of DTNB (5 mmol) and that of DTNB was determined at a saturating concentration of NADPH (0.2 mmol). Optimal pH for deinococcal TrxR activity was unable to be determined, since the reduction of FAD in TrxR by reduced pyridine nucleotide is deeply affected by the proton concentration in an assay solution. pH stability of the enzyme was investigated after 30 min of incubation in a Britten-Robinson buffer which covers a pH range of 2-10. Optimal temperature for TrxR activity was determined between 4°C and 90°C. TrxR thermostability was monitored by measuring activity in the standard assay condition after 30 min incubation at the same temperature range (Bollag and Edelstein, 1996).

Effect of group-specific reagents and chemicals on TrxR activity

Effects of a number of group-specific reagents (1 mM and 5 mM) for amino acids at the active site of enzyme were investigated. The reagents include 1-chloro-2, 4-dinitrobezene (DNCB), iodine, glyoxal, N-ethylmaleimide, and ρ-diazobenzene sulfonic acid. Effects of some chemicals (i.e. $MgSO_4$, $MnCl_2$, $ZnSO_4$, $AgNO_3$, $HgSO_4$, $CdCl_2$, and $CuSO_4$) on TrxR activity was also investigated, as chemicals that are readily reduced or oxidized by proton transfer can affect the activity of the redox enzymes.

UV/visible absorption spectrum of TrxR

For the confirmation of FAD as a prosthetic group of TrxR, absorbance scanning of the purified TrxR was performed in the range of 260 nm to 700 nm.

Analysis of amino acid composition and N-terminal sequencing of TrxR

An analysis of amino acid composition of the purified TrxR was made with phenylisothiocyanate derivatives of amino acids that were obtained from HCl hydrolysate of the enzyme by HPLC chromatography (Biocore 2000/HP 1100 Series & pico-TAG, Pharmacia Biosensor AB, Sweden/Waters, HP, USA). This work was carried out by Korea Basic Science Institute (Korea). The reverse phase HPLC (G 1017A, Agilent, 600 Controller, Waters, USA) was performed with the acidified products of the purified TrxR, which was obtained by using the Edman degradation method, to sequence the N-terminal amino acid of the enzyme. This work was carried out at the Central Lab. of Kangwon National University.

Results and Discussion

The thioredoxin and glutaredoxin systems, including the glutathione system, are indispensable for living organisms since they are the major thiol redox systems for maintaining intracellular redox potential homeostasis. Thioredoxin and glutaredoxin are relatively small proteins that containing redox disulfide at their active site. These small redox proteins supply protons to a number of reductases, e.g., ribonucleotide reductase, PAPS reductase, and methionine sulfoxide reductase. Besides, thioredoxin and glutaredoxin are also known to be involved in various cellular functions, such as the detoxification of H_2O_2, ascorbate recycling, cell growth regulation, and signal transduction (Holmgren, 1989; Becker *et al*., 2000).

Generally, most organisms possess both thioredoxin and glutaredoxin systems, although the cellular level of thioredoxin is comparably higher to that of glutaredoxin (Prinz *et al.*, 1997). However, some organisms such as *Drosophila melanogaster* and *Helicobacter pylori* lack a glutaredoxin system (Kanzok *et al*., 2001; Comtois *et al*., 2003). Interestingly, study of the whole genome sequence of *Deinococcus radiodurans* has revealed that this bacterium lacks genes for glutathione reductase, but possesses genes for the thioredoxin system (Makarova *et al*., 2001). Therefore, one may assume that thioredoxin system is an essential component for redox homeostasis in *Deinococcus* species.

Thioredoxin reductase (TrxR) is the essential enzyme in recycling of thioredoxin; recycling can be achieved by reducing the oxidized form of thioredoxin using either NADH or NADPH as the donors of reducing power. In *Deinococcus radiophilus*, iso-types of TrxR, which were distinguishable by their mobilities on polyacryamide gel and the type of electron donor used, i.e. NADH or NADPH, were documented. The iso-type that is active with both NADPH and NADH seems to be major enzyme and more important enzyme than the iso-type enzyme active only with NADPH, since the activity of the former iso-type TrxR is exceedingly higher than that of the latter one (Seo and Lee, 2006).

The purification of the major TrxR showing specificity for both NADH and NADPH from a cell-free extract by a step-wise process of ammonium sulfate fractionation, 2'5' ADP Sepharose 4B affinity chromatography, and Sephadex G-100 gel filtration is summarized in Table 1. The purified TrxR that showed activity with both NADPH and NADH is seen in Fig. 1B and 1C. During the purification process of deinococcal TrxRs, the precipitation of the major TrxR at 45-95% saturation of ammonium sulfate suggested that this iso-type TrxR seems to be highly polar. As suggested by others (Pigiet and Conley, 1977; Gromer *et al*., 1998; Horecka *et al*., 1998), 2'5' ADP Sepharose 4B affinity chromatography, which provided substantial increase in the purification fold from the previous step, was very useful for the purification of TrxR. The presence of FAD as a prosthetic group in deinococcal TrxR was confirmed with the absorption peaks at 385 and 460 nm, which is a typical flavoprotein absorption spectrum (Thelander, 1967; Brown *et al*., 1996). The isoelectric point of this purified TrxR

Table 1. Purification steps of TrxR active with both NADPH and NADH from *D. radiophilus*

Purification step	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Yeild (%)	Purification fold
Cell-free extract	726.7	942.5	0.77	100	1
45-95% sat. ASF	481.86	334.94	1.42	66.3	1.84
2'5' ADP-Sepharose 4B	169.15	0.55	307.55	23.3	399.42
Sephadex g-100	132.38	0.36	367.71	18.2	477.55

One unit of TrxR is defined as an amount of enzyme that is able to yield 1 μmol of TNB from DTNB per min at room temperature. Table 1 was based on TrxR activity with NADPH.

was ca. 4.5, which seems to be much more acidic than the isoelectric points of TrxRs reported in not only prokaryotes (Windle *et al*., 2000) but also Eucaryoic cells (Brown *et al.*, 1996).

The molecular weight of the purified TrxR determined by nondenaturing PAGE was found to be ca. 63.1 kDa (data not shown), whereas that estimated by the superose HR 12 gel filtration was ca. 72.2 kDa (data not shown). Such discrepancy in the TrxR molecular weights seems to be attributed to the acidic nature of the enzyme. The SDS-PAGE of the purified enzyme preparation following protein staining revealed a single band of 37 kDa (Fig. 2), a bit larger than 35 kDa of *D. radiodurans* TrxR as well as *E. coli* TrxR (Obiero *et al*., 2010). *D. radiophilus* TrxR is a homedimeric enzyme, and definitely

belongs to the L-TrxR (35-37 kDa subunit) which is widely distributed among Archaea, Bacteria, Fungi, and some Eukaryotes including *Arabidopsis thaliana* and intracellular parasites, such as *Entamoeba histolytica*, *Trichomonas vaginalis* etc. (Ellis *et al*., 1994; Bruchhaus and Tannich, 1995; Brown *et al*., 1996; Dai *et al.*, 1996).

The major TrxR iso-type of *D. radiophilus* that can utilizes both NADH and NADPH for activity is very peculiar, since most of the TrxR so far reported are known to be NADPH specific (Luthman and Holmgren, 1982; Brown *et al*., 1996;

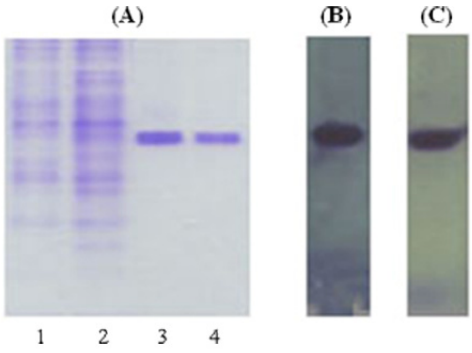

Fig. 1. Resolution of proteins during purification steps. (A) Proteins on native-PAGE, (B) TrxR activity with NADPH, (C) TrxR activity with NADH. 1. Cell-free extract (10 μg of protein), 2. 45-95% sat. ASF (20 μg of protein), 3. 2'5' ADP Sepharose 4B affinity chromategraphy (5 μg of protein), 4. Sephadex G-100 gel filtration (3 μg of protein).

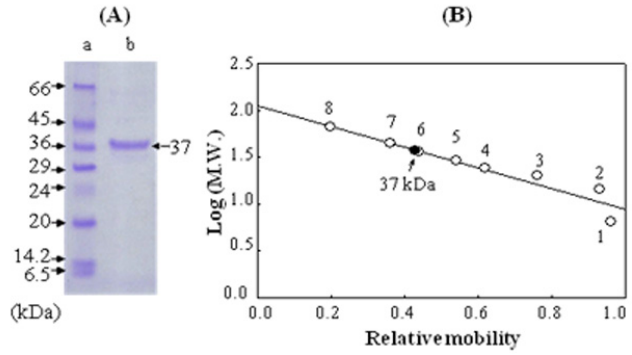

Fig. 2. Molecular weight determination of TrxR on SDS-polyacrylamide gel. (A) TrxR and protein markers on 0.1% SDS-12% polyacrylamide gel: a, size markers; b, purified TrxR. (B) Determination of molecular weight of TrxR on SDS-PAGE. Standard markers: 1, aprotinin (6.5 kDa); 2, bovine -lactalbumin (14.2 kDa); 3, soybean trypsin inhibitor (20 kDa); 4, bovine trypsinogen (24kDa); 5, bovine erythrocytes carbonic anhydrase (29 kDa); 6, rabbit muscle glyceroldehyde-3-phosphate dehydrogenase (36 kDa); 7, chicken egg albumin (45 kDa); 8, bovine serum albumin (66 kDa), TrxR (●).

Watanabe *et al*., 1999; Kanzok *et al*., 2001). The purified recombinant *D. radiodurance* TrxR utilizing only NADPH for activity has been recently documented (Obiero *et al*., 2010). The Km of *D. radiophilus* TrxR for NADPH was 12.5 μM (Fig. 3), whereas for NADH, it was 30.2 μM (Fig. 4). The saturation kinetics of this TrxR for NADPH was a hyperbolic curve, whereas a sigmoidal curve was observed for NADH. The latter result suggests that this deinococcal TrxR seems to be behaved as an allosteric enzyme when NADH was used as the cofactor. The Km value of *D. radiophilus* TrxR for NADPH (12.5 μM) was slightly different from those of other TrxRs, such as cytosolic TrxR of rat liver (6.0 μM), *Drosophila melanogaster* TrxR (6.5 μM), and *Giardia duodenalis* TrxR (8 μM). However, all of these eukaryotic TrxRs are NADPH-specific-enzymes (Luthman and Holmgren, 1982; Brown *et al*., 1996; Kanzok *et al.*, 2001). The Km value of the major deinococcal TrxR for DTNB in the presence of NADPH was 463 μM (Fig. 5). This Km value of the deinococcal TrxR was nearly the

Table 2. Effect of group-specific reagents on TrxR activity

Reagent	Relative activity (%)		Remark
	1 mM	5 mM	
None	100	100	
1-chloro-2,4-initrobenzene	92	107	selenocysteine modifier
Iodine	94	86	tyrosine modifier
Glyoxal	93	93	arginine modifier
N-ethylmaleimide	61	46	cysteine modifier
ρ-iazobenzene sulfonic acid	72	63	lysine modifier

Enzyme activity was measured in the standard assay condition: 50 mM potassium phosphate buffer (pH 7.0) 1 mM EDTA, 5 mM DTNB, and 0.2 mM NADPH. Relative activity was calculated as 100× (enzyme activity in the presence of chemical/ enzyme activity in the absence of chemical).

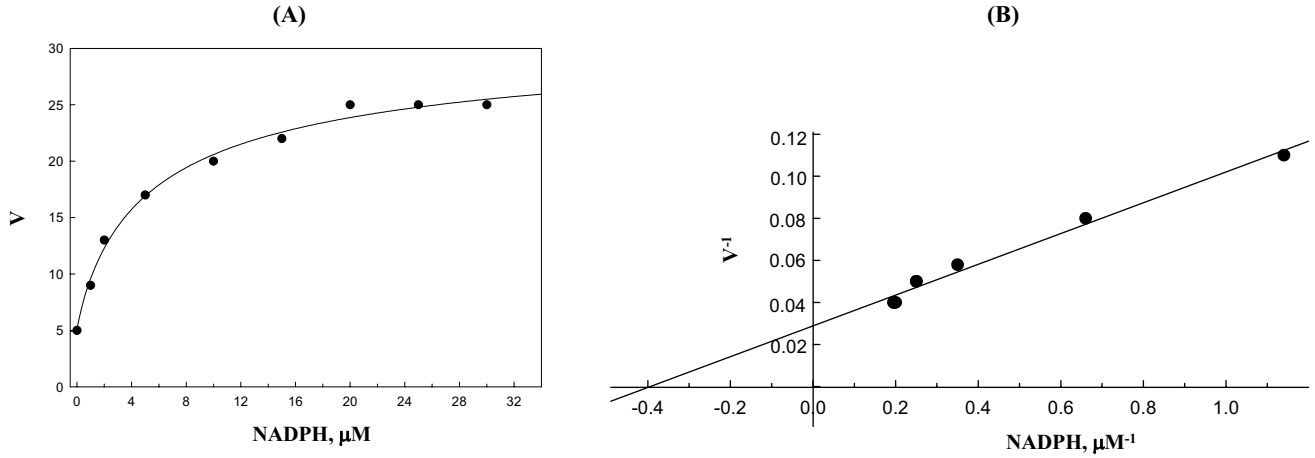

Fig. 3. (A) Substrate saturation curve. (B) Lineweaver-Burk plot of TrxR for NADPH. The Km and Vmax of the purified TRxR for NADPH were 12.5 μM and 25 μM/min, respectively.

same as that of *Plasmodium falciparum* H-TrxR (465 μM) (Kanzok *et al*., 2000), but is slightly lower than those of cytosolic and mitochondrial H-TrxRs occurring in rat liver (660 μM and 530 μM, respectively) (Watanabe *et al*., 1999). A drastic inhibition of TrxR activity by N-ethylmaleimide (cysteine modifier) suggests that cysteine is an essential amino acid located at the active site of *D. radiophilus* TrxR (Table 2). A similar report on inhibitory effect of N-ethylmaleimide at cysteine residue was made with L-TrxR of *Giardia duodenalis* (Brown *et al*., 1996). One would also speculate that lysine residue in TrxR seemed to be important for the activity, since ρ-diazobenzene sulfonic acid (lysine modifier) caused a considerable reduction of activity. However, DNCB (1-chloro-2,4-dinitrobenzene) which is known to be an inhibitor of mammalian TrxR (Arnér *et al*., 1995; Watanabe *et al*., 1999) did not exhibit any effect on deinococcal TrxR. The insensitivity of *D. radiophilus* TrxR (L-TrxR) to DNCB is rather conceivable since mammalian TrxR is a member of H-TrxR group and the inhibitory effect of DNCB on mammalian TrxR is due to the chemical modification of selenocysteine at its active site (Nordberg *et al*., 1998). TrxR activity was significantly decreased by metal ions including Ag^{1+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, and Cd^{2+}, but not by Mg^{2+} and Mn^{2+}. TrxR of *D. radiophilus* was stable at temperatures ranging from 4°C to 70°C for 30 min. However, the enzyme activity was drastically reduced at 80°C and completely inactivated at 90°C. The purified TrxR was stable at pH 6-7 and relatively stable even at pH 8-9, but quite unstable below pH 5. Stabilities of *D. radiophilus* TrxR at the wide range of temperature and pH are not surprising, since thermo- and acid stability of TrxR has been frequently employed for TrxR purification in many organisms (Williams *et al*., 1967; Luthman and Holmgren, 1982; Harms *et al*., 1998; Rigobello *et al*., 1998). The amino acids distribution in *D. radiophilus* TrxR was insignificantly different from those of various TrxRs found in other prokaryotic and eukaryotic organisms. The N-terminal 21 amino acid sequence of the deinococcal TrxR was H_2N-SEQAQMYDVIIVGGGPAGLTA-COOH. In a comparasion of the N-terminal amino acid sequence of *D. radiophilus* TrxR with those of other TrxRs (based online database in National Center for Biotechnology and Information, NCBI), there is 87% similarity to those of TrxRs of *Methanosarcina mazei*, *Archaeoglobus fulgidus*, and *Tthermoanaerbacter tengcongensis* and 77% similarity with *D. radiodurans* TrxR. The clustering of *D. radiophilus* TrxR with

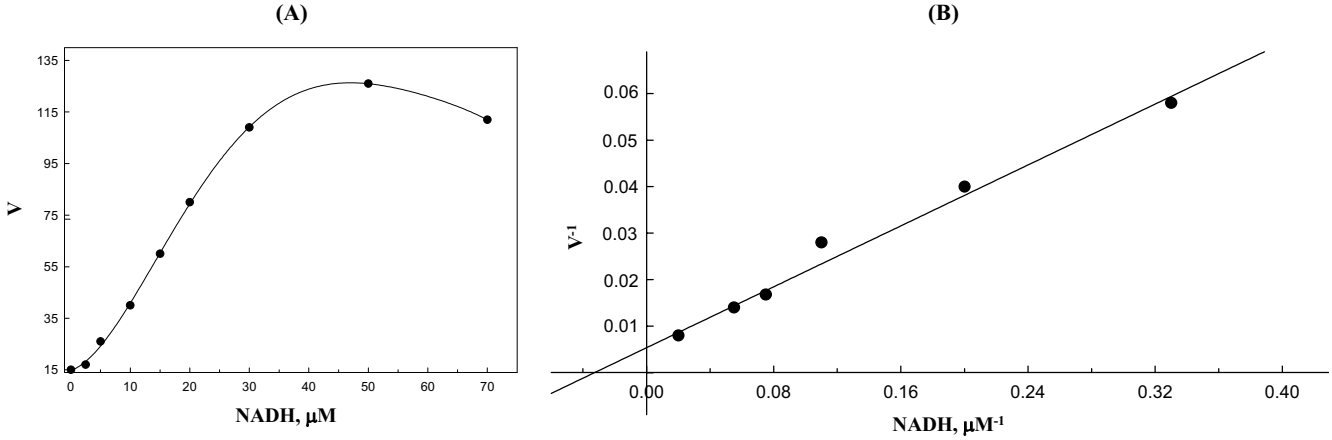

Fig. 4. (A) Substrate saturation curve. (B) Lineweaver-Burk plot of TrxR for NADH. The Km and Vmax of the purified TRxR for NADH were 30.2 μM and 192 μM/min, respectively.

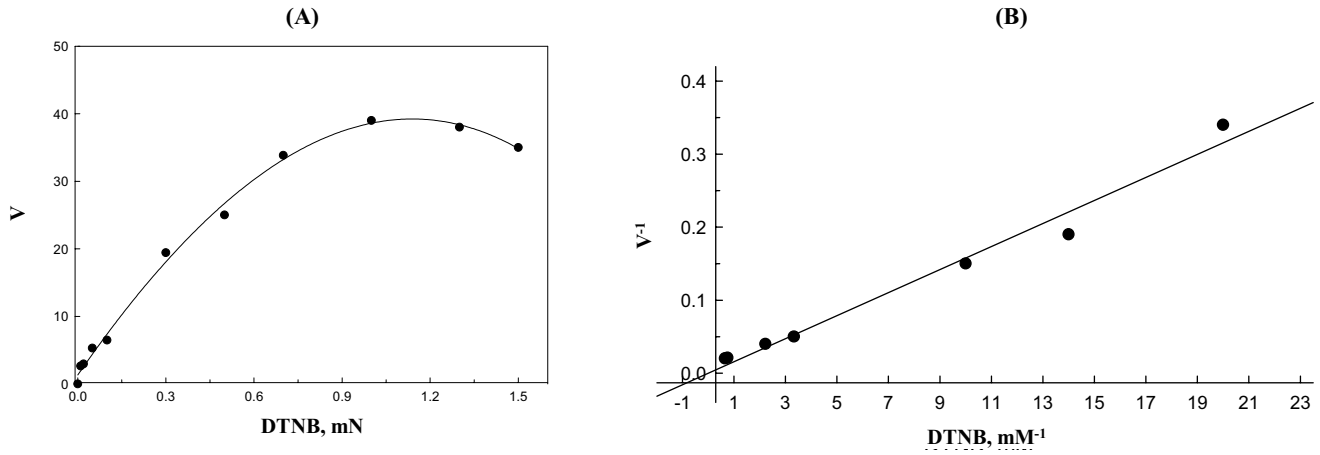

Fig. 5. (A) Substrate saturation curve. (B) Lineweaver-Burk plot of TrxR for DTNB. The Km and Vmax of the purified TRxR for DTNB were 463 μM and 756 μM/min, respectively.

M. mazei, *A. fulgidus*, and *T. tengcongensis* in a phylogenetic tree based on deduced amino acid sequences of various TrxRs suggests that TrxR of *D. radiophilus* probably shares a common ancestral lineage with those of *Archaea*.

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