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The isozymes of mammalian thioredoxin reductase (TrxR) contain the penultimate selenocysteine residue (SeCys) in the redox-active C-terminal tetrapeptide, -Gly-Cys-SeCys-Gly (end). A mutant form of the mammalian enzyme TrxR-X498C in which SeCys is replaced with Cys shows a dramatically decreased catalytic activity, suggesting that SeCys residue plays an integral role in the catalysis. In contrast, TrxR of the fruit fly, *Drosophila melanogaster*, has no selenium in the corresponding C-terminal redox sequence, which instead of SeCys has flanking serine residues in the terminal sequence, -Ser-Cys-Cys-Ser (end). Because the catalytic activity of Dm-TrxR is comparable to that of the mammalian selenoenzyme, we introduced the serine residues at the corresponding positions of the recombinant TrxR-X498C and mimicked the redox center of the fruit fly TrxR. However, the catalysis remained as low as the Cys mutant of the selenoenzyme, suggesting that the additional structural features are still required for the tetrapeptide to function as a redox center. MOPAC calculation suggested that the complete motif might involve the hexapeptide sequence, which includes a proline residue, -Pro-X-Ser-Cys-Cys-Ser (end). The proline-containing motif is conserved among other insect TrxRs such as those of honeybee and fruit fly.

Key words : Thioredoxin reductase, *Drosophila melanogaster*, MOPAC

Introduction

Selenium is an essential trace element for mammals, birds, amphibians, fishes and some bacteria. Mammalian thioredoxin reductase (TrxR) isozymes are among the most important selenoproteins, and they carries out several anti-oxidative and regulatory roles in cells besides the classical role in deoxyribonucleic acid synthesis, which has long been established for the microbial counterparts. The catalytic activity of mammalian thioredoxin reductase (TrxR) depends on a redox-active Cys-SeCys dipeptide in the C-terminal tetrapeptide motif, -Gly-Cys-SeCys-Gly (end)[1-3]. In general, selenium is more reactive than sulfur, and it also has a longer bond length, which appears to contribute to the selenium-sulfur bridge formation during the catalytic cycle[4-8]. A mutant form of mammalian TrxR, TrxR1-U498C, in which SeCys is substituted for Cys showed a low catalytic turnover[7-9]. In fruit fly *Drosophila melanogaster*, TrxR is closely related to mammalian TrxR in the sequence but it carries a redox-active C-terminal Ser-Cys-Cys-Ser (end) motif that has been proposed to facilitate the transient thiolate formation at the C-terminal redox active cysteines[10]. In this study we have introduced serine residues in the human lung TrxR-U498C to mimic the Dm-TrxR sequence, but the catalytic activity was not improved. Our results were consistent with the recent report that the serine-containing tetrapeptide of Dm-TrxR was not sufficient to replace the Cys-SeCys dipeptide in the rat selenoenzyme[11]. In the present study, semiempirical molecular orbital calculation was used to look for the missing structural features which mammalian selenoprotein does not share with the insect orthologues, and we here suggest that a functional redox motif in Dm-Trx might involve the hexa-peptide sequence with two more amino acid residues, Pro-Xaa-Ser-Cys-Cys-Ser. The proline residue appears to facilitate the disulfide bridge formation between the vicinal cysteine residues according to our molecular orbital calculation for the enthalpy change by the redox states.

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Materials and Methods

Bacterial strains and DNA manipulation

E. coli BL21 CodonPlus (DE3) RIL [F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* +Tet^r (DE3) *endA* (*argU ileY leuW* Cm^R)] (Stratagene) was used as an expression host for the mutant enzymes, and pET32a (Novagen) was used as the expression vector. *E. coli* TOP10 (Invitrogen) was used for cloning of mutant genes. General DNA manipulation was performed as described by Sambrook and Russel[12]. Restriction enzymes and DNA polymerase were purchased from TOYOBO (Osaka, Japan). Plasmid DNA was isolated using GFX Micro Plasmid Prep Kit from Amersham Bioscience (Tokyo, Japan). DNA sequencing was performed using a DYEnamic ET terminator cycle sequencing kit (Amersham) and a model 310 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Mutagenesis, expression, and purification of C-terminal mutants

Mutations were designed to yield enzymes with the following C-terminal tetrapeptide sequences: GCCG, GCCS, SCCS. The site-directed mutagenesis was carried out with the recombinant human lung thioredoxin reductase gene, which has been cloned in the pET32a vector at the *Bam*HI and *Hin*dIII sites (Novagen). The recombinant *E. coli* CodonPlus was grown in 200 ml of CIRCLE GROW medium containing 50 μg/ml of ampicillin at 28°C for 16 h, and the protein was expressed in the presence of 0.3 mM IPTG at 18°C for 22 h. The cells were disrupted by sonication, and the cell debris and membrane fraction were removed by centrifugation and ultracentrifugation. The recombinant protein was purified as the Trx-His_6-tagged fusion protein with nickel-NTA His-Bind column. Trx-tag was removed by enterokinase digestion (Novagen) and the tag and remaining fusion protein were removed by passing the solution through a Ni-NTA column.

DTNB assay

The enzyme assay of TrxR involves the NADPH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoate) (DTNB). The assay mixture (1 ml) contained 500 mM potassium phosphate, pH 7.0, 50 mM KCl, 10 mM EDTA, 0.24 mM NADPH, bovine serum albumin (0.2 mg/ml), and 2.5 mM DTNB (50 μl of a 50 mM solution in ethanol). An aliquot of enzyme was added to the mixture, and the change in the optical density at 412 nm was monitored over 2 min at the room temperature. Activity is defined as micromoles of NADPH oxidized per min by ΔA_{412}/13.6x2, since 1 mole of NADPH yields 2 moles of thionitrobenzoate.

Molecular orbital calculations

A personal computer, Endeavor Pro900 (EPSON), implemented with Pentium IV 1.4 GHz CPU and 1028 MB memory, was installed with the operating system Windows XP Home edition (Microsoft, USA). CS Chem3D Pro (CombridgeSoft Corporation, USA) was used to construct the initial structure of model peptides. The oxidized peptide models were made by erasing hydrogen atoms on the sulfur atoms and by connecting the two sulfurs by a single bond. The geometry of the oxidized peptide was optimized by molecular mechanistic optimization MM2 on Chem3D and further optimized by the molecular orbital calculation at the semiempirical levels of AM1[13] on WinMOPAC 3.5 Pro (FUJITSU, Japan). Then the geometry was optimized for the reduced form. The disulfide bridge was modified to the corresponding dithiol form on the computer screen, then run the MO calculation by AM1 Hamiltonian. The heat of formation for hydrogen molecule was summed up to that of the oxidized peptide, and the enthalpy difference between oxidized (Hox+H_{H2}) and reduced (Hred) forms were represented by the subtraction, $\Delta H = Hox + H_{H2} - Hred$[14].

Results and Discussion

Two rounds of site-directed mutagenesis on the TrxR-U498C gene have modified the C-terminal motif of the human lung TrxR to mimic the C-terminal Ser-Cys-Cys-Ser sequence of the non-selenoprotein counterpart of Dm-TrxR. The first round of mutation has made the sequence in which only the terminal Gly was replaced with Ser. It has been reported that both of the flanking Ser residues are important for activating the Cys residues in Dm-TrxR, and substituting the terminal one with Gly reduced the Dm-TrxR activity more significantly than changing the other[10]. Mutant genes were made from recombinant human lung TrxR gene using KOD-plus DNA polymerase (TOYOBO, Osaka) and the set of primers 5'-CCTCCAggCTggCTgCTgTAgTTTA AAgCTTgCggCCgC-3' and 5'-gCggCCgCAAgCTTT TAACTACAgCAgCCAgCCTggAgg-3'. The first round of mutagenesis made the G499S mutation. The second round of mutagenesis upon the G499S mutant accomplished to introduce the *Drosophila* tetrapeptide -SCCS using the set of primers 5'-AgCTTTTAACTAC AgCAgCTAgCCTggAggATgCTTgCC-3' and 5'-ggCAAgCATCCTCCAggCTAgCTgCTgTAgTTA AAAgCT-3'. An aliquot of the PCR product was digested with *Bam*HI for the confirmation (Fig. 1).

TrxR-SCCS and TrxR-GCCS were expressed in *E. coli* BL21 CodonPlus at exponential growth phase with 0.5 mM IPTG at 17°C for 16 h. The *Drosophila*-mimic of

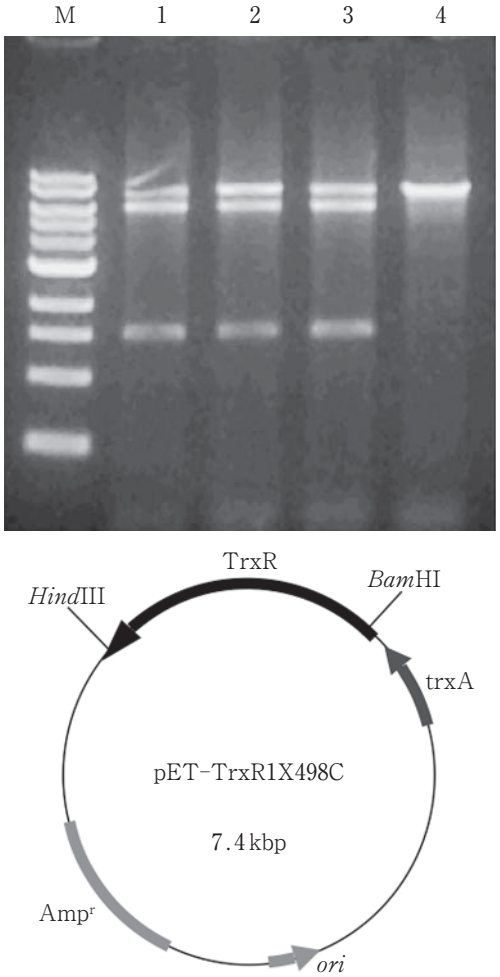

Fig. 1 Site-directed mutagenesis mimicking *Drosophila* tetrapeptide.
The recombinant plasmid of 7.6-kb was used as the template for the site-directed mutagenesis. The annealing temperatures were 60°C (Lane 1), 61°C (Lane 2) and 62°C (Lane 3), and their PCR products were digested with *Bam*HI for confirmation. Lane 4 contains the PCR product before the *Bam*HI digestion.

the mammalian enzyme was purified by Ni-NTA His-Bind column (Table 1). The one-serine mutant and the two-serine mutant showed the similar specific activity, 55.1 and 56.4 mU/mg, repectively by the DTNB assay, and the activity was as low as the Cys-mutant enzyme whose specific activity was 50 mU/mg (Fig. 2). Accordingly, the flanking serine residues appeared to have no effect on activating the Cys mutant. A recent report on the mutational study also introduced the Dm-TrxR sequence to the C-terminal tetrapeptide of the rat enzyme, and the mutant showed the activity as low as 0.5% of the original selenoenzyme. Kinetic studies and spectroscopic investigation on the *Drosophila*-mimic of the rat enzyme have led to the conclusion that the flanking Ser residues are not sufficient to alleviate the need for SeCys in the rat selenoenzyme. The tetrapep-

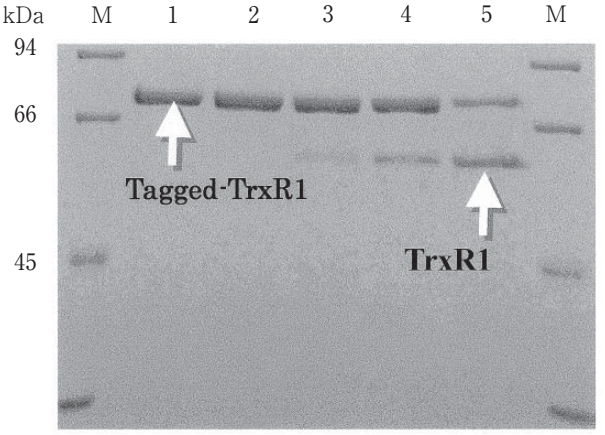

Fig. 2 Enterokinase digestion of the fusion protein.
An aliquot of purified Trx-His_6-tagged protein (73 kDa, 1.5 mg) was treated with 0 U (Lane 1), 0.063 U (Lane 2), 0.125 U (Lane 3), 0.25 U (Lane 4) and 0.5 U (Lane 5) of enterokinase at 20°C for 1 h. Lane 5 shows the optimal conditions employed in this study.

Table 1 Purification Summary

GCCS	Total activity (mU)	Protein (mg)	Specific Activity (mU/mg)	Purificaiton (fold)
Crude Extract[a)]	58.0	15.7	3.7	1
Trx-His_6-tagged[b)]	71.4	2.2	33.1	8.9
TrxR-GCCS[c)]	16.4	0.3	55.1	14.9

SCCS	Total activity (mU)	Protein (mg)	Specific Activity (mU/mg)	Purificaiton (fold)
Crude Extract[a)]	19.9	21.8	0.91	1
Trx-His_6-tagged[b)]	9.9	1.9	5.3	5.8
TrxR-GCCS[c)]	43.0	0.76	56.4	62.0

a) Cell debris and membrane were removed from the sonic extract by centrifugation and ultracentrifugation.
b) Trx-His_6-tagged fusion protein was purified by Ni-NTA His-Bind column chromatography.
c) Trx-His_6-tag was removed by enterokinase digestion.

tide motif Ser–Cys–Cys–Ser may require an additional structural feature, which is not shared by mammalian enzyme.

Semiempirical molecular orbital calculation was used for searching for the missing structural requirement, which confers the Ser–Cys–Cys–Ser sequence the competent redox catalysis. Since the selenosulfide bridge formation between SeCys–Cys pair appears to be much favored than disulfide bridge formation in the mammalian enzyme, we calculated the enthalpy change by disulfide bridge formation between the adjacent Cys residues using a formula previously reported[14]. Accordingly, the proline residue at the position two amino acids ahead of the terminal tetrapeptide –SCCS in Dm–TrxR turned out to be an interesting candidate (Table 2). The peptide model that mimics Dm–TrxR sequence bearing Pro–X–Ser–Cys–Cys–Ser motif gave a large negative ΔH value upon the dithiol oxidation, suggesting that the Pro-containing peptide would be much stable in the oxidized form than in the reduced form. However, the alleviation effect of proline may not work without the flanking serine residues as illustrated by the series of model peptides shown on the left column of the Table 2. In fact, missing either one of the flanking serine residues would make the redox center much less likely to be oxidized even in the presence of proline, and missing two serines makes the C–terminal Cys–Cys–pair as unfavorable as that of the Gln-containing model peptide. Furthermore, the larger positive ΔH values calculated for the peptides that mimicked only the tetrapeptide motif represents the difficulty of the adjacent Cys pair to be oxidized, the prediction may agree with our results and the study on the mutant rat enzyme (11). In addition, it is interesting to note that the hexapeptide motif is highly conserved among insect TrxR orthologues such as those of *Apis millifera* (honeybee: AY329357), *Drosophila pseudoobscura* (fruit fly: Q29FR6_DROPS).

Acknowledgements

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Table 2 Redox-dependent enthalpy change (ΔH) of model peptides

Sequence	kcal/mol	Sequence	kcal/mol
Ac–SIL–Pro–A– SCCS	−79.32698	Ac–SIL–Gln–A–SCCS	95.43838
Ac–SIL–Pro–A– SCCG	10.51758	Ac–SIL–Gln–A–SCCG	98.20615
Ac–SIL–Pro–A– GCCS	8.6996	Ac–SIL–Gln–A–GCCS	36.08184
Ac–SIL–Pro–A– GCCG	70.0677	Ac–SIL–Gln–A–GCCG	8.20471

The enthalpy change ΔH is defined to give the larger negative values when the disulfide bridge is favored over the reduced form. The proline-containing peptide increases the tendency to be oxidized as the number of serine content is increased within the tetrapeptide sequence. In contrast, serine residues makes it more difficult to form the oxidized form without the proline, suggesting that the SCCS tetrapeptide needs the proline residue at this position to function as an active redox-motif.

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キイロショウジョウバエ由来のチオレドキシン還元酵素のC末端テトラペプチド配列は，ヒト肺由来のチオレドキシン還元酵素では酸化還元活性を示さない

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ほ乳類チオレドキシン還元酵素はC末端配列-Gly-Cys-SeCys-Gly（end）の後ろから2番目にセレノシステイン（SeCys）残基を持つ．SeCys をシステインに変換すると酵素の活性は大きく低下するので，SeCys 残基が触媒活性に必須であることが分かる．これに対してキイロショウジョウバエのチオレドキシン還元酵素（Dm-TrxR）のC末端配列にはセレンが含まれず，システイン残基の対が2つのセリンに挟まれた配列-Ser-Cys-Cys-Ser（end）を持つ．それでも Dm-TrxR はほ乳類のセレン含有酵素と同程度の触媒能を示す．われわれはヒト肺チオレドキシン還元酵素に Dm-TrxR のC末端テトラペプチド配列を導入してその効果を調べた．しかし，酵素活性はまったく上昇せず，Dm-TrxR のC末端のテトラペプチド配列-Ser-Cys-Cys-Ser だけでは Cys 残基のチオール基を活性化する効果はなかった．そこで，分子軌道計算 MOPAC を用いて酸化還元機能を担うためのC末端配列モチーフを探索した．その結果，テトラペプチドにさらに2つ先のプロリンまでを含めた Pro-X-Ser-Cys-Cys-Ser（end）により初めて酸化還元モチーフとして機能する可能性が示唆された．Pro を含むこの配列モチーフはミツバチや蚊などほかの昆虫の TrxR でも保存されていた．