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Communications

Characterization of Human Cytosolic Thioredoxin Mutants with Increasing Side Chain Volumes at Residue-33

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Thioredoxin (Trx) is a small protein that exists in most living organisms, ranging from bacteria to mammals, and acts as a hydrogen donor for ribonucleotide reductase in *Escherichia coli*.[1] In higher organisms, Trx functions as a general dithiol-disulfide oxidoreductase that is involved in a variety of biological functions, such as protein disulfide reduction, H_2O_2 reduction, hydrogen donor for ribonucleotide reductase and methionine sulfoxide reductase, regulation of chloroplast photosynthetic enzymes, and redox regulation of transcription factors.[2] Trx basically catalyses the reduction of disulfides in proteins and becomes oxidized. Trx reductase reduces the oxidized Trx with the help of NADPH.

Most Trxs from a range of sources have highly conserved three-dimensional structures as shown in Figure S1, which shows the crystal structure of human cytosolic Trx.[3] The consensus sequence of the active disulfide bond region of human cytosolic Trx (Trp-Cys-Gly-Pro-Cys) between residue-31 and 35 is highly conserved in most Trxs from a range of sources, as listed in Table 1. The amino acid sequence between Cys-32 and Cys-35 forming the active disulfide bond is normally Gly-Pro. The active disulfide bond should be reduced easily to break the bond and oxidized to reform the bond during Trx function as a general dithiol-disulfide oxidoreductase. The Gly-Pro between Cys-32 and Cys-35 should provide the optimum structural environment for the opening and reclosing the active disulfide bond during the reducing function of Trx. Among the 72 Trxs searched from different sources in this study, Pro-34 is absolutely conserved, whereas two Trxs from *Corynebacterium nephridii* and *Fusarium culmorum* contain different amino acids (Ala and Pro, respectively) at residue-33 instead of Gly (more than 93% conservation). Pro-34 was site-specifically mutated to Ala or Val.[4] These mutations did not affect the activity of Trx significantly, whereas the substrate availability by Trx reductase was affected. A mutation of Pro-34 to a smaller amino acid, Ala, resulted in Trx being a less efficient substrate to its reductase, whereas a mutation of Pro-34 to an amino acid with similar size and flexibility, Val, made the mutant a more efficient substrate. On the other hand, this study did not include mutations to other amino acids larger than Val.

Table 1. Sequence comparison of the active disulfide bond regions of thioredoxins from a variety of sources. Among searched 72 thioredoxins, 11 thioredoxins are shown. Gly-33 in human and the corresponding residues in other sources are shown in bold italic letters

Source	Amino acid sequence
Human	ATWC***G***PCKMI
Rattus norvegicus	ATWC***G***PCKMI
Drosophila melanogaster (thio1)	ATWC***G***PCKEM
Staphylococcus aureus	ATWC***G***PCKMI
Escherichia coli	AEWC***G***PCKMI
Bacillus subtilis	APWC***G***PCKMI
Haemophilus influenzae	APWC***G***PCKMI
Chlamydia trachomatis	AEWC***G***PCKML
Rickettsia conorii	AEWC***G***PCKML
Corynebacterium nephridii (thio2)	AGWC***A***PCKAI
Fusarium culmorum	ADWC***P***PCKAI

The presumed role of Gly-33 involves providing the smallest side chain volume to achieve the optimum environment for the function of Trx. The effects of increasing side chain volumes at residue-33 of human cytosolic Trx on its function are unclear. A previous study of Gly-33 to Asp or Lys mutations in *E. coli* Trx, which introduced negative or positive charges at residue-33, reported that the introduction of charged amino acids at residue-33 did not affect the redox potential of the active disulfide bond of *E. coli* Trx, whereas the mutations affected the interactions with other proteins.[5,6] The present study examined the effects of increasing side chain volumes at residue-33 of human cytosolic Trx on its function to understand the relationship between the side chain volumes at residue-33 and Trx function at the protein level.

Site-directed mutagenesis is a useful tool for performing a structure-function study of many proteins.[7-9] Gly-33 in human cytosolic Trx was mutated separately to Ala, Val and Tyr to introduce amino acids with increasing size at residue-33. The amino acid volume of Gly, Ala, Val and Tyr is 60.1 Å^3,

88.6 Å^3, 140.0 Å^3 and 193.6 Å^3, respectively.[10] Mutations with these amino acids at residue-33 will result in an additional volume of 28.5 Å^3, 79.9 Å^3 and 133.5 Å^3, respectively. Site-directed mutagenesis and the construction of mutant expression vectors were carried out using common procedures with mutagenic primers, as listed in Table S1. PCR was carried out using the human Trx expression vector, pPROEX-1:Trx, as a template in a programmable PCR machine. The mutations were confirmed by DNA sequencing. The expressed Trxs were purified using a nickel affinity column. SDS-PAGE gel revealed highly purified mutants (Figure S2).

The dithiothreitol (DTT)-dependent insulin reduction method is the most commonly used assay for measuring the Trx activity.[11] This method involves the reduction of insulin by DTT and Trx, which results in the cleavage of two interchain disulfide bonds at similar overall rates. As reduction proceeds, a white insoluble precipitate is formed mainly from the free B chain of insulin. The time for complete precipitation of the free insulin B chain aggregates is longer than the rate of free chain production, resulting in a delay in precipitation. As listed in Table 2, the Ala-33 mutant showed a 14% shorter time to precipitation and 8.5% higher rates of precipitation. This indicated that the Gly-33 to Ala mutation enhanced the DTT-dependent insulin reduction activity of Trx. On the other hand, the Val-33 and Tyr-33 mutants exhibited longer times to precipitation (181% for Val-33 mutant and 209% for Tyr-33 mutant) and lower precipitation rates (71% for Val-33 mutant and 86% for Tyr-33 mutant). The times to precipitation represent the reducing catalytic activity of Trx in the insulin reduction assay. The approximately 2-fold longer times to precipitation indicated that the DTT-dependent insulin reduction activity of Trx was suppressed substantially by these mutations.

The apparent k_{cat}/K_m values were estimated using rat liver Trx reductase to determine the efficiency of mutated Trxs as a substrate for mammalian thioredoxin reductase.[12] The k_{cat}/K_m value of the Ala-33 mutant was 21% higher than that of normal Trx, whereas the k_{cat}/K_m value of the Val-33 and Tyr-33 mutants was approximately 39% and 61% lower, respectively (Table 2). This suggests that the side chain volume at residue-33 should be smaller than the side chain of Val to allow efficient Trx reduction by its reductase. If the side chain volume is larger than that of Val, the efficient interaction between Trx and its reductase can be inhibited due to its large side chain and the efficient reduction of the oxidized Trx can be disturbed. Figure S3 shows the structures of the increasing side chain volumes at residue-33 according to the mutations of Gly-33 (a) to Ala (b), Val (c) and Tyr (d). The side chain volumes at residue-33 increase in the surface direction, suggesting that a large side chain volume at residue-33 may deteriorate the interactions between the active disulfide bond region of Trx and the substrate binding site of Trx reductase.

Table 2. Activity of human cytosolic thioredoxins catalyzed insulin reduction by dithiothreitol and apparent k_{cat}/K_m values of normal and mutated thioredoxins with rat liver thioredoxin reductase

Trx	Time to precipitation (sec)	Rate of precipitation ($A_{650} \times min^{-1}$)	k_{cat}/K_m ($min^{-1}/\mu M$)
Normal (Gly-33)	162 ± 2	0.153 ± 0.003	175 ± 9
Ala-33 mutant	142 ± 5	0.166 ± 0.011	211 ± 36
Val-33 mutant	294 ± 19	0.108 ± 0.009	107 ± 4
Tyr-33 mutant	339 ± 35	0.132 ± 0.016	69 ± 1

Values are mean ± S.D. from three separate measurements.

The effects of the increasing side chain volumes at residue-33 of human cytosolic Trx on its function were examined using site-directed mutagenesis, DTT-dependent insulin reduction activity measurements and a mammalian Trx reductase assay. The insulin reduction activity of the Ala-33 mutant was enhanced and its apparent k_{cat}/K_m value to Trx reductase was also improved. This indicates that a mutation of Gly-33 to Ala, which is a relatively small amino acid with an additional methyl group, in human cytosolic Trx improves its DTT-dependent insulin reduction activity as well as its efficiency as a substrate for the reductase. This explains why *Corynebacterium nephridii* Trx contains Ala instead of Gly-33. On the other hand, Gly-33 mutations to Val and Tyr resulted in lower activities for insulin reduction, indicating that side chain volumes of three methyl groups or larger at residue-33 can affect the conformational environment of the active disulfide bond region. The k_{cat}/K_m values of the Val-33 and Tyr-33 mutants to Trx reductase were also decreased. This indicates that mutations of Gly-33 to larger amino acids, such as Val and Tyr, deteriorate the efficient substrate and enzyme interactions between Trx and its reductase. From these results, the following conclusions can be drawn. Human cytosolic Trx exhibits its enhanced reducing activity in a DTT-dependent insulin reduction assay and improved efficiency as a substrate for Trx reductase when its residue-33 is Ala. On the other hand, to maintain efficient reducing activity and be an efficient substrate for Trx reductase, the residue-33 of human cytosolic Trx should not be mutated to larger amino acids, such as Val and Tyr.

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