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Characterization of Two Site-Specific Human Cytosolic Thioredoxin Mutants (Pro-34 to Ala and Val)

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Thioredoxin (Trx) is a small protein performing multifunctional roles in various organisms from bacteria to mammalians. It has been found as a hydrogen donor for ribonucleotide reductase in *Escherichia* coli.[1] In higher organisms, it is involved in various biological functions as a general dithiol-disulfide oxidoreductase.[2] Its basic function is the catalytic reduction of disulfides in proteins. The oxidized Trx is reduced by Trx reductase and NADPH. In addition, the various physiological roles in many organisms have been reported.[3] They include the hydrogen donor for ribonucleotide reductase and methionine sulfoxide reductase, protein disulfide reduction, H_2O_2 reduction, regulation of chloroplast photosynthetic enzymes and redox regulation of transcription factors. It is also found at different subcellular compartments such as cytosol, nuclei, and mitochondria in higher organisms. Human cytosolic Trx is composed of 104 amino acids.[4] All Trxs from various sources have highly conserved three-dimensional structures.[5] The active site of Trx is unique as shown in Figure 1. It is a protruding loop from the end of the second β-sheet strand instead of the cleft, which is more commonly the case.

The consensus sequence of the active disulfide bond of the human cytosolic Trx is Trp-Cys-Gly-Pro-Cys between residue-31 and 35. This sequence is highly conserved in most Trxs from various sources as shown in Table 1. Among 72 Trxs searched from different sources, Pro-34 is absolutely conserved. The site-directed mutagenesis method has been a useful tool for the structure-function study of various proteins.[6-10] To examine the importance of this absolutely conserved Pro-34 residue in human cytosolic Trx function, the Pro-34 residue was mutated to Ala and Val. Site-directed mutagenesis was performed using a mutagenesis kit according to the provided protocol. PCRs were carried out using the human Trx expression vector pPROEX-1:Trx as a template in a programmable PCR machine with the mutagenic primer shown in Table 2. The whole DNA sequence of human Trx coding region was sequenced to verify the integrity of DNA sequences and the mutations. The expressed Trxs were purified using a nickel affinity column according to the provided protocol. SDS-PAGE revealed that the mutants were highly purified (data not shown).

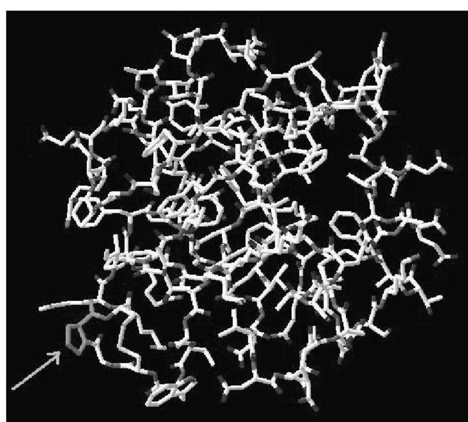

Figure 1. The location of Pro-34 in human cytosolic thioredoxin. The Pro-34 residue of the active site is indicated by an arrow.

The quantitative assay method for obtaining steady state kinetic parameters of Trx activity has not been established yet. The most well established assay method for Trx activity is the dithio-

Table 1. Sequence comparison at active site region of thioredoxins from various sources. Among searched 72 thioredoxins, 10 thioredoxins are shown here. The Pro-34 residue in human and the corresponding residues in other sources are highlighted in bold italic letters

Source	Amino acid Sequence
Human	ATWCG***P***CKMI
Bos Taurus	ATWCG***P***CKMI
Mus musculus	ATWCG***P***CKMI
Drosophila melanogaster (thiol)	ATWCG***P***CKEM
Chlamydia pneumoniae	AEWCG***P***CKML
Escherichia coli	AEWCG***P***CKMI
Haemophilus influenzae	APWCG***P***CKMI
Rickettsia conorii	AEWCG***P***CKML
Mycobacterium tuberculosis	ATWCG***P***CKMV
Fusarium culmorum	ADWCP***P***CKAI

Table 2. Primers for the site-directed mutagenesis. The mismatched bases are underlined

Mutations/ Primers	Primer Sequences
Pro-34 to Ala mutation	
Sense	5'-GCCACGTGGTGTGGG<u>G</u>CTTGCAAAATGATCAAG-3'
Antisense	5'-CTTGATCATTTTGCAAG<u>C</u>CCCACACCACGTGGC-3'
Pro-34 to Val mutation	
Sense	5'-GCCACGTGGTGTGGG<u>GT</u>TTGCAAAATGATCAAG-3'
Antisense	5'-CTTGATCATTTTGCAA<u>AC</u>CCCACACCACGTGGC-3'

Table 3. Rate of human cytosolic thioredoxins catalyzed insulin reduction by dithiothreitol and apparent k_{cat}/K_m values of normal and mutated thioredoxins to rat liver thioredoxin reductase

Trx	Time to precipitation (sec)	Rate of precipitation ($\Delta A_{650} \times min^{-1}$)	k_{cat}/K_m ($min^{-1}/\mu M$)
Normal(Pro-34)	162 ± 2	0.153 ± 0.003	175 ± 9
Ala-34	186 ± 5	0.222 ± 0.006	81 ± 4
Val-34	215 ± 14	0.151 ± 0.008	615 ± 24

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

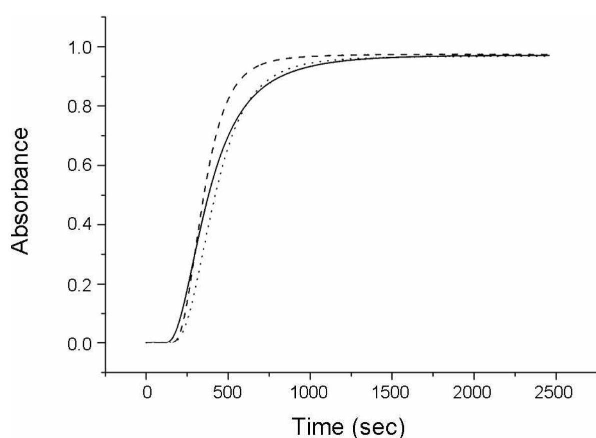

Figure 2. Thioredoxin-catalyzed reduction of insulin by dithiothreitol. The absorbance at 650 nm is plotted against time. The incubation mixture contained 100 mM potassium phosphate buffer (pH 7), 2 mM EDTA, 0.13 mM bovine insulin and 5 μM of thioredoxins in a final volume of 500 μL. Normal Pro-34 Trx (solid line), Ala-34 mutant Trx (dashed line) and Val-34 mutant Trx (dotted line).

threitol (DTT)-dependent insulin reduction method.[11] Reduction of insulin by DTT and thioredoxin leads to cleavage of the two interchain disulfide bonds at similar overall rates. As reduction proceeds, a white precipitate is formed mainly from the free B chain of insulin which is insoluble. The time for the complete precipitation of the free insulin B chain aggregates may be long, compared with the rate of the production of free chain resulting in a delay of precipitation as shown in Figure 2. As shown in Figure 2 and Table 3, the activities of the Ala-34 and Val-34 mutants were similar to that of normal Trx. The active sites of enzymes are usually located in a cleft. However, the active site of Trx is unique since it is a protruding loop instead of the cleft as shown in Figure 1. This may contribute to the flexibility of mutations at the residue-34 in the case of the insulin reduction activity of Trx. The mutations to Ala and Val can be affordable at this site since they are located at the protruding outsider of Trx. Due to the environment with less steric constrains, the mutations at Pro-34 to Ala and Val may not significantly affect the insulin reduction activities of the mutated Trxs.

To examine the efficiency of the mutated Trxs as a substrate of mammalian thioredoxin reductase, the apparent k_{cat}/K_m values was estimated using rat liver Trx reductase.[12] The k_{cat}/K_m value of Ala-34 mutant was significantly decreased about 2.2-fold as shown in Table 3. This indicated that the mutation to Ala at residue-34 made the Trx a less efficient substrate to its reductase. The decreased side chain volume at residue-34 probably affected the efficient interactions between the Trx and its reductase. The k_{cat}/K_m value of Val-34 mutant was significantly increase about 3.5-fold as shown in Table 3, indicating that the mutation to Val at residue-34 made the Trx a more efficient substrate to its reductase. Pro is unique since it is an imino acid. This feature gives an exceptional conformational rigidity compared to other amino acids. The mutation of the Pro-34 to Val may give an additional conformational freedom to Val-34 mutant to interact more efficiently with the Trx reductase.

In this study, the effects of the mutations of Pro-34 to Ala and Val on the function of human cytosolic Trx were examined using site-directed mutagenesis, the DTT-dependent insulin reduction activity measurement and mammalian Trx reductase assay. The insulin reduction activity of human cytosolic Trx was not significantly affected by the mutations of the Pro-34 to Ala and Val. This result could be related to the nature of the protruding active site of human cytosolic Trx. The k_{cat}/K_m value of Ala-34 mutant to the Trx reductase was significantly decreased, indicating that the mutation of Pro-34 to a smaller amino acid made the Trx a less efficient substrate to its reductase. This decreased k_{cat}/K_m value of Ala-34 mutant may be due to decreased volume of the side chain, which may result in inefficient interactions between the Ala-34 mutant and the Trx reductase. The Val-34 mutant showed the increase k_{cat}/K_m value to the Trx reductase, indicating that the mutation of Pro-34 to amino acid with similar size and flexibility made the mutant a more efficient substrate. This result indicates that the Pro-34 residue of the Trx should not be mutated to a smaller amino acid to act as a proper substrate to its reductase. The mutation of Pro-34 to amino acid with more conformational freedom and similar size may help Trx act as a more efficient substrate.

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