行政院國家科學委員會專題研究計畫成果報告
大腸桿菌 Thioredoxin 含活性區序列 CVPC 是否於 thioredoxin reductase 所催化的反應中具有功能?

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一、中文摘要

Thioredoxin 在細胞中催化重要的氧化還原反應，例如核甘酸的還原。此蛋白之活性區含一帶雙胱氨酸(bis(cysteinyl))的一致性(consensus)序列 Cys-Gly-Pro-Cys。然而, 此一致性序列在 *Clostridium litorale* 的 thioredoxin 竟轉變為 Cys-Val-Pro-Cys，且在大腸桿菌 thioredoxin reductase 所催化的氧化還原系統中這個 thioredoxin 並無活性。為了探討其問題是出在 thioredoxin reductase 所催化的反應動力學上，蛋白質的氧化還原電位上，亦或其他原因，並了解氧化還原蛋白主題結構 CXXC 中之第一個 X 的重要性，我們將 *E. coli* thioredoxin 活性區 Gly 突變為 Val。令我們驚訝的是在 *E. coli* thioredoxin reductase 所催化的氧還原系統中，此突變株 thioredoxin 只比野生蛋白的 k_{cat}/K_m 下降 1.7 倍。其主因在於 K_m 的上升。而在氧化還原性質方面，其硫醇的有效濃度(C_{eff})略微高於野生種，約是 1.2 倍。突變株與野生株間的直接平衡常數比(K_{12})為 1.4。因此突變株氧化還原電位比野生株降低約 3.5 mV。這些研究結果顯示活性區 Gly→Val 的改變並不造成 thioredoxin reductase 所催化的反應動力學或蛋白質的氧化還原性質上大幅的變化。*Clostridium litorale* thioredoxin 之所無法於大腸桿菌 thioredoxin reductase 催化的氧化還原系統具有活性應還有其他地方氨基酸的改變影響之。

關鍵詞：Thioredoxin、突變、Thioredoxin reductase、氧化還原

Abstract

Thioredoxin plays important roles in the cellular oxidoreduction reactions, such as the reduction of ribonucleotides. The catalytic centers of these proteins are contained within thiol/disulfide segments, which exhibit the consensus bis(cysteinyl) sequence Cys-Gly-Pro-Cys. However, a Val for Gly substitution takes place in this consensus sequence in *Clostridium litorale* thioredoxin. Moreover, this protein manifests null activity in the oxidation-reduction system catalyzed by *E. coli* thioredoxin reductase. To investigate whether the inactivation is attributed to a kinetic effect of the Gly→Val on the thioredoxin reductase catalyzed reaction, to the alteration of the redox potential of the protein by Gly to Val replacement, or to other reason, we substituted Val for Gly at position 33 in the active site of *E. coli* thioredoxin. Surprisingly, in the redox reaction catalyzed by thioredoxin reductase, the G33V mutation only lowers k_{cat}/K_m by 1.7 folds, which is mainly due to a raise of K_m. The thiol

effective concentration (C_{eff}) of the mutant is slightly higher, approximately 1.2 times, than that of the wild-type protein. Direct redox equilibrium ratio (K_{12}) between the mutant and the wild-type proteins is about 1.4. Hence, the mutation decreases the redox potential by approximately 3.5 mV. These results demonstrate that the Gly→Val substitution does not have substantial effects on the kinetics of the thioredoxin reductase catalyzed reaction or the redox properties of the protein. Therefore, the loss of function of the *Clostridium litorale* thioredoxin must be attributed to other amino acid changes in this protein.

Keywords: Thioredoxin, Mutation, Thioredoxin reductase, Oxidoreduction

二、緣由與目的

Thioredoxin is a small protein with two redox-active half-cystines in the active site. The function of thioredoxin includes reduction of methionine sulfoxide and sulfate (1), hydrogen donor for ribonucleotide reductase (2), regulatory factors for photosynthetic enzymes such as spinach chloroplast fructose bisphosphatase and NADP-dependent malate dehydrogenase (3, 4) etc.. For thioredoxin to be functional in these reactions, the protein needs to be reduced by NADPH in a reaction catalyzed by thioredoxin reductase as follows (5),

$$Trx\text{-}S_2 + NADPH + H^+ \leftrightarrow Trx\text{-}(SH)_2 + NADP^+ \quad (1)$$

Where $Trx\text{-}S_2$ and $Trx\text{-}(SH)_2$ refer to the oxidized and reduced thioredoxin, respectively.

From bacteria to human, the active site region is highly conserved with an amino acid sequence of Cys-Gly-Pro-Cys. The dipeptide sequence encompassed by two half-cystines is thought to be important in modulating the redox properties of the thiol-disulfide oxidoreductase of thioredoxin enzyme family that possesses Cys-X-X-Cys motif (6). Our previous studies (7) showed that thioredoxin with CDPC active site reduces catalytic efficiency of thioredoxin reductase by approximately 10 folds. Therefore, amino acid at position 33 (follows *E. coli* sequence) of thioredoxin also seems to be important for the catalysis of equation. 1 by thioredoxin reductase.

A deviation of the consensus sequence was reported by Kreimer et al. (8) for the thioredoxin from *Clostridium litorale*. The active site has a sequence of Cys-Val-Pro-Cys. These authors showed that this thioredoxin was inactive in heterologous enzymatic assays with thioredoxin reductase from *E. coli*. The loss of function, in principal, could be a consequence of deficiency in kinetics of catalysis performed by thioredoxin reductase on *C. litorale* thioredoxin or/and the redox potential of the protein. Moreover, in view of the high conservation of the active site sequence, the loss of enzymatic activity of *E. coli* thioredoxin reductase with *C. litorale* thioredoxin could be due to the amino acid substitution from Gly to Val at the active center.

In this study, we generated Gly→Val substitution in *E. coli* thioredoxin to investigate the effects of Val for Gly substitution on the redox properties and kinetic behavior of *E. coli* thioredoxin. The results demonstrate that the substitution does not impart substantial change in either kinetics or redox properties. Therefore, the inactivation of the *C. litorale* thioredoxin in the heterologous enzymatic system is not caused by the variation of the active site consensus sequence.

三、結果

Site Directed Mutagenesis for G33V Thioredoxin and Purification of Protein

Sequential PCR method was used to obtain G33V mutation. A plasmid, pET/trx, carries the wild-type thioredoxin gene was used as template. The first reaction uses a 5' primer of sequence 5'TAATACGACTCACTATAGGG3' (T7) and a 3' primer of sequence 5'TTGCACGGAACGCACCACTCTGC3' (G33V). Underline indicates the position of the amino acid substitution from Gly to Val. For the second reaction, an oligonucleotide of sequence 5' TGATGGTGCATAAGGCCTGAACCAGA TCAG3' was used as 3' primer, and the product of the first reaction was used as 5' primer. The product of the second reaction was ligated to pGEM-T Easy vector. After transforming *E. coli* DH5α, white colonies were selected from X-gal and ampicillin plates. The size of the plasmid was checked, and the mutation the gene was confirmed by DNA sequencing. The plasmid was scissored with *Xba*I and *Eco*RI, and the mutant gene was ligated between the same sites of pET to yield pET/G33V. The mutant protein was purified by DEAE and G50 chromatography. Electrophoresis of the protein on SDS-polyacrylamide gel showed a single band.

Measurements of C_{eff}

C_{eff} of the wild-type and the G33V mutant protein were measured using glutathione as the reference. The measured Ceff of wild-type protein was 13 M, and 16 M for G33V mutant protein (Table 1). The value for the mutant protein is only a little higher than the wild-type protein. However, we think that this increase is significant.

Direct Equilibrium Ratio

Direct redox equilibrium ratio (K_{12}) between G33V thioredoxin and the wild-type thioredoxin was measured. A value of 1.4 was obtained. This demonstrates that G33V is somewhat more reductive than the wild-type protein.

Oxidoreduction Catalyzed by E. coli Thioredoxin Reductase

E. coli thioredoxin reductase catalyzes the electron transfer between NADPH and thioredoxin. Kinetic parameters of this reaction when using wild-type and G33V mutant proteins as electron acceptors were studied. G33V substitution does not significantly affect k_{cat}, whereas it increases K_m by 1.8 folds. Therefore, the catalytic efficiency decreases approximately 1.7 folds compared to the wild-type protein (Table 2).

四、討論

Direct equilibrium ratio between G33V and wild-type proteins showed that Gly to Val replacement generates a more reductive protein. C_{eff} measurements also demonstrate this trend, although the difference is very small. Other mutations at this position will be performed in the future to look into this minute change. The Gly→Val mutation lowers that redox potential by 3.5 mV as demonstrated by direct equilibrium and C_{eff}. Such change is perhaps even better suited for thioredoxin itself with regard to its reductive functions in many cellular events. However, as the reduction of thioredoxin in vivo is catalyzed by thioredoxin reductase, it is also necessary to consider this part of the electron transport. The transport of electrons from NADPH to G33V thioredoxin catalyzed by thioredoxin reductase is somewhat hampered by the 1.8-fold increase compared to the wild-type protein, while the k_{cat} remains unaffected. The overall catalytic efficiency of thioredoxin reductase decreases by 1.7 folds when using the mutant thioredoxin.

From our results, it is clear that the Val for Gly substitution does not provoke a substantial change in the redox potential or the kinetics of thioredoxin reductase catalyzed reaction. The incompatibility of *C. litorale* thioredoxin with *E. coli* thioredoxin reductase is therefore not caused by the G33V replacement of the consensus sequence. *C. litorale* thioredoxin also contains other residues that differ from *E. coli* thioredoxin. It is likely that some of these alterations contribute to the observed incompatibility.

五、參考文獻

1. Black, S., Harte, E. M., Hudson, B., and Wartofsky, L. (1960) *J. Biol. Chem. 235*, 2910-2916.
2. Laurent, T. C., Moore, E. C., and Reichard, P. (1964) *J. Biol. Chem. 239*, 3436-3444.
3. Clancey, C. I., and Gilbert, H. F. (1987) *J. Biol. Chem. 262*, 13545-13549.
4. Scheibe, R., Fickenscher, K., and Ashton, A. R. (1986) *Biochim. Biophys. Acta 870*, 191-197.
5. Williams, C. H., Jr. (1976) *Enzymes 13,* 89-173.
6. Huber-Wunderlich, M., and Glockshuber, R. (1998) *Fold Des. 3*, 161-171.
7. Lin, T.-Y. (1999) *Biochemistry 38*, 15508-15513.
8. Kreimer, S., Sohling, B., and Andreesen, J. R. (1997) *Arch. Microbiol*, 168, 328-337.

Figure 1. The kinetics of G33V and wild-type thioredoxin as substrates of thioredoxin reductase. The assay mixture contains 3.6 nM of thioredoxin reductase, 0.1 to 5.3 μM of wild-type or G33V thioredoxin, 0.24 mM NADPH, 0.1 mg/ml BSA, 0.5 mM DTNB in 0.1 M sodium phosphate buffer, 2 mM EDTA, pH 7. , wild-type thioredoxin; O, G33V thioredoxin.

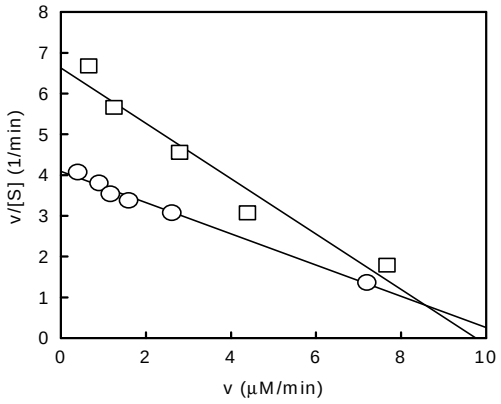

Table 1. Redox Properties of G33V and Wild-type Thioredoxin

Trx	C_{eff} (M)	Direct equilibrium ratio between G33V and wild-type (K_{12})	$\Delta E^{o'}$ (mV)
G33V	16±1	1.4±0.1	-3.5
Wild-type	13±2		

Table 2. Kinetic Parameters of the Thioredoxin Reductase Reaction with Thioredoxin at pH 7, 25 ^{o}C

Trx	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}min^{-1}$)
G33V	1340±120	2.7±0.2	496
Wild-type	1250±100	1.5±0.1	833