行政院國家科學委員會專題研究計畫　成果報告

CXXC 主題結構的第一個 X 對氧化還原性質調控的研究

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一、中文摘要

屬於 thioredoxin 酵素家族的成員具有 thioredoxin 的折疊型式，且在它們活性區內含有 Cys-X-X-Cys 的主題結構(motif)。然而此家族中的各成員卻具備決然不同的氧化還原性質。為何結構很類似的蛋白質卻可擁有殊異之氧化還原電位長久以來一直是令人不解的問題。近年來，一般採納的觀念是 Cys-X-X-Cys 主題結構中之 X-X 序列調控這些酵素的氧化還原性質(例如，Huber-Wunderlich and Glockshuber 1998, A single dipeptide sequence modulates the redox properties of a whole enzyme family. *Fold. & Des., 3*:161-171.)。然而，我們先前對第一個 X 所做的研究結果卻不甚符合此一見解。為了更清楚明瞭這個問題，在這一年的計畫中，我們將針對此一酵素家族的原始蛋白 *E. coli* thioredoxin，進行進一步之定點突變以研究第一個 X 如何影響酵素的氧化還原性質。我們將第一個 X 由 Gly 突變 Ile，發現突變蛋白仍具有與野生蛋白質相同的氧化還原性質。突變株的硫醇有效濃度(C_{eff})為 12 M，野生種 C_{eff} 為 10 M。突變株與野生株間的直接平衡常數比(K_{12})為 0.8。突變株與野生株間的氧化還原電位($\Delta E^{o'}$)無明顯的差異(2.9mV)。圓形(CD)光譜及螢光研究結果顯示活性區 Gly→Ile 的改變並不造成無論是氧化態或還原態 thioredoxin 結構的變化。也正符合了 Gly→Ile 不會影響到氧化還原性質之結果。因此第一個 X 由一個 Gly 改為疏水性的 Ile 並不會改變蛋白質的氧化還原電位或結構。這些結果更顯示第一個 X 在氧化還原電位上的調控功能是相當局限的。

關鍵詞：Thioredoxin、突變、氧化還原 thioredoxin、氧化還原酵素

Abstract

Enzymes of the thioredoxin superfamily of thiol-disulfide oxidoreductases share the thioredoxin common fold. Their active sites possess a Cys-X-X-Cys motif. Intriguingly, the individual members vary strongly in their redox properties. Why proteins with very similar structure exhibit the enormous range of redox potential has been a puzzling question for long time. A generally adopted concept recently is that the X-X dipeptide sequence modulates the redox properties of these enzymes (e.g. Huber-Wunderlich and Glockshuber 1998, A single dipeptide sequence modulates the redox properties of a whole enzyme family. *Fold.& Des., 3*:161-171.). Our previous investigation in the first X however does not quite reconcile with this notion. To clarify this point, in

this one-year project, we performed additional site-directed mutagenesis, Gly33→Ile, on the prototype protein of this family, *E. coli* thioredoxin to investigate how the first X would modulate the redox potential. We found that the mutation does not significantly affect the redox properties of the protein. The effective concentration of the dithiols (C_{eff}) for the mutant and the wild-type proteins are 12 M and 10 M, respectively. Equilibrium constant obtained from pair-wise reaction between the mutant and the wild-type proteins shows a value of 0.8, indicating that the replacement does not significantly affect the thiol-disulfide redox equilibrium. The redox potentials of the mutant and the wild-type proteins do not differ significantly (2.9 mV). CD and fluorescence spectroscopies show that the secondary and the tertiary structures of the mutant proteins do not vary from those of the wild-type, either in the oxidized or the reduced states, which are in accord with the results of redox measurements. Therefore, substitution of a hydrophobic Ile for Gly at the first X does not affect the redox potential or structure of thioredoxin. These results further substantiate that the modulating function of the first X in redox potential is quite limited.

Keywords: thioredoxin, mutation, redox potential, oxidoreductase

二、緣由與目的

Thioredoxin superfamily of thiol-disulfide oxidoreductases are wide spread enzymes that catalyze the redox reactions in all living organisms. They carry out functions such as oxidation of proteins, reduction of proteins, orchestration of protein folding, and maintenance of redox environments within cells.

Thiol-disulfide oxidoreductases have common thioredoxin fold and possess Cys-X-X-Cys motif that is essential for their catalysis of redox reactions (Laboissière et al., 1995; Walker et al., 1996). The Cys-X-X-Cys motif is located in the active site and capable of forming a disulfide bond between the two cysteines. An intriguing feature of proteins in this superfamily is that their reduction potentials vary widely. *E. coli* thioredoxin has a reduction potential ($E^{o'}$) of –0.270 V (Moore et al., 1964). A homologous enzyme, protein disulfide isomerase, has an $E^{o'}$ of –0.147 to –0.180 V (Lundström and Holmgren, 1993; Darby and Creighton, 1995). *E. coli* DsbA has an $E^{o'}$ of –0.09 to –0.11 V (Wunderlich and Glockshuber, 1993; Grauschopf et al., 1995). The broad spectrum of the reduction potential engenders these thiol-disulfide oxidoreductases diverse roles in living cells.

Considerable effort has been devoted to finding out the determinants for the reduction potential of the thioredoxin superfamily of thiol-disulfide oxidoreductases. The general thought to date is that the X-X dipeptide sequence modulates the redox potential of the thiol-disulfide oxidoreductase family (Huber-Wunderlich and Glockshuber, 1998).

Thioredoxin is the prototype of this superfamily of proteins. It is a 12 kDa protein that can serve as a reducing agent for ribonucleotide reductase and other proteins (Holmgren, 1985; Buchanan et al., 1994; Powis and Monfort, 2001). The active site of thioredoxin is composed of Cys32-Gly33-Pro34-Cys35, which is located at the N-terminus of α2 helix near the central portion of the β-sheet of the protein (Holmgren et al., 1975; Katti et al., 1990).

In the past years, we investigated the role of the first X of C-X-X-C motif in the redox properties of thioredoxin. Contrary to the general consensus, our results did not show any mastery of the first X over the redox potential of thioredoxin. We are quite curious about the decisiveness of the first X in the redox potential. In this report, we constructed another mutation that has an Ile replacement for Gly33 to investigate the determination of the first X on the redox

properties of the protein.

三、結果

Site Directed Mutagenesis for G33I

G33I was generated by site-directed mutagenesis using a sequential PCR method (Kammann et al., 1989; Russel et al., 1988). A plasmid carries thioredoxin gene, pET/trx, was used as the template. The first reaction used a 5' primer of sequence 5' TAATACGACTCACTATAGGG 3' (T7) and a 3' primer of sequence 5' TTGCACGGAATGCACCACTCTGC 3' (G33I). Underline indicates the position of alteration. For the second reaction, the product of the first reaction will be used as the 5' primer and the 3' primer (ndemsc/stu) has a sequence of 5' TGATGGTGCATAAGGCCTGAACCAGAT CAG 3'. IUB code is used herein. The mutant gene, harboring on pGEM-T vector, was confirmed by dideoxysequence. The correct plasmid was then digested with *Xba*I and *Eco*RI to give an approximately 400 bp *trx*A fragment. It was ligated with pET/trx vector that has been treated with the same restriction enzymes. The resultant plasmid was called pET/G33I.

Purification of the mutant and the wild-type Proteins

G33I mutant protein was overexpressed from pET/G33I in *E. coli*. The wild-type protein was expressed from *E. coli* SK3987. The proteins were purified by DEAE and G50 chromatographies. The detail procedure follows that of Lin (1999). Electrophoresis of the purified proteins on a SDS-polyacrylamide gel showed a single band in both cases.

Measurements of effective concentration of thiols (C_{eff})

C_{eff} (Page, 1973; 1977; Moore and Jenks, 1982, Creighton, 1983) of the wild-type and the G33I mutant proteins were measured using glutathione as the reference (Lin and Kim, 1989). The measured C_{eff} of wild-type protein was 10 M. C_{eff} was 12 M for G33I mutant protein (Table 1), which is close to the value of the wild-type protein.

Direct Equilibrium Ratio

Direct redox equilibrium ratio (K_{12}) (Aslund et al., 1997) between the G33I mutant and the wild-type thioredoxin was measured. K_{12} was 0.8 (Table 1), demonstrating that G33I mutation has little effect on the redox equilibrium ratio.

Redox potential of the mutant protein

Redox potentials of G33I mutant thioredoxin was -267 mV, which does not varied significantly from that of the wild-type thioredoxin (-270 mV) (Table 1). The result indicates that the redox potential does not change significantly by a long-chain hydrophobic residue at the first X position.

Structural studies by CD

The structures of the oxidized and the reduced proteins of both the wild-type and the mutant proteins were studied by CD. The mutant protein displayed a spectrum similar to the wild-type protein, either in the oxidized or reduced forms (data not shown), in the far UV region. These results showed that the mutation does not alter the secondary structure of the protein.

Structural Studies by fluorescence

The tertiary structure of the mutant and the wild-type proteins were compared by fluorescence spectroscopy. The fluorescence intensity of the oxidized G33I protein was a little lower than that of the wild-type protein in the oxidized state (Figure 1). Reduced form of the G33I protein showed an increase in fluorescence relative to the oxidized form, and its intensity resembles that of the reduced wild-type thioredoxin (Figure 1). The results indicate that the mutation does not alter the tertiary structures of the protein in the reduced state.

四、討論

Thioredoxin have been implicated in a large number of cell events. Its oxidation and reduction functions take part in many cellular reactions. The C-X-X-C motif in its active site is essential in carrying out such redox functions. The central residues in the motif are considered to be a determinant of the redox potential. In this study, we performed a Gly→Ile substitution at the first X position of *E. coli* thioredoxin. C_{eff} measurements demonstrate that the mutant protein has a value similar to that of the wild-type protein. The redox equilibrium between the G33I mutant and the wild-type protein shows a value of 0.8, again showing that G33I dose not significantly interfere with the thiol-disulfide equilibrium. The redox potential difference between the mutant and the wild-type proteins is merely 2.9 mV. All thee results indicate that a long hydrophobic side chain at the first X position does not vastly change the redox properties of thioredoxin.

CD spectra indicate that the structure of either oxidized or reduced thioredoxin is not altered by the G33I substitution. The tertiary structure of the reduced G33I is similar to that of the wild-type protein. However, the oxidized G33I exhibits a lower intensity in the fluorescence spectra. The tryptophan residues (residue 28 and 31) are in close proximity to the active site. The lowering of intensity could be due to an increase in the quenching of tryptophan intensity. We will further investigate this point. Nevertheless, the relatively inefficient of the Ile in altering the redox properties of thioredoxin suggests that the control of redox potential by the first X is limited.

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Table 1. Redox Properties of G33I and Wild-type Thioredoxin

Trx	C_{eff} (M)	Direct equilibrium ratio between G33V and wild-type (K_{12})	ΔE^o (mV)	$\Delta E^{o'}$ (mV)
G33I	12.3±0.5	0.8±0.1	-267	2.9
Wild-type	10±1		-270	

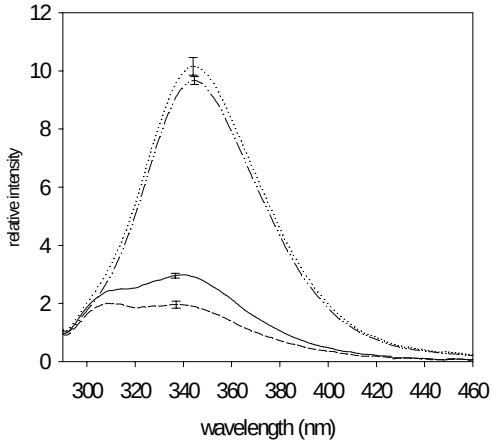

Figure 1. Fluorescence of G33I and Wild-type thioredoxin. ─, oxidized wild-type; ·····, reduced wild-type; ------, oxidized G33I; –··–, reduced G33I.