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N-Terminus Deletion Affecting the Preparation of Soluble Cyanobacterial Glutaredoxin in *Escherichia coli*

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Abstract—Glutaredoxin has been implicated in maintenance of a normal cellular thiol/disufide ratio and the regeneration of oxidatively damaged proteins. In order to obtain more information about these important regulatory proteins in cyanobacteria, we have previously cloned and expressed the first cyanobacterial glutaredoxin gene *ssr2061* in *Escherichia coli*. In this work, the second glutaredoxin gene *slr1562* was studied. About 90% of Grx2061 coded by *ssr2061* was produced in a soluble form while 90% of Grx1562 coded by *slr1562* was found in inclusion bodies. To improve the production of soluble Grx1562, we constructed two mutants: Grx1562NC with cysteines in conserved site substituted by serines, and Grx1562M with N-terminus hydrophobic region deletion. Only the latter mutant was successfully expressed in soluble form with increased glutaredoxin activity and showed less sensitivity in oxidative stress. Spectroscopic analysis shows that the structure of Grx1562M with less hydrophobic nature could give more opportunity for protein solubility and could improve the substrate catalytic efficiency. These results suggest that hydrophobic N-terminus determines the insolubility of Grx1562 and may provide another strategy for increasing expression level of soluble heterologous proteins in *E. coli*.

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Glutaredoxins (Grxs) are known to be a ubiquitous multifunctional family of glutathione (GSH)-dependent disulfide oxidoreductases [1]. They play important roles in reduction of ascorbate, protection against reactive oxygen species, regulation of the DNA binding activity of nuclear factors, and sulfur metabolism [2]. Grx contains a disulfide/dithiol in the conserved active site sequence, [CPY/FC], which belongs to the thioredoxin fold superfamily based on their three-dimensional structures [3]. Thiol/disulfide interchange reaction catalyzed by Grx is crucial for intracellular redox homeostasis, especially under oxidative stress. A specificity of Grx is its involvement in protein glutathiolation processes. Indeed, an oxidative stress can induce either a reversible oxidation of cysteine residues into sulfenic acid or an irreversible oxidation into sulfonic or sulfinic acids. A mixed disulfide bridge between a given target protein and glutathione could prevent irreversible oxidation of active-site cysteine residue. As Grx can also efficiently perform deglutathiolation, it could play a major role in the regulation and maintenance of protein activities [4, 5].

Grxs in several organisms such as *Escherichia coli*, yeast, plant, and mammalian cells have been studied. In *E. coli*, Grxs vary in size from 82 to 215 amino acid residues [6] but in most other species the average size is around 100 amino acids [7]. Cyanobacterium *Synechocystis* sp. PCC 6803 is a prokaryotic photosynthetic microalgae, which is often used as a model organism for the study of photosynthesis and other important physiological processes. Our previous work has focused on an examination of the expression level and antioxidative stress characterization of the first-known cyanobacteria Grx, Grx2061, which shows high sequence conservation with other well-known Grxs [8]. GenBank database searching indicates that an open reading frame in *Synechocystis* sp. PCC 6803 genome, *slr1562*, was presumed to encode another Grx with 63% sequence identity to Grx2061. Since *Synechocystis* Grx is not abundant *in*

Abbreviations: Grxs) glutaredoxins; GSH) glutathione; GST) glutathione-S-transferase; HED) 2-hydroxyethyl disulfide; IBs) inclusion bodies; IPTG) isopropyl-β-D-thiogalactopyranoside.

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vivo, it might be difficult to isolate the protein directly from *Synechocystis* for further structure and function studies. Thus, to use molecular biology techniques to obtain enough recombinant protein is of importance.

The cloning and sequencing of Grx from a number of prokaryotic and eukaryotic organisms has allowed the possibility of its expression in *E. coli* [9, 10]. However, high-level expression of heterologous proteins in *E. coli* is often associated with inactive aggregates, known as inclusion bodies (IBs). The formation of IBs is mainly attributed to the overexpression of proteins in the cell lacking the required accessories for its folding to the native form [11, 12]. Although refolding of IBs *in vitro* is widely studied, there are some inherent limitations including low activity yield, high concentration of denaturing agents, limited choice of chromatographic media, etc., which restrict its application in many cases [13]. Soluble expression becomes a good alternative for expression of proteins of interest because of less activity loss and facilitation of purification. Improvement in soluble expression could be achieved with some success by manipulation of the induction conditions [14], the introduction of deletions or substitutions of amino acid residues, fusion with partner proteins like glutathione-S-transferase (GST), as well as expression element changes in the vector itself [15].

In the present study, another Grx gene, *slr1562*, from *Synechocystis* was cloned into the same vector but found to be expressed in insoluble form. Deletion of its N-terminus hydrophobic regions was found to be very helpful for protein production in *E. coli*, while replacement of cysteines with serines in active sites could not improve its solubilization. Structure analysis using spectroscopic methods revealed that the deletion of Grx1562 N-terminus has a little effect on its secondary structure, but increase the hydrophilicity of the active site, which may account for the solubilization feasibility of the protein.

MATERIALS AND METHODS

Materials. GSH, NADPH, 2-hydroxyethyl disulfide (HED), glutathione reductase from yeast, and culture media were from Sigma (USA). Hitrap chelating column and Superose-6 column were from GE Healthcare Life Science (USA). Plasmid pET-21b and *E. coli* strain BL21(DE3) were from Invitrogen (USA). Restriction enzymes, ligase, and pfu were from Takara Biotech (Japan). All general laboratory reagents were of the highest grade available unless otherwise noted.

Construction of plasmids for expression of Grx1562 and its two mutants. A sequence of an open reading frame, *slr1562*, coding for a protein of 109 amino acids (Grx1562) with a dithiol active site (CPFC) was amplified by PCR using genomic DNA of *Synechocystis* sp. strain PCC 6803 as a template. The primers for *slr1562* were 1562F (5′-GGCGCATATGGCTAATTTGTTCAACTGG-3′) and 1562R (5′-TTATCTCGAGGGCTGGGTTAG-GAGGAGTG-3′). Two restriction sites (underlined) *Nde*I and *Xho*I were designed to allow directional subcloning into expression vector pET-21b. The plasmid containing *slr1562* was named pET-1562 and was used as templates for site-directed mutagenesis and N-terminal deletion.

Point mutation of specific amino acids in Grx1562 was performed by a two-step PCR mutagenesis technique. Briefly, a new reverse primer that converts the codons for cysteine in its active site to those for serine and the original forward primer 1562F were introduced in the first PCR amplification. Then the mutated PCR products were used as the forward primer in the second PCR amplification. These final mutated PCR products were also ligated into pET-21b to produce recombinant plasmid pET-1562NC. The new reverse primer for the first sep PCR had the following sequences (mutated bases in bold), 1562NCR: 5′-GTTTCGCCCGGATGC**T**AAAA-GGGC**T**AGTTTGCCAAG-3′.

The N-terminus of the *slr1562* gene was deleted by PCR amplification of the shortened gene with new forward primers, 1562MF: 5′-GTAGCATATGCCCCTC-CTCAGTGG-3′. The deletion primer was designed to create a deletion of seven residues in the N-terminus of Grx1562. One restriction site (underlined) *Nde*I was introduced to allow further cloning in the pET-21b expression vector. This recombinant plasmid was named pET-1562M.

Expression and purification of recombinant proteins. For gene overexpression, BL21(DE3) host bacteria were transformed with the recombinant plasmids pET-1562, pET-1562NC, and pET-1562M. These transformants were grown overnight at 37°C in 1 liter of Luria–Bertani medium supplemented with ampicillin (100 μg/ml). The recombinant genes were expressed in exponentially growing cells by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of incubation at 37°C, the cells were collected by centrifugation and stored at −70°C until use. The pellets were resuspended in 50 ml of 20 mM Tris buffer, pH 7.5, 10 mM imidazole, and 1 mg/ml lysozyme. The bacterial suspensions were sonicated and cleared by centrifugation at 12,000*g* for 20 min.

The supernatants were filtered through a 0.45-μm filter and applied onto a 1-ml Ni^{2+} Hitrap chelating column. The bound proteins were eluted with 500 mM imidazole. A further purification step was performed by gel filtration chromatography using prep grade column Superose-6. After the two-step purification process, the size and purity of recombinant proteins were judged by 15% SDS-PAGE and by size exclusion chromatography. The purity of proteins for enzymatic and spectroscopic analysis was no less than 95%.

Enzymatic activity assay. The assay for glutaredoxin is based on its GSH-disulfide trans-hydrogenase (thioltransferase) activity as described by Holmgren [1]. A mix-

ture containing 1 mM GSH, 0.2 mM NADPH, 2 mM EDTA, 0.1 mg/ml BSA, and 0.5 unit (U) of yeast glutathione reductase was prepared. To 500 μl of the mixture, HED was added to a final concentration of 0.7 mM. After 2 min, protein samples, Grx1562 or Grx1562M, were added to the mixture and the Grx activity was determined by measuring the decrease in A_{340} for 3 min at 25°C with molar extinction coefficient of 6200 $M^{-1}\cdot cm^{-1}$. An identical cuvette without Grx sample provided the background control. One activity unit was defined as the amount of the enzyme providing oxidation of 1 μmol NADPH per min.

Response of *E. coli* to oxidative stress. Overnight cultures of *E. coli* BL21(DE3) cells transformed with either pET-21b as a control or pET-1562 and pET-1562M plasmids were grown in fresh LB medium containing 100 μg/ml ampicillin under continuous shaking at 37°C. When the OD_{600} reached a value of 0.6, the desired concentrations of H_2O_2 (0, 2.5, 5, 10 mM) and 0.5 mM IPTG were added into culture solution simultaneously. Growth rates of cells were further measured after 120 min.

Hydrophobicity prediction. A computer-assisted analysis of the surface hydrophobicity of Grx1562 and Grx2061 along their amino acid sequence based on the Kyte–Doolittle hydropathy scale [16] was carried out using the DNASTAR Protean analysis software. A window of size $N = 9$ was run along the length of protein segments; for each window, the hydropathy values of the nine amino acids were summed and divided by nine to obtain the average hydrophobicity per residue for the window.

Spectroscopic analysis. A circular dichroism (CD) spectrum of the purified protein was obtained at 25°C in the wavelength rage of 250-190 nm using a Jasco J-715 (Japan) spectropolarimeter. The CD spectra were generated using a 1.0-mm quartz cuvette at a protein concentration of 0.3 mg/ml in 10 mM sodium phosphate, pH 8.0, containing 10 mM KCl and scanned three times for data accumulation (the average spectrum was reported as molar ellipticity per residue [θ]).

Intrinsic fluorescence emission spectra were measured at room temperature using a Hitachi F-4500 (Japan) fluorescence spectrophotometer with slit width of 5 nm. For the intrinsic fluorescence measurements, the excitation wavelengths were 295 nm for tryptophan. The emission spectra were recorded from 310 to 400 nm. Each purified protein (0.3 mg/ml) was dissolved in 20 mM Tris-HCl, pH 8.0.

RESULTS

Sequence analysis and expression of recombinant proteins in *E. coli*. The open reading frame sequence *slr1562* from *Synechocystis* probably coding for another Grx was amplified and expressed in *E. coli* to study protein structure and function. Figure 1 indicates that Grx1562 has an extended N-terminus and shows 63% sequence similarity to Grx2061. These two proteins have the same conserved active site (Cys-Pro-Phe-Cys), hydrophobic surface area, and a GSH or ribonucleotide reductase-binding site (underlined).

After induction with IPTG, recombinant Grx1562 and its mutants Grx1562M and Grx1562NC were expressed compared with control cells harboring pET-21b as shown in Fig. 2a. Expression patterns of Grx1562 (~14.5 kD) and Grx2061 (~12 kD) are also shown in Fig. 2b, in which 90% of Grx2061 was produced in the supernatant fraction with large amounts while 90% of Grx1562 was almost only in the pellet fraction. To further study activity, more soluble Grx1562 was needed for purification. Although we improved fermentation conditions, induction with different final concentrations of the inducer IPTG from 0.05 to 0.5 mM and cultivation temperature from 16 to 37°C showed no improvements in either the overall expression or the soluble production level of Grx1562 (data not shown). Interestingly, most of the recombinant Grx2061 could be easily obtained in soluble form under normal fermentation conditions.

Effect of N-terminal deletion on protein production. It is noticeable that Grx2061 was easily expressed in soluble form while Grx1562 was prone to be produced as inclusion bodies. We compared the surface hydrophobicity of

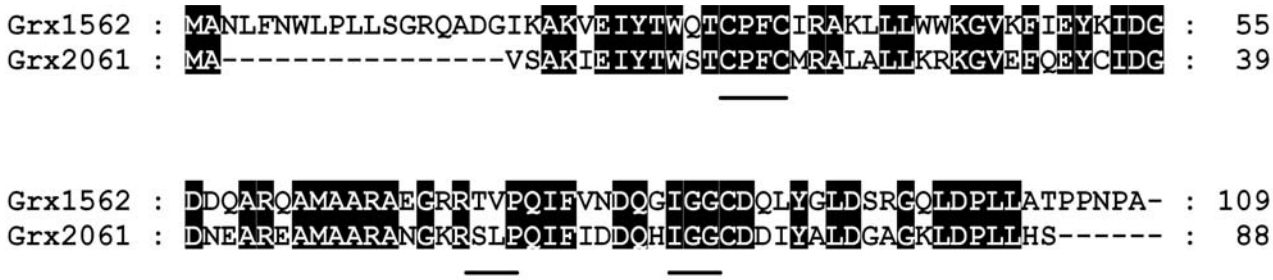

Fig. 1. ClustalX alignment of two *Synechocystis* glutaredoxin sequences. GenBank accession numbers are as follows: NP_442889 (Grx1562 of *Synechocystis* sp. PCC 6803) and NP_440852 (Grx2061 of *Synechocystis* sp. PCC 6803). Conserved residues are shown by dark gray background. The active site, hydrophobic surface area, and a GSH binding site are underlined.

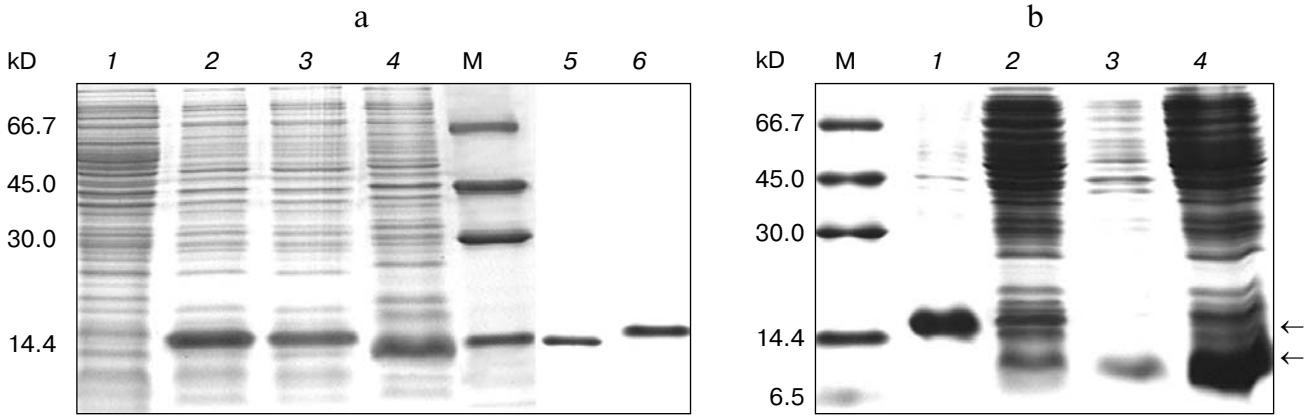

Fig. 2. Expression profiles of different constructs. a) 15% SDS-PAGE analysis of Grx1562 and its mutants expressed in *E. coli*. Protein samples (10 μl) were visualized by staining with Coomassie blue. Lanes: M) molecular weight markers; *1-4*) the whole lysate of the cells after induction transformed with pET-21b, pET-1562, pET-1562NC, and pET-1562M plasmid, respectively; *5*, *6*) purified Grx1562M and Grx1562. b) 15% SDS-PAGE analysis of Grx1562 and Grx2061 expressed in *E. coli*. Lanes: M) molecular weight markers; *1*) pellet fraction of pET-1562-transformed cells; *2*) supernatant fraction of pET-1562-transformed cells; *3*) pellet fraction of pET-2061-transformed cells; *4*) supernatant fraction of pET-2061-transformed cells. The arrows indicate the position of Grx1562 (upper) and Grx2061 (lower).

Grx1562 and Grx2061 along their amino acid sequence using the DNASTAR Protean analysis software. The results in Fig. 3 indicate that the extended N-terminal of Grx1562 shows highly hydrophobic nature, whose amino acids sequence is different from that of Grx2061. Based on this difference we deleted seven hydrophobic amino acids (ANLFNWL) at its N-terminal to generate a mutant construct, Grx1562M, to see whether these hydrophobic amino acids have positive effects on soluble expression of Grx1562. In addition, another mutant protein, Grx1562NC, in which cysteines at positions 31 and 34 were both replaced by serines, was constructed to detect if the formation of disulfide bond could affect expression patterns (Fig. 4a).

Expression level of two mutants and Grx1562 in suspension culture after IPTG induction was then compared using Coomassie stained gel. As indicated in Fig. 4b, the deletion of the N-terminus of the protein further enhanced the soluble expression of Grx1562M so that around 80% of it existed in the soluble fraction, while there was no improvement for Grx1562NC in comparison to the expression pattern of the full-length protein Grx1562.

Activity of Grx1562 and Grx1562M. Purified recombinant Grx1562 and its N-terminus deletion Grx1562M

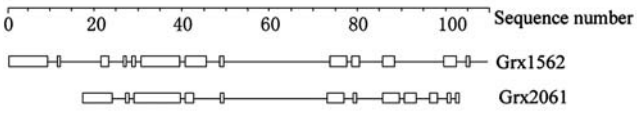

Fig. 3. Kyte–Doolittle hydropathy profile of Grx1562 and Grx2061. The hydrophobicity profile was obtained using the Kyte–Doolittle scheme of sliding window average over nine neighboring residues. Hydrophobic regions of Grx1562 and Grx2061 are boxed.

were tested for their ability to catalyze thiol-disulfide oxidoreduction reactions by means of the usual standard HED assay of glutaredoxin as shown in Fig. 5a. Interestingly, the HED catalytic activity of Grx1562M (18.0 U/mg) is slightly higher than that of Grx1562 (14.0 U/mg), indicating that the N-terminal extension might have an effect on the activity. This change might result from the conformation difference between the two proteins and the conserved activity site of Grx1562M could approach the substrates and catalyze the reaction with more efficiency.

Antioxidative property of Grx1562 and Grx1562M. To further investigate the role of Grxs in protection against oxidative stress, the growth rate of *E. coli* was examined in the presence of hydroperoxide. Resistance to H_2O_2 was determined following a 120 min exposure (Fig. 5b). Elevating the levels of Grx1562 and Grx1562M by transforming pET-1562 and pET-1562M into *E. coli* cells increased the growth rate of the *E. coli* strain against H_2O_2 stress compared with the vector alone. The average growth rate increase in *E. coli* expressed Grx1562M is 15% higher than that of *E. coli* expressed Grx1562.

Structure analysis of recombinant proteins using spectroscopic methods. CD spectra and tryptophan fluorescence were used as a tool to analyze of structural changes in a protein upon some perturbation, or in comparison of the structure of an engineered protein to the parent protein. To examine the effect of N-terminus deletion on the conformation of Grx1562, full-length Grx1562 and Grx1562M were purified to homogeneity (Fig. 2a) and subjected to spectroscopic analysis. As shown in Fig. 6a, the far UV-CD spectra corresponding to the recombinant Grxs are dominated by strong minima at 208 and 222 nm, indicating their highly helical nature. We also found that there is a little difference between the

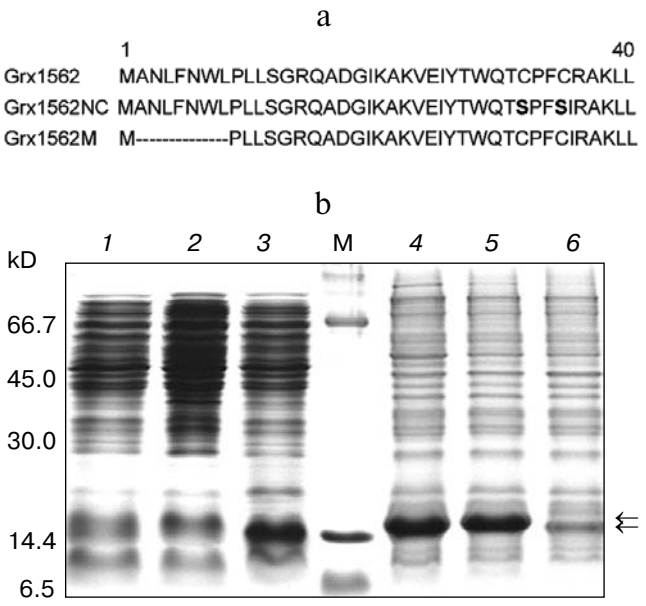

Fig. 4. Sequence alignment and SDS-PAGE analysis of full length Grx1562 and its mutants. a) Alignment of Grx1562 and its mutants. In Grx1562NC, the cysteines in conserved sites were replaced by serines (bold) to enhance the soluble expression levels in *E. coli*. In Grx1562M, the N-terminal sequence of the full-length protein Grx1562 was removed in order to enhance the soluble expression levels in *E. coli*. b) 15% SDS-PAGE analysis of recombinant Grx1562 and its mutants expressed in *E. coli*. Protein samples (10 μl) were visualized by staining with Coomassie blue. Lanes: M) molecular weight markers; *1-3*) supernatant fractions of pET-1562-, pET-1562NC-, and pET-1562M-transformed cells, respectively; *4-6*) pellet fractions of pET-1562-, pET-1562NC-, and pET-1562M-transformed cells, respectively. The arrows indicate the position of Grx1562 and Grx1562NC (upper) and Grx1562M (lower).

spectra of Grx1562 and its mutant Grx1562M, which indicates that the N-terminal deletion of Grx1562 has no significant effect on the secondary structure of the full-length protein.

Changes in protein structure upon mutation may have been too subtle to be observed by CD spectroscopy. Thus, methods based on measurement of tryptophan fluorescence were employed to look for structural variations [17]. There are four tryptophan residues at positions 7, 28, 42, and 43 at the N-terminus and close to the active site of Grx1562, which are buried in a hydrophobic environment. Changes in the intrinsic tryptophan fluorescence spectra of the excitation at 295 nm reflect changes in the local environment of the tryptophan residue. In Fig. 6b, purified Grx1562 and Grx1562M were excited at 295 nm and a shift of intrinsic tryptophan fluorescence appeared from green (Grx1562, wavelength of maximum emission is 342 nm) to violet (Grx1562M, wavelength of maximum emission is 350 nm). This result suggests movement of tryptophan residues of the N-terminus deletion was mutated to be in a more polar or hydrophilic environment. The fluorescence intensities of Grx1562M increase by 8%, also reflecting a difference in the environment of the fluorophore. These differences might contribute to enhance the high solubility of Grx1562M.

DISCUSSION

As a host of protein expression systems, *E. coli* has gained widespread application in the production of high-level recombinant proteins. However, the formation of IBs is often found due to the improper folding of the expressed

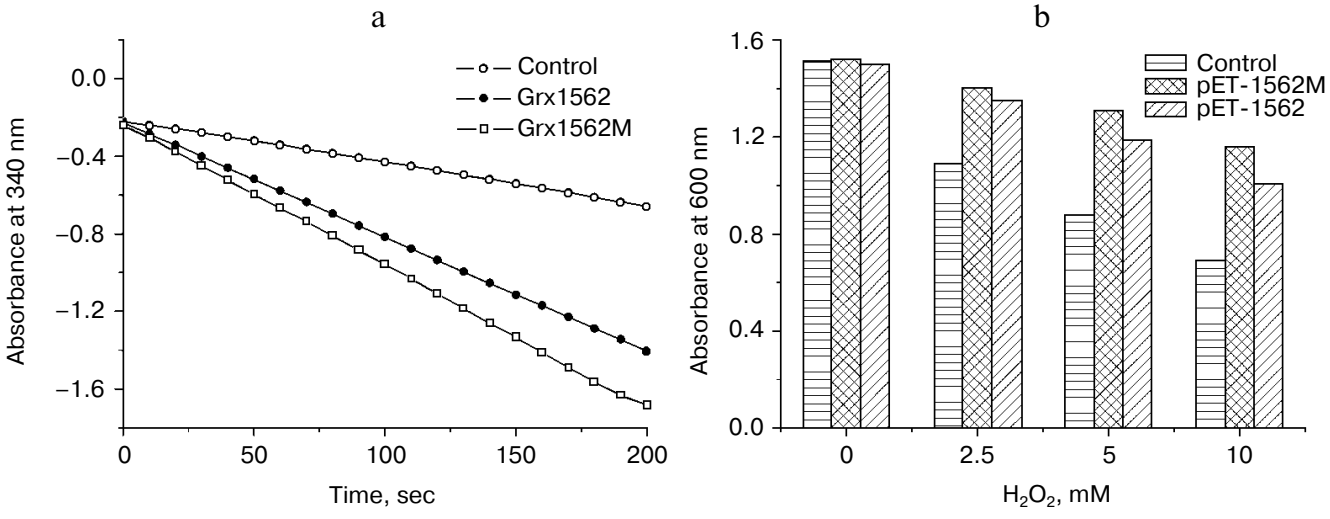

Fig. 5. Glutaredoxin activity and antioxidative stress property of Grx1562 and Grx1562M. a) Glutaredoxin activity assay of Grx1562 and 1562M. Time courses of the reduction HED by Grx1562 and Grx1562M were monitored as the couple NADPH oxidation at 340 nm as described in "Materials and Methods". b) *Escherichia coli* cells transformed with pET-21b (empty vector), pET-1562, and pET-1562M were cultivated to OD_{600} 0.6. Then IPTG and H_2O_2 at different concentrations were added and OD values were recorded as growth rates after 120 min of induction.

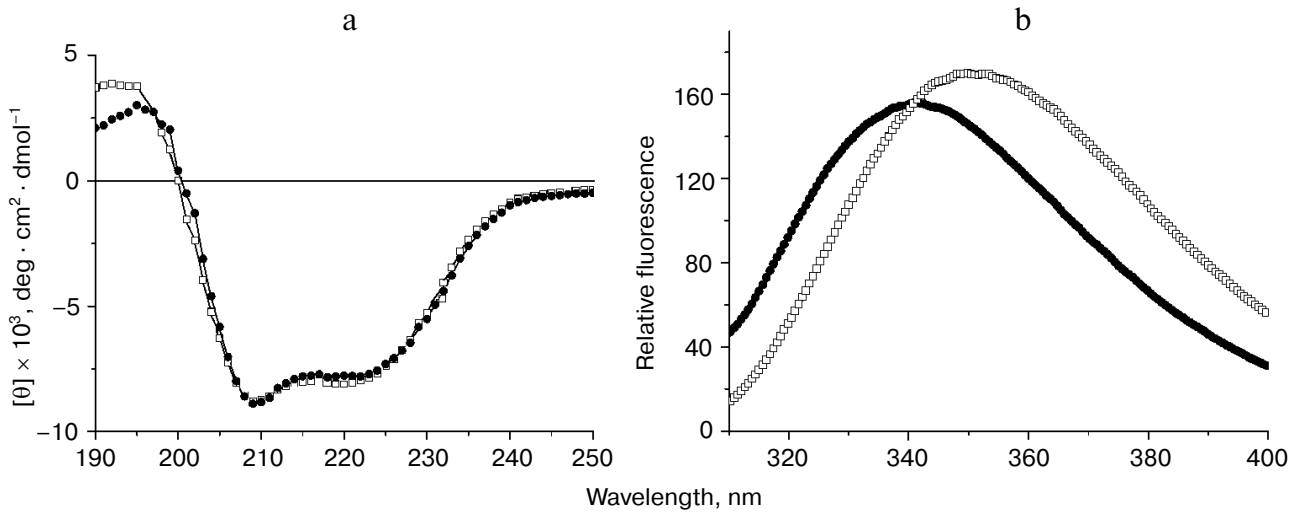

Fig. 6. Spectroscopic analysis of Grx1562 (closed symbols) and Grx1562M (open symbols). a) Circular dichroism spectrum of recombinant Grx1562 and Grx1562M (CD spectra were recorded as described in "Materials and Methods"). [θ] is the mean residual weight ellipticity. b) Room temperature fluorescence emission spectra of Grx1562 and Grx1562M. The protein concentration was 0.3 mg/ml in 20 mM Tris-HCl, pH 8.0. The excitation wavelength was 295 nm.

heterogeneous proteins [18]. Grxs are always thought of as heat-stable proteins and expressed as soluble protein in *E. coli*. Some of these recombinant proteins are prepared as a good molecular model for NMR structure studies [19, 20]. In this work, another cyanobacterial glutaredoxin Grx1562 with high sequence similarity to Grx2061 was studied, and it was shown that 90% of this recombinant protein was expressed as IBs. Analogous results were also observed in poplar Grx even though it co-transformed the strain BL21(DE3) with helper plasmid pSBET. The authors successfully prepared high purity proteins in a soluble form by changing the C-terminus sequence [21]. On one hand, disulfide bonds have been reported to be involved in protein misfolding when overexpressed in *E. coli* [22] and lead to difficult refolding process for inclusion bodies. On the other hand, very hydrophobic polypeptides will sometime limit protein solubility and thus are likely to aggregate in the native state [12, 23].

Therefore, we constructed two Grx1562 mutants to improve the expression level of target protein according to the analysis of cysteines and hydrophobic nature using the DNASTAR software. About 80% of Grx1562M was successfully expressed in soluble form while Grx1562NC had the same expression pattern as Grx1562. Thus, we conclude that the cause for the formation of inclusion body of Grx1562 is not the formation of disulfide bond but rather is a property of the N-terminus. Some other proteins such as thioredoxin *f* [24] and neurotoxin [25] have been reported to prevent IBs formation and promote accumulation of protein in soluble state after deleting the N-terminus or C-terminus hydrophobic region of the proteins.

Glutathionylation of protein is always observed under oxidative stress. Significant deglutathionylation of enzymes inactivated by glutathionylation can be efficiently catalyzed by glutaredoxins [26]. The antioxidative properties of the cyanobacterial Grx and its mutants were also investigated using *E. coli* as a model. Overexpression of the Grx genes in *E. coli* lead to dramatically enhanced resistance of *E. coli* against H_2O_2 (Fig. 5b). It may be noted that *E. coli* strains still contained endogenous Grx genes in their chromosomes, but the normal level of Grx was not sufficient to protect the cells against H_2O_2 mediated toxicity. Grx1562 and Grx1562M were calculated to contribute about 60 and 83% to total Grx activity, suggesting that Grx1562M is more active than Grx1562 under the stress condition.

CD spectra and tryptophan fluorescence are widely used in the analysis of structural changes in a protein upon some perturbation, or in comparison of the structure of an engineered protein to the parent protein [18]. So, spectroscopic methods could be helpful to understand the different expression patterns. The CD spectrum of Grx1562M is similar to that of the full-length Grx1562, which indicates that the N-terminus amino acids have little effects on its secondary structure distribution. If substantial structural changes occurred as the result of mutations such that tryptophan environments were altered, a shift of wavelength at maximum fluorescence intensity (λ_{max}) would be expected [27]. Compared with Grx1562 a red shift of λ_{max} was found for Grx1562M, from 342 to 350 nm, and some increase in fluorescence intensity at the maximum emission occurred, revealing that the tryptophan environment is perturbed upon mutation. The causes of changing in the fluorescence of the mutant include the increasing average exposure of its tryptophans to the aqueous phase and the loosening structure. In addition, removal of the hydro-

phobic domain improved the substrate binding and catalytic efficiency at conserved sites and led to the slight increase in Grx activity of Grx1562M.

In conclusion, we have found that deletion of the hydrophobic N-terminus leads to protein production that can be recovered largely from the soluble *E. coli* fraction without activity loss, while the existence of disulfide bond has no effect on soluble expression of Grx1562. This opens the way to substitute Grx1562 for further structural and biochemical studies, including the preparation of variants by site-directed mutagenesis to identify the targets of this protein in cyanobacteria, and provides another strategy for improving soluble expression level of recombinant proteins in *E. coli*.

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