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Peptide ligands specific to the oxidized form of *Escherichia coli* thioredoxin

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ABSTRACT

Thioredoxin (Trx) is a highly conserved redox protein involved in several essential cellular processes. In this study, our goal was to isolate peptide ligands to *Escherichia coli* Trx that mimic protein–protein interactions, specifically the T7 polymerase-Trx interaction. To do this, we subjected Trx to affinity selection against a panel of linear and cysteine-constrained peptides using M13 phage display. A novel cyclized conserved peptide sequence, with a motif of C(D/N/S/T/G)D(S/T)-hydrophobic-C-X-hydrophobic-P, was isolated to Trx. These peptides bound specifically to the *E. coli* Trx when compared to the human and spirulina homologs. An alanine substitution of the active site cysteines (CGPC) resulted in a significant loss of peptide binding affinity to the Cys-32 mutant. The peptides were also characterized in the context of Trx's role as a processivity factor of the T7 DNA polymerase (gp5). As the interaction between gp5 and Trx normally takes place under reducing conditions, which might interfere with the conformation of the disulfide-bridged peptides, we made use of a 22 residue deletion mutant of gp5 in the thioredoxin binding domain (gp5Δ22) that bypassed the requirements of reducing conditions to interact with Trx. A competition study revealed that the peptide selectively inhibits the interaction of gp5Δ22 with Trx, under oxidizing conditions, with an IC50 of ~10 μM.

1. Introduction

Thioredoxin (Trx) is an evolutionarily conserved protein that is involved in a multitude of cellular processes including transcription regulation, cell division, energy transduction, several biosynthetic pathways, detoxification, and apoptosis [1]. Trx has a highly conserved active site motif, CXXC, which is found in all species. Trx typically participates in redox reactions through the reversible oxidation of its active site dithiol, to a disulfide, and catalyzes dithiol–disulfide exchange reactions [2]. In the cell, it is generally reduced by flavoenzyme Trx reductase using NADPH [3,4]. The biochemical functions of Trx can be described as 1) an electron carrier for the catalytic cycles of biosynthetic enzymes such as ribonucleotide reductases, methionine sulfoxide reductases, and sulfate reductases, and 2) to protect cytosolic proteins from aggregation or inactivation via oxidative formation of intra- or intermolecular disulfides [5].

Escherichia coli thioredoxin is a ~12 kDa single polypeptide chain containing 108 amino acids, and an evolutionarily conserved 3-D structure which has been determined to 1.68 Å resolution [1,2,6]. Acidic residues are commonly located in the N-terminal region of the molecule, whereas the C-terminus is mainly hydrophobic [2]. The structure of oxidized *E. coli* Trx contains a single twisted β-sheet composed of five strands flanked by four α-helical segments [6–8]. The active site, Cys-Gly-Pro-Cys (CGPC, residues 32–35), is located in a unique protrusion between the middle strand of the β-pleated sheet and an α-helix [2].

Thioredoxin has also been found to play a role in the propagation of bacteriophage M13, T4, and T7 in *E. coli* [9–11]. Reduced Trx is required for the assembly of filamentous phages f1 and M13 [4]. In the case of T4 phage, Trx is a substrate for host *E. coli* thioredoxin reductase and specific donor for T4 ribonucleotide reductase [4]. Phage T7 DNA polymerase (gp5, ~80 kDa) forms a 1:1 non-covalent complex (K_D=5 nM) with reduced Trx and is an essential subunit of the viral DNA polymerase. In fact, *E. coli* Trx is a T7 DNA polymerase processivity factor that allows gp5 to incorporate thousands of nucleotides per polymerization cycle, in contrast to the incorporation of only a few nucleotides when Trx is absent. Trx also increases the 3′–5′ double stranded DNA exonuclease activity of the polymerase, but does not affect the single-stranded DNA exonuclease activity [12]. The crystal structure of the T7 DNA replication complex (gp5/Trx) has been solved and reveals that Trx binds to gp5 in an extended loop, located between helices H and H1 in the thumb of T7 DNA polymerase [13]. This 71 amino acid loop is referred to as the Trx Binding Domain (TBD) and is not present in other type I polymerases. Upon binding to the TBD, Trx's active site cysteines are blocked [13]. While Cys-32 of Trx forms a hydrogen bond with Thr-327 of T7 DNA polymerase, studies have shown that mutating the active site cysteines still permits maximal DNA polymerization rates to be achieved; however, the affinity of active site mutants of Trx for T7 DNA polymerase is lowered

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[12]. Furthermore, Trx also increases the affinity of gp5 for both gp4 and gp2.5, both of which are essential for the coordination of DNA unwinding. In this complex the gp4 acts as a helicase/primase and gp2.5 is a single-stranded DNA (ssDNA) binding protein [14].

Phage display is a useful technology that can yield peptide ligands to proteins of interest [15,16]. One key feature of phage display is the physical connection between the displayed sequences and their genetic codes, which allows the rapid isolation and identification of peptide ligands. In this study, linear and cyclized libraries displayed on the surface of bacteriophage M13 [17] were screened by affinity selection to isolate peptides to *E. coli* Trx. Twenty-four peptides exhibited a highly conserved disulfide-constrained sequence, spaced by four residues, and no linear peptides were isolated. Such constrained peptides do not bind to mutants of Trx in which both active site cysteines were mutated to alanine. However, Cys-32 is required for the interaction between the Trx peptide ligands and *E. coli* Trx. We also discovered when using a mutant of gp5, which bypassed the requirement of the wild-type gp5 for reducing conditions to interact with Trx, the peptides (under oxidizing conditions) block the interaction of Trx with the mutant gp5, thus decreasing DNA polymerization activity. Biochemical experiments demonstrate that one synthetic peptide binds to Trx and competes for binding to gp5 with an IC50 of ~10 μM.

2. Materials and methods

2.1. Reagents

All plasmids were propagated and proteins were expressed in the *E. coli* strains XL1 Blue F′ Tet (Stratagene, La Jolla, CA) and BL21 (DE3) (Novagen, Madison, WI), respectively. Phage were propagated in *E. coli* strain SS320 [18]. The pET32a plasmid and pBirA plasmid were purchased from Novagen and Avidity (CO), respectively. Recombinant Trx from *E. coli*, human, and spirulina were purchased from Sigma Chemical Co. (Saint Louis, MO). Nickel-NTA agarose was purchased from Qiagen (Germantown, MD). Advantage cDNA polymerase, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and HRP-conjugated anti-M13 antibody were purchased from BD Biosciences-Clontech (Palo Alto, CA), Sigma, and Amersham Biosciences (Piscataway, NJ), respectively. Proteins were biotinylated and quantitated using the EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ Biotin Quantitation Kit from Pierce Chemical Co. (Rockford, IL). DNA sequencing and oligonucleotide synthesis service were performed by MWG Biotech (High Point, NC). Synthetic Trx peptides were synthesized, oxidized, and purified at the University of Chicago Peptide Core Facility (Chicago, IL).

2.2. Protein expression and purification

Expression of proteins in BL21(DE3) pBirA was achieved by growing one 1 L cultures in 2xYT (2xYT per liter: 16 g tryptone, 10 g yeast extract, and 5 g NaCl) with antibiotics, and inducing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) final concentration at an optical density at 600 nm wavelength (O.D. 600 nm) of 0.6. Proteins were expressed for 4 h at 37 °C and cells were harvested by centrifugation. Biotinylated proteins were cloned and expressed according to Scholle et al. [19]. Recombinant proteins were purified by immobilized metal affinity chromatography using Ni-NTA agarose. Purity and concentration was determined using an Agilent Bioanalyzer 2100 (Palo Alto, CA).

2.3. Affinity selection of phage

Combinatorial peptide libraries displayed as N-terminal fusions to the protein product of gene III of bacteriophage M13 were utilized for affinity selection experiments. The libraries consisted of X_{20}, Z_{10}, CX_4C, and CX_6C peptides, where X is encoded by NNK or NNT codons, Z is encoded by VNK or VVK codons, and C represents cysteine [17]. For the affinity selections, 5 μg of *in vivo* biotinylated Trx [18] was incubated with streptavidin-coated magnetic beads for 30 min, blocked with 1 μM biotin, and tumbled with 10^{12} phage (100 library equivalents) for 2 h at room temperature. Beads were washed three times with phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 1.5 mM KH_2PO_4) supplemented with 0.05% Tween 20 (PBST), magnetically separated, and bound phage eluted by adding 100 μL of 50 mM glycine (pH 2.0) for 5 min. The pH of the solutions containing released phage particles was neutralized by adding 10 μL of 1 M Tris–HCl (pH 8.0). Phage were added to 200 μL of SS320 (Tet^R) overnight culture and amplified overnight in 5 mL of 2xYT broth with tetracycline (final 5 μg/mL) at 37 °C with shaking. After two additional rounds of selection, the recovered phage was serially diluted and plated to obtain single plaques. Phage enzyme-linked immunosorbent assays (ELISA) were performed using a previously described protocol [19]. The DNA inserts of phage yielding positive ELISA signals were amplified by the polymerase chain reaction (PCR) using pIII (5′-TTTTTTTAGGAGATTTTCAACGTG-3′) and -96 (5′-CCCTCATAGTTAGCG-TAACG-3′) oligonucleotides. PCR products were purified using Qiagen PCR Purification Kit and sequenced.

2.4. Construction of Trx mutants

Trx mutants were constructed using pET32 as the DNA template for PCR mutagenesis of the Trx active site. Mutants were constructed using primers to introduce AGPC (5′-CGA TTT CTG GGC AGA GTG GGC TGG TCC GTG CAA AAT GAT CGC CCC GAT TCT G-3′), CGPA (5′-CGA TTT CTG GGC AGA GTG GTG CGG TCC GGC TAA AAT GAT CGC CCC GAT TCT G-3′), and AGPA (5′-CGA TTT CTG GGC AGA GTG GGC TGG TCC GGC TAA AAT GAT CGC CCC GAT TCT G-3′) mutations in the active site. A common reverse primer (5′-CCA CTC TGC CCA GAA ATC G-3′), and two flanking primers with ligation independent compatible overhangs (5′-TAC TTC CAA TCC AAT GGC AGC GAT AAA ATT ATT CAC CTG AC-3′ and 5′-TCC ACT TCC AAT GGA GCC AGA ACC AGA ACC GGC-3′) were used for pull-thru PCR and cloning into pMCSG16 according to Scholle et al. [19].

2.5. Thioredoxin activity assay

The activity of Trx and inhibition in the presence of peptides were measured as described by Holmgren and Björnstedt [20]. The following peptides were added to Trx at 0, 1, 10 and 50 μM concentrations to test inhibition: Trx peptide ligand TP-2 (NH_2-SRNVNCNASVCTIPDRLITDNPGSGS-$CONH_2$), APC1300 [19], and Src SH3 ligand (NH_2-SSRPLPPLPGS-$CONH_2$). Peptides were incubated with Trx for 1 h before testing activity.

2.6. Trx peptide ligand-gp5 binding and competition assay

A total of 1 μg of Trx (Sigma) was immobilized by hydrophobic adsorption onto high-binding polystyrene plates (Costar; Corning, NY) in PBS overnight at 4 °C. Wells were blocked for 1 h at room temperature using 1% bovine serum albumin (BSA) in PBS. Wells were washed three times with PBST. Binding assays used biotinylated TP-2 peptide added to wells in concentrations ranging from 5.7 nM to 22.4 μM. The competition assay used a total of 2.0 μM of biotinylated TP-2 peptide ligand in PBST was added to each well containing a dilution of gp5, lysozyme, and BSA and incubated at room temperature for 2 h. Wells were washed with PBST, streptavidin alkaline phosphatase (1:5000 dilution in PBST) was added, and incubated for 30 min at room temperature. Wells were washed thoroughly with PBST and alkaline phosphatase activity monitored using *p*-nitrophenyl phosphate and read spectrophotometrically at 405 nm.

2.7. Mutagenesis of T7 gene 5 and protein expression and purification

A mutant of T7 gene 5 (gp5) was constructed in which 66 nucleotides in the sequence encoding the Trx-binding domain were deleted. The amino acid residues deleted were 297–318 (gp5Δ22). Mutagenesis was carried out using PCR to obtain the desired deletion in the gene 5 plasmid, pGP5-3 [21]; the identity of the clone was confirmed by DNA sequencing. The gp5 and gp5Δ22 proteins were overproduced in a *E. coli* strain A307 (DE3) that lacks Trx [12], and then purified as previously described [21]. Trx was purified as described by Tabor et al. [21].

2.8. DNA polymerase activity assay

The T7 DNA polymerase activity assay was performed using M13 mGP1-2 ssDNA to which the primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) was annealed, in a reaction mixture containing 40 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 0.2 mg/mL BSA, 500 μM each of dATP, dCTP, dGTP, dTTP, and [α-^{3}H] dTTP (3 cpm/pmol), 16 nM primed-M13, and the indicated concentrations of Trx, gp5 or gp5Δ22 in the Results section. The wild-type gp5 or gp5Δ22 was incubated with Trx on ice for 10 min and the remaining components were added and reactions initiated once transferred to 30 or 37 °C. The reactions were stopped after 10 min when carried at 30 °C and 4 min when carried at 37 °C with EDTA to a final concentration of 150 mM. Aliquots were placed on DE81 filter paper, washed with 0.3 M ammonium formate (pH 8.0), and then the amount of radioactively labeled synthesized DNA bound to the DE81 filter was measured with scintillation counter. In the competition experiments, peptides are serially diluted in PBS and the total amount of the peptide buffer was held constant in all samples.

3. Results

3.1. Trx peptide ligands isolated by phage display

Phage-displayed combinatorial peptide libraries [17] were affinity selected according to Materials and methods, using *E. coli* Trx that was *in vivo* biotinylated at the N-terminus as an AviTag fusion [18]. The phage libraries used in these selection experiments display peptides at the N-terminus of the minor coat protein 3 (pIII) of bacteriophage M13. After three rounds of affinity selection, 96 phage clones were screened using a phage enzyme-linked immunosorbent assay (ELISA). A total of 24 phage clones bound specifically to *E. coli* Trx in which three sequences were represented twice, one sequence three times, and 15 unique sequences (Fig. 1A). A comparison of the peptide sequences revealed a disulfide-constrained motif in which the cysteines are spaced by four residues (Fig. 1A). (One exception, TP-19, was shown to have two cysteines spaced by six residues.) We tested the requirement for this cysteine spacing in the peptide by running phage ELISAs with clones from two fixed cysteine peptide libraries (CX_4C and CX_6C [17]); however, no clones exhibited binding to Trx (data not shown). This suggests that more than a disulfide bond spaced by four residues is required for the peptide to interact with Trx.

A LOGO plot of the selected Trx peptide ligands is shown Fig. 1B. The plot reveals that positions 9 and 10 (after Cys-1) are commonly acidic, whereas positions 12 and 15 have a strong preference for hydrophobic residues. Such a motif suggests that the interaction with Trx is possibly both charged and hydrophobic. Additionally, a proline residue is prevalent at position 16 or 17 in 71% of the isolated peptides. The occurrence of the proline suggests that a turn in the peptide's secondary structure assists in the presentation on phage or binding.

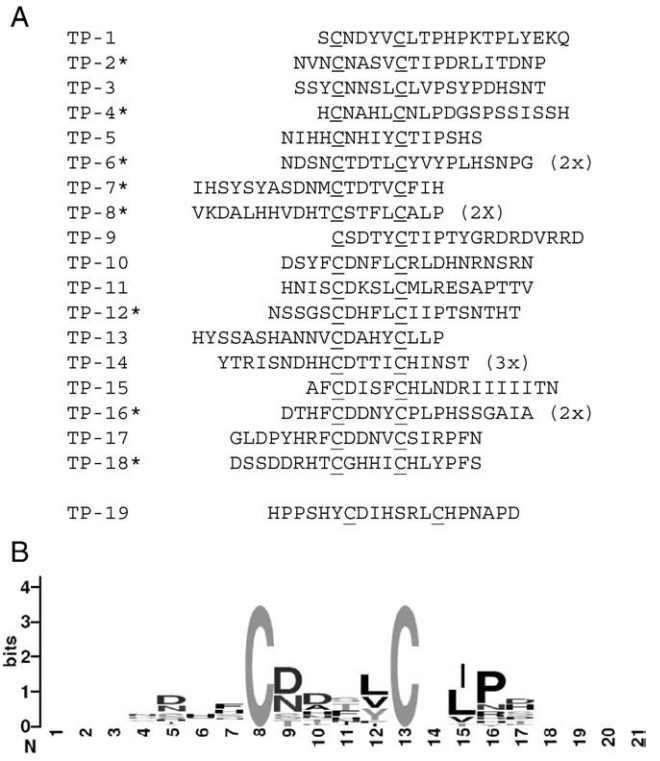

Fig. 1. *E. coli* thioredoxin (Trx) peptide ligands. (A) An alignment of 24 disulfide-constrained peptides that bind to Trx. All peptides were represented once except those noted in parenthesis. Cysteine residues are underlined and spaced by four residues with one exception with a six-residue spacing. (B) LOGO plot of 23 Trx peptide ligands (minus TP-19) showing conserved residues. The peptides were aligned at the conserved cysteine residues. The font size (in bits) is proportional to the occurrence of the residues at each position.

3.2. The Trx peptide ligands bind selectively to E. coli Trx

Eight of the phage-displayed peptides were chosen based on the residue that follows the N-terminal cysteine (CN, CT, CS, CD, CG). These peptides were subjected to a phage ELISA (Fig. 2A) to test binding specificity. Peptide binding activity was assayed on immobilized *E. coli*, human, and spirulina (blue-green algae) Trx proteins that retained redox activity (data not shown). Binding was specific to the *E. coli* Trx, with no cross-reactivity to the other species. Similarly, the Trx peptide ligands did not bind to unrelated proteins including: human Src SH3 domain, streptavidin, and BSA immobilized onto polystyrene plates. While molecular weights of the three Trx proteins are very similar (i.e., 12 kDa for *E. coli* and spirulina, and 13 kDa for human), the sequence identity of human and *E. coli* Trx is only 38%, with identical active sites (CGPC) between the two proteins. Human Trx also differs by three additional cysteines, of which Cys-73 has been shown to assist in the dimerization of human Trx1 [22]. The spirulina Trx sample is non-recombinant, with a purity of ~70%. (The sequence identity of spirulina Trx could not be analyzed, since the primary structure is unknown.) Overall, the Trx-selected peptides bind in a species-specific manner to *E. coli*, with similar affinity when compared to the ~5 μM K_D interaction of Src SH3 and its peptide ligand [23].

3.3. Trx peptide ligands binding to Trx active site mutants

Three Trx active site mutants were constructed by PCR, expressed, and purified according to Materials and methods. The active site cysteines at positions 32 and 35 were replaced with alanine to yield CGPA, AGPC, and AGPA sequences. The biotinylated proteins were immobilized onto streptavidin-coated polystyrene plates and subjected to a phage ELISA using the eight phage-displayed Trx peptide

ligands (Fig. 2B). These peptides exhibit approximately 20–50% lower binding affinity to the CGPA (C35A) mutant, when compared to wild-type (Fig. 2B). The CGPA mutant replaces the buried cysteine residue at position 35 with alanine, which appears to have a modest effect on the peptide-Trx interaction. The AGPC (C32A) and AGPA (C32A; C35A) mutants both have the solvent-exposed cysteine at position 32 mutated to alanine. These mutants exhibit very low binding for the Trx peptide ligands, suggesting that the peptide binding site to be in close proximity to, or involving, the Cys-32 residue. Furthermore, the alanine substitutions of the active site cysteines, which mimic the reduced state of Trx, may cause a conformational shift in the structure of Trx [24]. We hypothesize that this shift may cause residues within the peptide binding site to also shift resulting in a reduction of peptide affinity.

3.4. Inhibition of the Trx peptide ligand-thioredoxin interaction by gp5

Thioredoxin's role in protein–protein interactions [1] led us to hypothesize that the Trx peptides might mimic the way cellular proteins interact with Trx and thus should inhibit interactions with binding partners. However, upon careful analysis of the Trx peptide ligand sequences, we found no match with the sequences of interacting partners reported by Kumar et al. [1]. Nevertheless, we decided to focus on one model Trx-protein interaction and examine the ability of the Trx ligands to perturb it. Given the number of studies on the interaction of Trx and T7 DNA polymerase (gp5) [9,12–14,21,25,26], we tested the ability of gp5 to compete for binding to Trx in the presence of one Trx peptide ligand. A serial dilution of synthetic biotinylated peptide (TP-2) was made and binding detected using streptavidin conjugated to alkaline phosphatase (SA-AP). The 50% binding concentration of the peptide was 2.0 μM, which is an estimate of the K_D (Fig. 3A). Next, the peptide concentration was held constant at 2.0 μM (Trx at 1 μg per well) and varying concentrations of BSA, lysozyme, and gp5 were mixed in solution (Fig. 3B). The addition of higher concentrations of BSA or lysozyme had little effect on the binding of TP-2 to Trx. Conversely, a concentration of ~1 μM gp5 protein competed for nearly 90% of the Trx peptide binding. This result reveals that the TP-2 peptide indeed competes for binding to Trx at the same surface as the gp5 protein. It is noteworthy to mention that BSA, lysozyme, and gp5 proteins were stored in 1 mM DTT. Therefore, residual DTT in the competition may be sufficient to allow gp5-Trx interaction and yet maintain the binding properties of the oxidized TP-2 peptide. We also determined that the peptide binding is reduced by >50% in the presence of

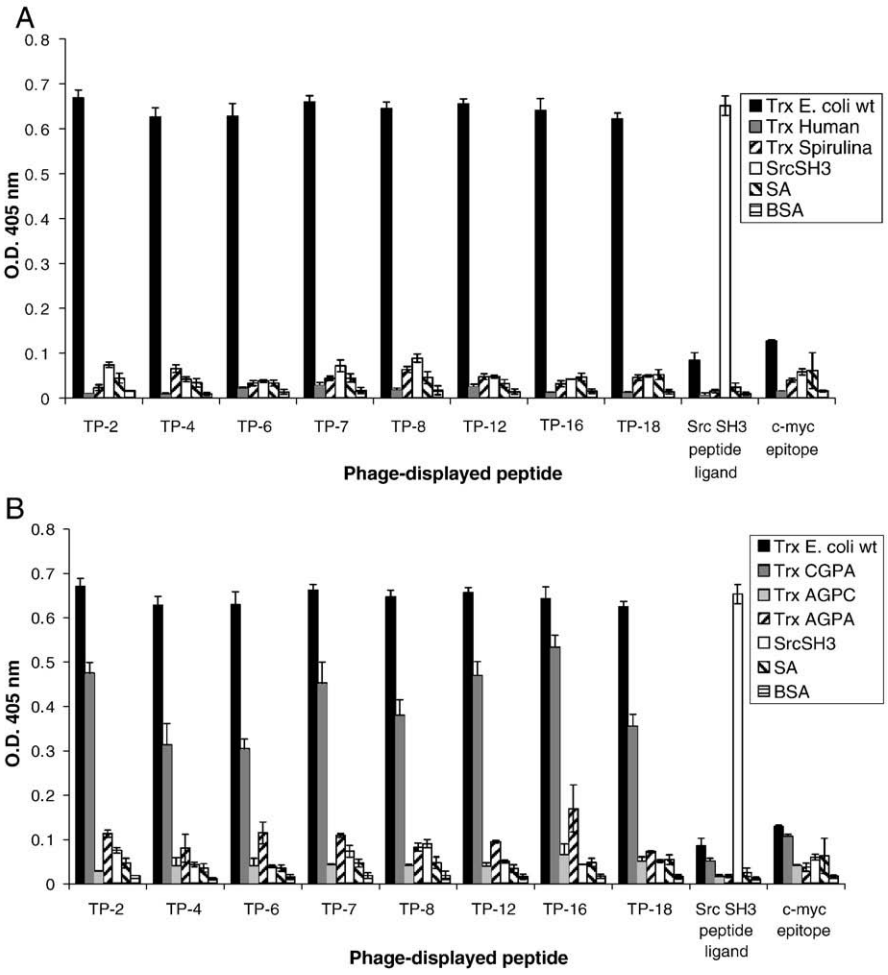

Fig. 2. Trx peptide ligand specificity. (A) Eight phage-displayed Trx peptide ligands were subjected to a phage enzyme-linked immunosorbent assay (ELISA). Biotinylated target proteins were immobilized on high-binding polystyrene plates by hydrophobic adsorption. Phage binding was detected with anti-M13 antibody horseradish peroxidase (HRP) conjugate and binding activity is measured at O.D. 405 nm. Target proteins include wild-type *E. coli* Trx, human Trx, spirulina Trx, human Src SH3 domain, streptavidin, and BSA. Trx peptide ligands bind specifically to *E. coli* Trx, whereas the Src SH3 peptide ligand binds to Src SH3 domain as a positive control. Binding activity of the peptides was measured in triplicate wells. (B) Eight phage-displayed Trx peptide ligands were subjected to a phage enzyme-linked immunosorbent assay. Target proteins were immobilized on streptavidin-coated Costar high-binding polystyrene plates. Phage binding was detected with anti-M13 antibody-HRP conjugate and binding activity is measured at O.D. 405 nm. Target proteins include wild-type *E. coli* Trx and Trx active site mutants CGPA, AGPC, and AGPA. The human Src SH3 domain was used as a positive control for binding to Src SH3 peptide ligand display on phage. Streptavidin and bovine serum albumin were used as negative binding controls. Binding activity of the Trx peptide ligands was measured in triplicate wells.

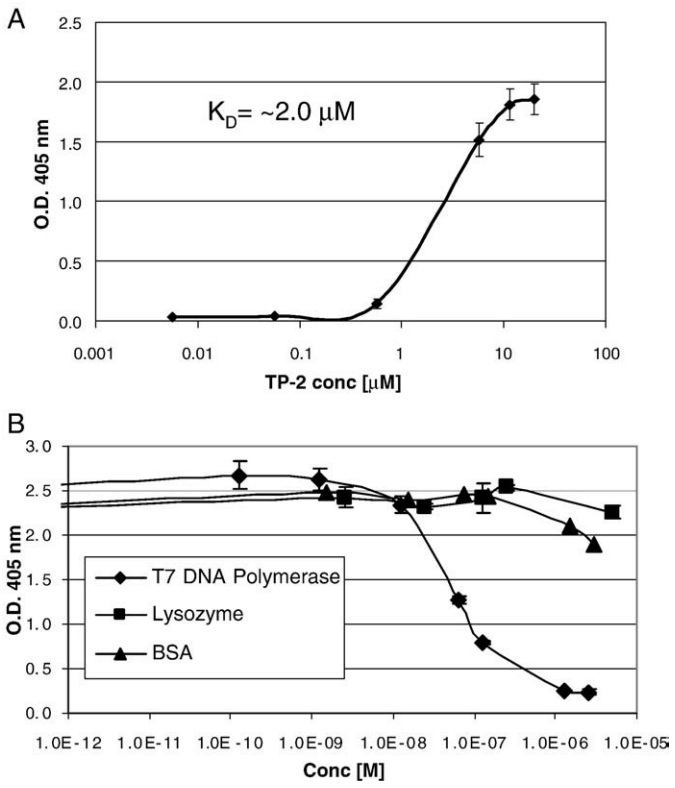

Fig. 3. Binding affinity measurement and inhibition of the Trx peptide ligand-Trx interaction. (A) Biotinylated TP-2 peptide (Biotin-SRNVNCNASVCTIPDRLITDNPGSGS-$CONH_2$) was subjected a dilution binding assay. Concentrations of the synthetic peptide are 0.0057, 0.057, 0.57, 5.7, 11.4, 22.8 μM. Binding of Trx peptide ligand to Trx was monitored by streptavidin alkaline phosphatase and *p*-nitrophenyl phosphate. Signal was measured at O.D. 405 nm. The data presented are an average of three experiments. (B) Biotinylated TP-2 peptide was utilized in a competition assay in the presence of varying molar concentrations of gp5, lysozyme, and BSA. The TP-2 peptide concentration was 2.0 μM. *E. coli* Trx (non-biotinylated) was immobilized onto polystyrene plates by hydrophobic adsorption and blocked with BSA. Binding of Trx peptide ligand to Trx was monitored using streptavidin alkaline phosphatase as in A. The data presented are an average of three experiments.

10 mM DTT (data not shown), which likely reduces the disulfide bond in the peptides.

3.5. Effect of Trx peptide ligands on activity of gp5-Trx complex

The interaction of gp5 and Trx is a high affinity interaction and requires the active site Cys residues of Trx to be in their reduced form, as oxidized Trx does not bind to the polymerase [12]. This interaction stimulates the polymerase activity of gp5 and increases its processivity from few nucleotides to thousands in a single DNA binding event [21,26]. The observed binding constant, K_D, of the interaction between Trx and gp5, when measured by the stimulation of different concentrations of Trx to the polymerase activity of gp5 on primed single-stranded (ss) M13, is calculated to be 3.7 nM (Fig. 4). To study the interaction between the TP-2 and Trx, we carried out a competition experiment by measuring the ability of Trx to stimulate the polymerase activity of gp5 on primed-ssM13 in the presence of different concentrations of TP-2. If the peptide interacts with Trx, a decrease in the stimulation of the polymerase activity of gp5 by the presence of Trx will be observed. The peptide failed to inhibit the interaction of Trx and gp5, even under conditions where Trx was limiting and peptide is in excess (data not shown). This could be a result of either the inability of the peptide to compete the nM range binding affinity between Trx and gp5 and/or the dependence of the interaction of the peptide and Trx on the redox state of the cysteine residues in the peptide and Trx. In order to

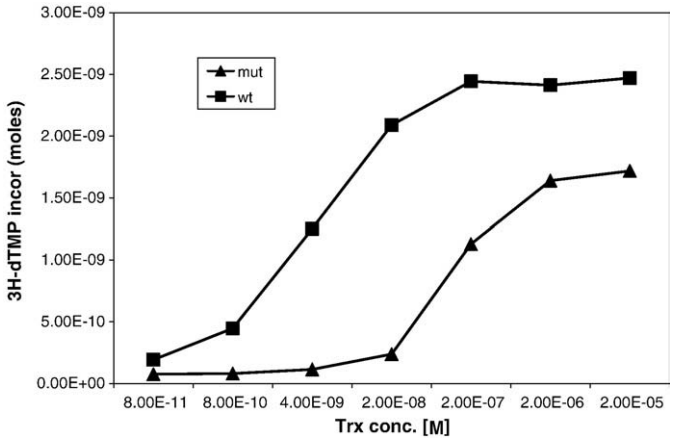

Fig. 4. Observed binding constants (K_D) for the interaction of Trx with wt-gp5 and gp5Δ22. In these experiments, the concentrations of Trx used are 20,000, 2000, 200, 20, 4, 0.8, and 0.08 nM, and the concentration of gp5 and gp5-Δ22 is 400 nM. Samples were incubated in the presence of DTT at 30 °C for 10 min and monitored as described in Materials and methods. K_D is determined using non-linear curve fit of one binding site (Michaelis–Menton equation), provided by the software package, Origin.

address these two possibilities, we used a mutant of gp5 that lacks 22 residues in the TBD (gp5Δ22) [14]. The polymerase activity of this mutant is stimulated by Trx, but binds nearly 30-fold weaker than the wild-type gp5 (wt-gp5) (K_D = 108 nM) (Fig. 4). In addition, unlike the wt-gp5, the stimulation of the polymerase activity of gp5Δ22 by Trx is not fully dependent on the presence of DTT (Fig. 5), and therefore allowing the competition experiment to be conducted under oxidized conditions. In the presence of DTT, the peptide was not able to compete with gp5Δ22, even under conditions where the peptide is 40-fold in excess of Trx (Fig. 5). This finding indicates that the peptide indeed binds to Trx with very low affinity. However, it is possible that the interaction with Trx requires the cysteine residues in Trx and/or the TP-2 to be in their oxidized form. In order to test this, we conducted the competition experiment under oxidizing conditions. Interestingly, in the absence of DTT, the TP-2 competes with the binding of Trx to gp5Δ22 leading with an observed inhibition binding constant IC50 of ~10 μM (Fig. 5). The inhibition by the TP-2 is specific as shown by the ability of a homologous peptide to inhibit similarly (TP-7: NH_2-SRIHSYSYASDNMCTDTVCFIHGSGS-

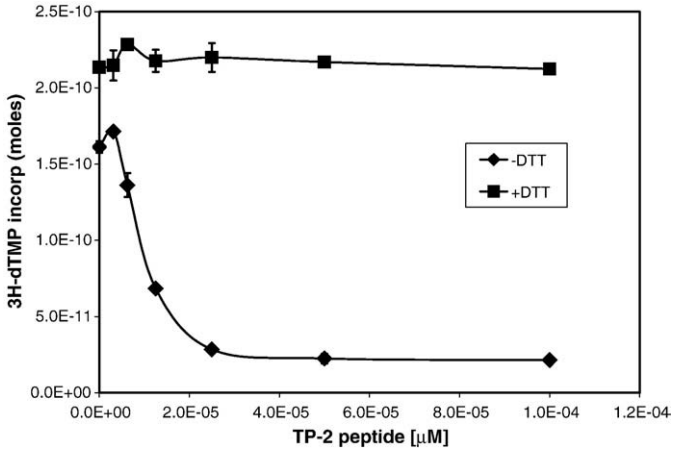

Fig. 5. Trx peptide ligand inhibits the gp5Δ22 interaction with Trx in the presence of DTT. Here concentrations of TP-2 peptide used are 0, 3.1, 6.2, 12.5, 25, 50, and 100 μM. Concentration of Trx is 2.5 μM (saturating condition) and gp5Δ22 is 50 nM. Reaction was done in the presence and absence of DTT at 37 °C for 4 min and monitored as described in Materials and methods. The observed IC50 was estimated as the point where the polymerase activity is half of that in the absence of peptide.

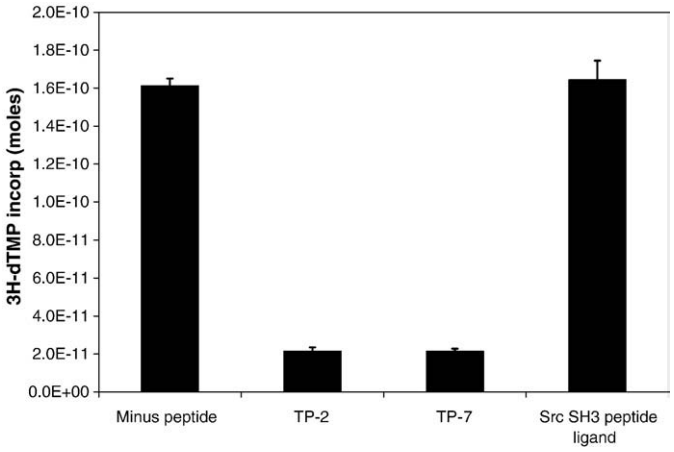

Fig. 6. Inhibition of the Trx-gp5 interaction is specific for the Trx peptide ligand. The concentration of peptide used is 100 μM. Concentration of Trx is 2.5 μM (saturating condition) and gp5Δ22 is 50 nM. The reaction was done in the absence of DTT, at 37 °C for 4 min, and monitored as described in Materials and methods.

$CONH_2$), whereas the SrcSH3 peptide ligand (NH_2-SSRPLPPLPGS-$CONH_2$) does not (Fig. 6).

4. Discussion

In the current study, our goal was to isolate peptides that mimic protein–protein interactions of *E. coli* thioredoxin (Trx). We isolated 19 unique peptide sequences using M13 phage display that specifically bind to Trx. The peptide sequence contains two cysteine residues spaced by four amino acids resulting in a consensus sequence of C(D/N/S/T/G)D(S/T)-hydrophobic-C-X-hydrophobic-P. Since the M13 phage coat proteins are translocated to the oxidizing environment of the *E. coli* periplasm, the displayed cysteine-containing peptides are in an oxidized state. The M13 displayed peptides thus form intramolecular disulfide-constrained loops. These constrained loops often reduce the free energy (ΔG) of binding when compared to linear (non-cysteine) peptide interactions. Furthermore, the M13 minor coat protein III also has three disulfide bonds, which when interfered by additional free cysteines greatly lower infectivity. The phage displaying the Trx peptide ligands did not exhibit a significant difference in infectivity (data not shown). Overall, it is concluded that the peptides are in an oxidized state and linear peptides cannot be isolated to either oxidized or reduced Trx.

The redox state of the two cysteine residues of the peptide and/or the two active site cysteine residues in Trx is an important factor for their interaction. When the active site cysteine residues in Trx are mutated, the peptide exhibits a reduced affinity to Trx (~50%). Additionally, the peptide and Trx interact with much higher affinity when the binding is carried out under oxidized conditions in comparison with reduced conditions (data not shown). This may be due to linearization of the peptide or a shift in conformation of the Trx in its reduced form. The binding of Trx peptide ligands to the CGPA Trx mutant (with the buried Cys-35 replaced by an alanine) reveals that Cys-32 is required for binding to Trx. This mutation may present Trx in a conformation that is amenable to binding or that an intermolecular disulfide is formed between the peptide ligands and Trx. Mass spectrometry analysis revealed that the peptides did not form an intermolecular disulfide bond, given the absence of a shift in mass when Trx and peptide were mixed (data not shown).

The Trx peptide ligands isolated from phage selections were subjected to a cross-reactivity assay with human and spirulina Trx. The human Trx sequence is known and exhibits a 38% identity when compared to *E. coli* Trx with a conserved active site between the two species (CGPC). The sequence of the spirulina Trx is currently unknown and cannot be compared. The phage ELISA revealed no cross-reactivity between *E. coli* and human or spirulina Trx. This suggests that the active site, which includes the cysteine residues, is not sufficient for binding to the Trx peptide ligands.

We utilized the well-characterized binding of gp5 to Trx to study the ability of this peptide to compete with this interaction. The crystal structure of the gp5-Trx complex shows that the active site Cys residues of Trx are buried within the gp5-binding interface. Previously described biochemical data clearly indicates that the interaction of gp5 and Trx requires a reduced environment [12]. However, under reduced conditions, the Trx peptide ligand was not able to compete for the high affinity interaction of Trx with gp5 (K_D ~ 4 nM). This indicates that the Cys residues in either the peptide and/or Trx are needed to be in their oxidized form for optimum binding. This is also in agreement with the finding that under reduced conditions, where the peptide interacts with Trx with a lower affinity when compared with its interaction under oxidized conditions. Therefore, we used a mutant of gp5 in which 22 residues within the TBD are deleted (gp5Δ22) that weakens the binding affinity between Trx and gp5 and allows the peptide to better compete with this interaction. The binding affinity of gp5Δ22 is 30-fold less than that of the wt-gp5. Interestingly, this mutant does not require DTT for the interaction with Trx, which provided an experimental condition to perform the competition study under reducing and oxidizing conditions. When carefully examining the sequence of the deleted portion from the TBD, we found that it contains one Cys residue (Cys 313). In fact, recent work has demonstrated that Cys 313 is required to prevent the aggregation of gp5 (Johnson, D.J. and Lee, S.J., unpublished data). This raises the possibility that the reduced condition required for the interaction of Trx with gp5 is more of a requirement for gp5 rather than for Trx. Interestingly, the peptide competes with the interaction of Trx and gp5Δ22 only under oxidized conditions with an observed IC50 of ~10 μM. The requirement of the peptide to be in its oxidized form may suggest that the Cys residues stabilize the peptide loop and are important for the peptide-Trx interaction.

In summary, novel *E. coli* thioredoxin peptide ligands were isolated by phage display with a conserved disulfide motif. These peptides exhibit the best binding to the wild-type Trx (CGPC) in which the K_D was measured to be approximately 2 μM. Peptide binding affinity was significantly reduced or no binding observed for the Trx active site mutants (CGPA, AGPC, and AGPA). The isolated ligands were synthesized as purified peptides and shown to block the thioredoxin-gp5 interaction with an IC50 of ~10 μM. Interestingly, the peptide sequences do not match other cellular interacting proteins, which is likely due to the disulfide bond that is not expected to occur in a cytosolic protein.

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