Promoter Recognition by a Complex of Spx and the C-Terminal Domain of the RNA Polymerase α Subunit

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Abstract

Background: Spx, an ArsC (arsenate reductase) family member, is a global transcriptional regulator of the microbial stress response and is highly conserved amongst Gram-positive bacteria. *Bacillus subtilis* Spx protein exerts positive and negative control of transcription through its interaction with the C-terminal domain of the RNA polymerase (RNAP) α subunit (αCTD). Spx activates *trxA* (thioredoxin) and *trxB* (thioredoxin reductase) in response to thiol stress, and bears an N-terminal C10XXC13 redox disulfide center that is oxidized in active Spx.

Methodology/Principal Findings: The structure of mutant Spx<sup>C10S</sup> showed a change in the conformation of helix α4. Amino acid substitutions R60E and K62E within and adjacent to helix α4 conferred defects in Spx-activated transcription but not Spx-dependent repression. Electrophoretic mobility-shift assays showed αCTD interaction with *trxB* promoter DNA, but addition of Spx generated a supershifted complex that was disrupted in the presence of reductant (DTT). Interaction of αCTD/Spx complex with promoter DNA required the cis-acting elements -45AGCA-42 and -34AGCG-31 of the *trxB* promoter. The Spx<sup>G52R</sup> mutant, defective in αCTD binding, did not interact with the αCTD-trxB complex. Spx<sup>R60E</sup> not only failed to complex with αCTD-trxB, but also disrupted αCTD-trxB DNA interaction.

Conclusions/Significance: The results show that Spx and αCTD form a complex that recognizes the promoter DNA of an Spx-controlled gene. A conformational change during oxidation of Spx to the disulfide form likely alters the structure of Spx α helix α4, which contains residues that function in transcriptional activation and αCTD/Spx-promoter interaction. The results suggest that one of these residues, R60 of the α4 region of oxidized Spx, functions in αCTD/Spx-promoter contact but not in αCTD interaction.

Citation: Nakano MM, Lin A, Zuber CS, Newberry KJ, Brennan RG, et al. (2010) Promoter Recognition by a Complex of Spx and the C-Terminal Domain of the RNA Polymerase α Subunit. PLoS ONE 5(1): e8664. doi:10.1371/journal.pone.0008664

Editor: Malcolm James Horsburgh, University of Liverpool, United Kingdom

Received November 13, 2009; Accepted December 19, 2009; Published January 13, 2010

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Funding: Research was supported by grants G-0040 from the Welch Foundation (to RGB) and GM045898 from the National Institutes of Health (to PJZ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Spx is a member of the ArsC (arsenate reductase) family of proteins and is a unique transcriptional regulator of Gram-positive bacteria [1]. In *Bacillus subtilis*, its primary function is in the global control of the thiol stress response [2]. A role for Spx in pathogenesis has been inferred from studies of *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus anthracis*, and *Streptococcus mutans*, in which it is produced upon host cell infection and serves to control the expression of virulence determinants [3,4,5,6,7]. *B. subtilis* Spx interacts with the C-terminal domain of the α subunit (αCTD) of RNA polymerase (RNAP), and in doing so, exerts both positive and negative control of gene transcription [2,8]. Genetic and biochemical studies showed that the G52 residue of Spx and the Y263 residue of αCTD form part of the contact interface between Spx and RNAP, which was confirmed by the x-ray structure determination of the αCTD/Spx complex [9]. The Y263 is conserved in Gram-positive bacteria and is not found in the Gram-negatives [1].

In vitro transcription experiments showed that Spx directly activates transcription of genes involved in thiol homeostasis, including *trxA* (thioredoxin gene) and *trxB* (thioredoxin reductase) [10,11]. Spx-dependent transcriptional activation is under redox control, requiring a CXXC disulfide center at the protein’s N-terminus [10]. The oxidized form of Spx activates transcription of genes under its control.

Upon oxidative stress, Spx concentrations increase due to enhanced transcription and decreased proteolysis. Increased *spx* transcription is attributed, in part, to inactivation of the PerR and YodR repressors that negatively control *spx* transcription during non-stress conditions [12,13]. Spx is also under proteolytic control requiring the ATP-dependent ClpXP protease [8,14], but Spx is able to escape from the proteolysis under oxidative conditions because both the ClpX ATPase subunit and YjbH, an Spx-specific adaptor protein involved in ClpXP proteolysis of Spx, are inactivated by oxidants [15,16,17]. YjbH, a Zn-binding protein, interacts with Spx and the interaction accelerates the proteolysis of Spx by ClpXP [15]. Oxidative stress releases Zn from YjbH and ClpX, which is thought to result in loss of YjbH-mediated proteolysis.

The tight transcriptional and post-translational control of intracellular Spx is logical from a physiological viewpoint, given that the Spx-dependent regulation is exerted over a genome-wide scale. When Spx levels increase, as a result of either a null
affinity column chromatography as previously described [9]. Purified, cleaved Spx was dialyzed into 25 mM PIPES, pH 6.5, 100 mM KCl for use in subsequent crystallization trials.

The CTD containing residues 245 to 314 of RpoA was cloned into the IPTG-inducible PET28a overexpression vector (Invitrogen), which contains an N-terminal histidine tag and thrombin cleavage site. The CTD-PET28a vector was transformed into Rosetta 2 competent cells (EMD Biosciences) for overexpression. The CTD was purified by Ni-NTA affinity chromatography (Qiagen). Following purification, the histidine tag was cleaved by incubation with thrombin protease (GE Healthcare). Purified, cleaved CTD was dialyzed into 25 mM Pipes, pH 6.5, 100 mM KCl. The Spx-CTD complex was formed by mixing equimolar amounts of purified C10S Spx and CTD. The complex was concentrated to 10 mg/ml.

Crystals of the reduced C10S Spx-CTD complex were grown by the hanging-drop vapor diffusion method. Two microliters of 0.5 mM C10S Spx-CTD was mixed with 2 μl of reservoir solution containing 25–30% polyethylene glycol 4000, 0.1 M sodium citrate pH 5.3 and 0.1 M MgCl₂. Crystals appeared within two to three days and reached maximum dimensions of 0.3 × 0.05 × 0.05 mm² in approximately one week. The crystals were cryoprotected in the mother liquor plus the addition of 15% glycerol and flash frozen in a nitrogen stream at −180 °C. X-ray intensity data were collected on beamline 0.3.1 at the Advanced Light Source and data were processed using MOSFLM [22] as implemented in the CCP4 suite of programs (CCP4, 2004) (Table 1). The crystals took the space group P1 with cell dimensions a = 29.4 Å, b = 32.2 Å, c = 50.6 Å, α = 106.1°, β = 91.6°, γ = 103.8°.

The structure of the C10S Spx-CTD complex was solved by molecular replacement using the oxidized Spx-CTD structure as the search model (Table 1). Manual fitting and adjustment of the

| Table 1. Selected Crystallographic Data and Statistics. |
|-----------------------------------------------|
| SpxC10S-CTD |
|-----------------------------------------------|
| **Data Collection** |
| Wavelength (Å) | 0.0000 |
| Resolution (Å) | 48.3–1.9 |
| No. Observed Reflections | 174,032 |
| No. Unique Reflections | 12,912 |
| Completeness (%) (last shell) | 95.1 (94.9) |
| l/σl (last shell) | 7.7 (2.9) |
| Rsym (%) (last shell) | 5.9 (23.0) |
| **Refinement** |
| Resolution (Å) | 48.3–1.9 |
| Reflections (working set/test set) | 12,250/662 |
| Protein Atoms (≠) | 1,465 |
| Solvent Molecules (≠) | 40 |
| Rwork/Rfree (%)b | 23.6/27.8 |
| RMSD bond lengths (Å) | 0.006 |
| RMSD bond angles (°) | 1.27 |
| Average B-factor (Å²) | 38.5 |

* |fobs| = \sum_\{Ihkl-Ihkl(0)\} ||Fcalc||/||Fobs||; where all Ihkl(0) is the observed intensity and Ihkl is the final average value of intensity.  
| Rwork | = \sum_\{Fcalc\} - \sum_\{Fobs\}\sum_\{Fobs\}; and | Rfree | = \sum_\{Fcalc\} - \sum_\{Fobs\}\sum_\{Fobs\}; where all reflections belong to a test set of 5% randomly selected data.  

doi:10.1371/journal.pone.0008664.1001

**Materials and Methods**

**Crystalization and Structural Analysis of Reduced Spx**

“Reduced” Spx, in which residue Cys10 was replaced by serine (C10S Spx), was overexpressed in E. coli and purified by Ni²⁺-NTA affinity column chromatography as previously described [9]. Purified, cleaved Spx was dialyzed into 25 mM PIPES, pH 6.5, 100 mM KCl for use in subsequent crystallization trials.

vitamin in ctpX or ctpP, or in response to oxidative stress, transcription of a large set of genes is reduced [2–6]. Most, if not all, of these genes are positively controlled by transcription activators. For example, ComA-dependent sfA transcription is repressed when Spx is overproduced. ComA binds to the regulatory region of sfA and recruits RNAP to the promoter region through the interaction of ComA and CTD [10,19,20]. ComA-dependent sfA transcription requires residues C265 and K267 located in the z1 helix of CTD [20]. Y263 is the residue of CTD that interacts with Spx, so it is also located in the z1 helix, indicating that Spx likely competes for binding of CTD with ComA [9,20]. The adverse effect of Spx on ResD-dependent ComA-dependent srfA transcription with RNAP, directly binds to a sequence within an Spx-activated promoter DNA. A previous study showed that oxidized Spx alone or Spx, when complexed with RNAP, and Spx [11]. Contact of Spx with the CTD, directs RNAP to Spx-activated promoters, either by altering the way in which RNAP engages the promoter, or by forming part of the binding surface which specifically recognizes Spx-activated promoter sequences. In the hope of obtaining direct evidence of a DNA-protein interaction in the transcription initiation complex at the trxB and trxA promoters, we carried out site-specific DNA and protein crosslinking using trxA and trxB promoter DNA, RNAP, and Spx [11]. Contact of Spx with promoter DNA was not detected by crosslinking at any of the nucleotide positions examined. The addition of Spx resulted in enhanced contact with the -10 region of the trxB and trxA promoters at 37°C. Similarly, Spx stimulated contact of the ββ’ subunits of RNAP with nucleotide base positions near the transcription start sites and around 21/-22 in both promoters. This study also uncovered evidence of a cis-acting element upstream of the core promoter sequences in Spx-controlled genes that is required for Spx-activated transcription [11].

In this work, we identify the cis-sequence in the trxB promoter that is essential for Spx-dependent transcriptional activation. As previously shown, Spx alone is unable to bind to the trxB promoter, but Spx is capable of generating a supershifted trxB-CTD-Spx complex in an electrophoretic mobility shift assay (EMSA). In parallel, structural studies identify a change in the conformation of a helix 4 of Spx when residue C10 of the redox disulfide center at its N-terminus is mutated to serine. When R60, a residue associated with the helix 4 region, is mutated to glutamate, Spx-dependent transcription of trxB in vivo and binding of the CTD-Spx complex to the trxB promoter in vivo are abolished.

**Materials and Methods**

**Crystallization and Structural Analysis of Reduced Spx**

“Reduced” Spx, in which residue Cys10 was replaced by serine (C10S Spx), was overexpressed in E. coli and purified by Ni²⁺-NTA affinity column chromatography as previously described [9]. Purified, cleaved Spx was dialyzed into 25 mM PIPES, pH 6.5, 100 mM KCl for use in subsequent crystallization trials.
model resulted in the placement of amino acid residues 1–115 of Spx and residues 245–311 of αCTD into the electron density map using O [23]. The initial protein model was subjected to rigid body refinement, followed by simulated annealing and positional and B-factor refinement using CNS [24]. Simulated-annealing omit maps were calculated to ensure the correct placement of all residues and to avoid model bias. The final model included residues 1–115 of Spx, residues 245–311 of αCTD, and 40 solvent molecules. The final Rwork and Rfree are 23.6% and 27.8%, respectively, to 1.9 Å resolution. The stereochemistry of the final model was assessed with PROCHECK [25], which revealed 89.7% of all ϕ/ψ angles in the most favored regions of the Ramachandran plot and none in the disallowed regions. The coordinates and structure factors have been deposited in the RCSB with accession number 3IHQ.

**Construction of Spx Amino Acid Substitution Mutants**

All bacterial strains and plasmids are listed in Table 2. The effect of Spx amino acid substitution was examined with the Spx construct carrying amino acid substitutions (AN to DD) at the carboxyl-terminal end, which renders Spx insensitive to CtxKp proteolysis [2]. The previously constructed plasmids pSN56 [2] and pZv14 [20] are pDR111 derivatives that carry spaDD and spaC/OA/DD, respectively. pDR111 is an amyE integration vector, and the cloned spa genes are transcribed from the IPTG-inducible Ppandkky promoter [26].

Three additional spa mutations conferring single amino acid substitutions were generated by two-step PCR-based mutagenesis using a pair of complementary mutagenic oligonucleotides – oMMN07-351 and oMMN07-352 for R60E, oMMN07-353 and oMMN07-354 for K62E, and oMMN07-355 and oMMN07-356 for K66E. Each oligonucleotide pair was used for the first PCR, together with either the upstream oligonucleotide oMMN01-173 or the downstream oligonucleotide oMMN01-174 and the plasmid pSN56 as template. The two PCR products carrying short complementary ends were annealed, filled-in by ExTaq polymerase (Takara Bio USA), and used as template for the second-round of PCR using oMMN01-173 and oMMN01-174. The PCR product was digested with Sall and HindIII and cloned into pDR111 and digested with the same enzymes to generate pMMN683 (spaDD::KO63E), pMMN684 (spaDD::KO62E), and pMMN685 (spaDD::KO66E).

Plasmid pMMN754 carrying spaDD::KO52R was generated as follows. The spa gene carrying the G52R mutation was amplified from chromosomal DNA isolated from ORB455 using oligonucleotides oMMN01-173 and oMMN01-174. The PCR product was digested with HindIII and BclI and the 5′-end of spa containing the G52R mutation was isolated. The 3′-end of spa carrying the DD mutation was isolated from pSN56 digested with BclI and Sall. The two fragments were cloned into pUC19 digested with HindIII and Sall by three fragment ligation to generate pMMN753. The spa fragment was isolated from pMMN753 digested with HindIII and Sall and cloned into the HindIII-Sall sites of pDR111 to generate pMMN754.

The effect of the R60E, K62E, and K66E mutations of Spx on txrB expression was determined by measuring lacZ expression driven by the txrB promoter (−115 to +47) using a transcription start site as previously described [10]. Plasmids pMMN683 (spaDD::KO63E), pMMN684 (spaDD::KO62E), and pMMN685 (spaDD::KO66E) were used to transform ORB14566 carrying spaC::neo and txrB-lacZ at thrC, and transformants were selected for spectinomycin resistance (Sp)c to generate ORB6895, ORB6896, and ORB6897, respectively. The transformants were screened for the amylase-negative phenotype, which is indicative of double-crossover recombination [27]. As a control, pSN56 carrying the wild-type SpxDD was used to transform ORB4566, and ORB6894 was obtained.

The strains carrying spaA-lacZ and the wild-type or mutant SpxDD were constructed as follows. The strain JH642 was transformed with pMMN683, pMMN684, and pMMN685, and the strains ORB6930, ORB6931, and ORB6932 were constructed as described above. Each strain was transduced with SPB phage carrying pMMN92-borne spaA-lacZ [18] to generate ORB6934, ORB6935, and ORB6936. A control strain, ORB6129, carrying the wild-type SpxDD at the thrC locus and the spaA-lacZ fusion was constructed as previously described [20].

**Construction of txrB Promoter Mutations**

All mutant txrB promoters are derivatives of pDYR9 [11], which carries the txrB promoter (−115 to +47) fused to lacZ. Base substitution mutations of the txrB promoter were constructed by two-step PCR using complementary mutagenic primer pairs in a procedure similar to that used for the amino acid substitutions of Spx. The sequences of mutagenic oligonucleotides are listed in Table 3, and the outside forward (oDYR07-52) and reverse primers (oDYR07-32) were previously described [11]. DNA fragments resulting from the second PCR were digested with EcoRI and HindIII and cloned into pDG793 [28] that had been digested with the same enzymes. pDG793 is a thrC integration plasmid and double-crossover recombination is selected by Thr+ phenotype. Each plasmid was used to transform ORB3834 (spaC::neo), and erythromycin-resistant (Erm+) Thr+ transformants were then transformed with chromosomal DNA isolated from strains carrying the wild-type and mutant spaDD at the amyE locus. All plasmids are listed in Table 2.

**Measurement of β-galactosidase Activity**

The effect of the Spx amino acid substitutions on the expression of txrB and spaA was determined by measuring β-galactosidase activity in cells carrying txrB-lacZ (ORB6894 to ORB6897) and spaA-lacZ (ORB6129, ORB6930 to ORB6932) in the presence and absence of IPTG. The strains were grown at 37°C overnight on DS agar plates [29] supplemented with spectinomycin and erythromycin (for txrB-lacZ) or spectinomycin and chloramphenicol (for spaA-lacZ). The overnight cultures were used to inoculate the same liquid medium at a starting optical density of 0.02 to 0.05. When the OD600 of the cultures reached 0.4 to 0.5, the cultures were divided into two flasks and 1 mM IPTG was added to one of the flasks. Samples were taken at 0.5- to 1-hr intervals to assay β-galactosidase activity, which was expressed as Miller units [30].

**Western Blot Analysis**

The strains ORB6894 (SpxDD), ORB6895 (SpxDD::KO6E), ORB6896 (SpxDD::KO62E), and ORB6897 (SpxDD::KO66E) were cultured in DS liquid medium supplemented with spectinomycin and erythromycin as described above. Each culture was divided into two tubes at an OD600 of 0.4 to 0.5, and 1 mM IPTG was added to one of the tubes. Two milliliter samples were harvested after a 1.5-hr incubation and resuspended with 0.5 ml of 20 mM potassium phosphate buffer pH 7.5, 15 mM MgCl2, 20% sucrose. Lysozyme (1 mg/ml) was added and the suspension was incubated by gently shaking at 37°C for 30 min. The protoplasts were collected by centrifugation at 7,000 x g for 5 min and washed once with the same buffer. The precipitated protoplasts were lysed by resuspending with 0.5 ml of lysis buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA) to obtain crude extract. Protein concentrations in the crude extract were determined using BioRad protein assay solution, and 15 μg of total protein was applied to an...
| Strain or plasmid | Relevant genotype or characteristics |
|-------------------|--------------------------------------|
| **B. subtilis strains** |                                      |
| JH642             | parental strain                      |
| ORB3834           | ΔspxBneo                              |
| ORB4028           | ΔspxBneo hisI3-rpoC                   |
| ORB4055           | spxBΔ192                              |
| ORB4566           | ΔspxBneo thrC::txbB(−510 to +190)-lacZ |
| ORB6129           | amyE::Pspankhy-spx^{D^200} srfA-lacZ   |
| ORB6894           | ΔspxBneo thrC::txbB(−510 to +190)-lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB6895           | ΔspxBneo thrC::txbB(−510 to +190)-lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB6896           | ΔspxBneo thrC::txbB(−510 to +190)-lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB6897           | ΔspxBneo thrC::txbB(−510 to +190)-lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB6930           | amyE::Pspankhy-spx^{D^200}            | This study |
| ORB6931           | amyE::Pspankhy-spx^{D^200}            | This study |
| ORB6932           | amyE::Pspankhy-spx^{D^200}            | This study |
| ORB6934           | amyE::Pspankhy-spx^{D^200} srfA-lacZ  | This study |
| ORB6935           | amyE::Pspankhy-spx^{D^200} srfA-lacZ  | This study |
| ORB6936           | amyE::Pspankhy-spx^{D^200} srfA-lacZ  | This study |
| ORB7271           | ΔspxBneo thrC::txbB(G-44C)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7272           | ΔspxBneo thrC::txbB(G-44A)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7273           | ΔspxBneo thrC::txbB(G-33A)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7274           | ΔspxBneo thrC::txbB(G-31A)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7275           | ΔspxBneo thrC::txbB(G-44T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7276           | ΔspxBneo thrC::txbB(lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7282           | ΔspxBneo thrC::txbB(lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB7316           | ΔspxBneo thrC::txbB(lacZ amyE::Pspankhy-spx^{D^200}C170A | This study |
| ORB7337           | ΔspxBneo thrC::txbB(lacZ amyE::Pspankhy-spx^{D^200}G128R | This study |
| ORB7322           | ΔspxBneo thrC::txbB(C-32A)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7342           | ΔspxBneo thrC::txbB(A-34T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7343           | ΔspxBneo thrC::txbB(A-34T)-lacZ amyE::Pspankhy-spx^{D^200} | This study |
| ORB7347           | ΔspxBneo thrC::txbB(A-34T)-lacZ amyE::Pspankhy-spx^{D^200}C170A | This study |
| ORB7348           | ΔspxBneo thrC::txbB(A-34T)-lacZ amyE::Pspankhy-spx^{D^200}G128R | This study |
| ORB7349           | ΔspxBneo thrC::txbB(C-32T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7356           | ΔspxBneo thrC::txbB(G-31C)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7368           | ΔspxBneo thrC::txbB(A-45T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7375           | ΔspxBneo thrC::txbB(A-42T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7426           | ΔspxBneo thrC::txbB(G-31T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7427           | ΔspxBneo thrC::txbB(A-42G)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7488           | ΔspxBneo thrC::txbB(C-43G)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7491           | ΔspxBneo thrC::txbB(C-43T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7540           | ΔspxBneo thrC::txbB(A-34G)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7541           | ΔspxBneo thrC::txbB(A-34C)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7592           | ΔspxBneo thrC::txbB(A-37C)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7593           | ΔspxBneo thrC::txbB(A-39C)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7594           | ΔspxBneo thrC::txbB(C-43A)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7595           | ΔspxBneo thrC::txbB(T-47G)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| ORB7596           | ΔspxBneo thrC::txbB(G-49T)-lacZ amyE::Pspankhy-spx^{D^0} | This study |
| **Plasmid**       |                                      |
| pCS228            | pDG793 with txB(G-31T)-lacZ            | This study |
| pCS229            | pDG793 with txB(A-42G)-lacZ            | This study |
| pDG793            | thrC integration vector with promoter-less lacZ | [28] |
SDS-polyacrylamide (15%) gel. The Western blot experiment was done as previously described using an anti-Spx antibody [31].

Protein Purification

RNAP was purified from ORB4028 (spx::neo his 10-rpoC) as previously described using a Ni-NTA affinity column, a heparin agarose, and a Bio-Rad High Q column [11,32,33]. The self-cleavable intein system (New England Biolabs) was used for overproduction and purification of a CTD and Spx. aCTD (residues 225–314) was overproduced in Escherichia coli BL21/pLysS carrying pSN37 [8] and purified using a chitin column and a BioRad High Q column.

A was overproduced using pSN64 in E. coli ER2566 (New England Biolabs) and purified using a chitin column and a High Q column. The wild-type Spx protein was overproduced from pMMN470 [31] in ER2566 and purified using a chitin column and a BioRad High S column. To overproduce the SpxR60E protein, pMMN712 was constructed as follows. The fragment, amplified by PCR using oligonucleotides oMMN01-135 and oMMN01-137 together with pMMN683 as template, was digested with NcoI and SmaI and then cloned into pTYB4 (New England Biolabs) digested with the same enzymes. ER2566 carrying pSN21 and pMMN712 were used to overproduce the SpxG52R [8] and SpxR60E mutant proteins, respectively, and the proteins were purified similarly to the wild-type Spx.

| Strain or plasmid | Relevant genotype or characteristics |
|------------------|-------------------------------------|
| pDYR9            | pDG793 with trxB-lacZ                |
| pDYR24           | pDG793 with trxB(A-37C)-lacZ         |
| pDYR25           | pDG793 with trxB(A-39C)-lacZ         |
| pDYR26           | pDG793 with trxB(C-43A)-lacZ         |
| pDYR27           | pDG793 with trxB(G-44T)-lacZ         |
| pDYR28           | pDG793 with trxB(T-47G)-lacZ         |
| pDYR29           | pDG793 with trxB(G-49T)-lacZ         |
| pDR111           | amyE integration vector with Pspanky promoter |
| pMMN92           | SPβ carrying srfA-lacZ               |
| pMMN470          | pTYB4 with spx                       |
| pMMN683          | pDR111 with spxDD-G52R               |
| pMMN684          | pDR111 with spxDD-K66E               |
| pMMN685          | pDR111 with spxDD-K62E               |
| pMMN738          | pTYB4 with spxβ                      |
| pMMN745          | pDG793 with trxB(G-44C)-lacZ         |
| pMMN746          | pDG793 with trxB(G-44A)-lacZ         |
| pMMN747          | pDG793 with trxB(G-33A)-lacZ         |
| pMMN748          | pDG793 with trxB(G-31A)-lacZ         |
| pMMN752          | pDG793 with trxB(C-32A)-lacZ         |
| pMMN753          | pUC19 with spxβ                      |
| pMMN754          | pDR111 with spxβ                      |
| pMMN755          | pDG793 with trxB(A-34T)-lacZ         |
| pMMN756          | pDG793 with trxB(C-32T)-lacZ         |
| pMMN757          | pDG793 with trxB(G-31C)-lacZ         |
| pMMN758          | pDG793 with trxB(A-45T)-lacZ         |
| pMMN759          | pDG793 with trxB(A-42T)-lacZ         |
| pMMN763          | pDG793 with trxB(C-43G)-lacZ         |
| pMMN764          | pDG793 with trxB(C-43T)-lacZ         |
| pMMN775          | pDG793 with trxB(A-34G)-lacZ         |
| pMMN776          | pDG793 with trxB(A-34C)-lacZ         |
| pSN21            | pTYB4 with spxβ                      |
| pSN37            | pTYB2 with spoA-CTD                   |
| pSN56            | pDR111 with spxDD                    |
| pSN64            | pTYB4 with sigA                      |
| pTYB4            | protein expression vector with intein tag |
| pUC19            | cloning vector                       |
| pZY14            | pDR111 with spxDD-CTD                |

Unless otherwise noted, trxB-lacZ contains a region between -115 and +47 of the trxB promoter.

doi:10.1371/journal.pone.0008664.t002

Table 2. Cont.
Table 3. Oligonucleotides used in this study.

| Oligonucleotide       | Sequence                              | Purpose                          |
|-----------------------|---------------------------------------|----------------------------------|
| oMMN01-135            | AGAGGAGTGAAAGATCCATGGTTACACTATAC     | spx forward                      |
| oMMN01-137            | TAATCCCCGGGTTTTTCAGCAGGTTTCTTTTCTTT | spx reverse                      |
| oMMN07-351            | GAAATCATCTCAACCGAGTCAAAGATTCCCAA    | spx R60E forward                 |
| oMMN07-352            | TGGATAATTTTCTCTGCTCCAACAGGATTCCCAA | spx R60E reverse                 |
| oMMN07-353            | ATCCAAACGGGTTGAGATTTCAAAAGGTTACAG   | spx K62E forward                 |
| oMMN07-354            | CAGTTTCTTCTCTGCAAGCAGGTTGAGATTTCCAA| spx K62E reverse                 |
| oMMN07-355            | TCAAAGATTTACAGAAGTGAATGGAACAGGTTA   | spx K66E forward                 |
| oMMN07-356            | AACGTTGACATTTCCAGAATACACTTTTGTGAG  | spx K66E reverse                 |
| oMMN08-405            | ATCGTGTTGAAACAAAAATATGCGTATAC      | trxB G-44A forward               |
| oMMN08-406            | TACACGCTATTTTTTTTTTTCACAGGAT       | trxB G-44A reverse               |
| oMMN08-407            | ATCGTGTTGACAAAAATATGCGTATAC       | trxB G-44C forward               |
| oMMN08-408            | TACACGCTATTTTTTTTTTTCACAGGAT       | trxB G-44C reverse               |
| oMMN08-409            | GAGCAAAAATATGCGTATACCATGAGA         | trxB G-33A forward               |
| oMMN08-410            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB G-33A reverse               |
| oMMN08-411            | GAGCAAAAATATGCGTATACCATGAGA         | trxB G-31A forward               |
| oMMN08-412            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB G-31A reverse               |
| oMMN08-413            | GAGCAAAAATATGCGTATACCATGAGA         | trxB C-32A forward               |
| oMMN08-414            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB C-32A reverse               |
| oMMN08-415            | GAGCAAAAATATGCGTATACCATGAGA         | trxB A-34T forward               |
| oMMN08-416            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB A-34T reverse               |
| oMMN08-417            | GAGCAAAAATATGCGTATACCATGAGA         | trxB C-32T forward               |
| oMMN08-418            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB C-32T reverse               |
| oMMN08-419            | GAGCAAAAATATGCGTATACCATGAGA         | trxB G-31C forward               |
| oMMN08-420            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB G-31C reverse               |
| oMMN08-421            | TTTATCGTTGTTGCAAACAAAAATACGGGTA    | trxB A-45T forward               |
| oMMN08-422            | CGCTATTTTTTTCGCCACACAGGATTAAA      | trxB A-45T reverse               |
| oMMN08-423            | TAATCGTTGTTGCTAABAAAAATAGGTA       | trxB A-42T forward               |
| oMMN08-424            | TACGCTATTTTTTTGCTCAACAGGATTAA      | trxB A-42T reverse               |
| oMMN08-436            | GAGCAAAAATATGCGTATACCATGAGA         | trxB G-31T forward               |
| oMMN08-437            | TCTCATGTTGATACAGTATTACATTCTTCTTTCT| trxB G-31T reverse               |
| oMMN08-438            | TAAATCGTTGTTGCAAACAAAAATACGGGTA    | trxB A-42G forward               |
| oMMN08-439            | TACGCTATTTTTTCGCCACACAGGATTAAA      | trxB A-42G reverse               |
| oMMN08-440            | AATCGTTGTTGAGAAAATAATAGTA          | trxB C-43G forward               |
| oMMN08-441            | CATTTTTTTTCTTCAACACGATT            | trxB C-43G reverse               |
| oMMN08-442            | AATCGTTGTTGAGAAAATAATAGTA          | trxB C-43T forward               |
| oMMN08-443            | CATTTTTTTTACTTCAACACGATT          | trxB C-43T reverse               |
| oMMN08-461            | GAGCAAAAATATGCGTATACCATGAGA         | trxB A-34G forward               |
| oMMN08-462            | TCTCATGTTGATACAGGCTTTTTTTTCGTC     | trxB A-34G reverse               |
| oMMN08-463            | GAGCAAAAATATGCGTATACCATGAGA         | trxB A-34C forward               |
| oMMN08-464            | TCTCATGTTGATACAGGCTTTTTTTTCGTC     | trxB A-34C reverse               |
| oMMN08-465            | TAATCGTTGTTGCAAACAAAAATACGGTATAC    | trxB (−56 to -21)               |
| oMMN08-466            | CATGTTGATACGCTTTTTTTTTTCGTCACACGATT| trxB (−21 to -55)               |
| oMMN08-473            | TAATCGTTGTTGCAAACAAAAATACGGTATAC    | trxB G-44A G-33A forward         |
| oMMN08-474            | CATGTTGATACGCTTTTTTTTTTTCGTCACACGATT| trxB G-44A G-33A reverse         |

doi:10.1371/journal.pone.0008664.t003

In Vitro Transcription

A linear trxB template was generated by PCR with oligonucleotides oDY07-32 and oDY07-52. The template is expected to produce a 66-base transcript. One nM of the template and 25 nM of RNAP together with 25 nM σ^3 were incubated without or with 7.5 nM Spx protein in 62.3 μl of 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM MgCl₂, and 50 μg/ml BSA. After 10-min incubation at 37°C, 7.7 μl of nucleotide mixture (700 μM ATP,
GTP and CTP, 35 μM UTP, 17.5 μCi [α-32P]UTP was added to each reaction. After incubation at 37°C for 2, 5, and 10 min, 20 μl of the reaction was withdrawn to mix with 10 μl of stop solution (1 M ammonium acetate, 0.1 mg yeast RNA, and 0.03 M EDTA). The mixture was precipitated with ethanol and resuspended with 5 μl of formamide-dye (0.3% xylene cyanol, 0.3% bromophenol blue, and 12 mM EDTA dissolved in formamide). The samples were heated at 90°C for 2 min and were applied onto an 8% polyacrylamide-urea gel. The gel was dried and autoradiographs were scanned on a Typhoon Trio scanner (GE Healthcare).

**Electrophoretic Mobility Shift Assay (EMSA)**

The probe used for EMSA was a fragment extending from −56 to −21 of the trxB promoter region, which was generated by annealing complementary oligonucleotides. The 36-mer 5′-OMNN08-465 was the template strand and the 35-mer 5′-OMNN08-466 was the non-template strand that lacks A (complementary to −56T) at its 3′-end. The two oligonucleotides (5 pmols each) were mixed in 20 μl of 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM MgCl2 and heated at 90°C for 5 min, then slowly cooled to room temperature. To radiolabel the non-template strand, Klenow fragment and 10 μCi of [α-32P]dATP (800 Ci/mmol) were added to the annealing reaction to fill-in the 3′-end. After incubation at room temperature for 15 min, unincorporated [α-32P]dATP was removed using a nucleotide purification kit (Qiagen). The mutant (G-44A G-33A) trxB and spoVG probes were generated in a similar manner, except using oligonucleotides 5′-OMNN08-466 and 5′-OMNN08-474, and 5′-OMNN09-477 and 5′-OMNN09-478, respectively.

Five μM of Spx and 5 μM of αCTD (unless otherwise stated) were incubated at room temperature for 10 min in 20 μl of 20 mM Tris-HCl pH 7.8, 50 mM NaCl, 5 mM MgCl2, 10% glycerol. The radiolabeled probe (2,000 cpm/reaction) was added to the preincubated mixture and further incubated at room temperature for 15 min. The reaction mixture was applied onto a pre-run 6% native polyacrylamide gel and run in TGE buffer (50 mM Tris, 0.38 M glycine, 2 mM EDTA) at 180V. The gel was dried and scanned on a Typhoon Trio variable mode imager.

**Results**

**The Wild-Type and Spx(C10S) Structure**

Formation of the disulfide bond between C10 and C13 is essential for the positive regulatory role of Spx, but not for its negative role [10,20,35]. We hypothesized that a conformational change caused by formation of the disulfide bond could provide a mechanism for how Spx is involved in the transcriptional activation of genes such as trxA and trxB. Therefore, the crystal structure of Spx (C10S), which mimics the reduced form, in complex with the αCTD was determined to 1.9 Å resolution (Figure 1A).

The structure of the reduced αCTD-Spx complex is quite similar to that of the oxidized αCTD-Spx complex [9,36] and an overlay of 156 corresponding Cα atoms of both complexes, excluding residues on helix α4 of Spx, results in a root mean square deviation of 0.6 Å. As seen previously the αCTD contains four core α helices (α1–α4) and a somewhat extended N-terminal helix designated α1′ (Figure 1A). The reduced C10S Spx protein is a mixed α/β protein with its secondary structural elements arranged: β1α2β2α1α3(310)β3αβ4α2. (Figure 1A). Thus, Spx retains most of the secondary structure that is found in oxidized Spx. Importantly, the αCTD-Spx interface of the reduced complex is identical to that of the oxidized αCTD-Spx complex indicating that the biological effects of thiol stress readout by these proteins are not a consequence of a radically different structure of this complex.

Interestingly, the loss of the disulfide linkage between residues C10 and C13 does not result in a significant change in the local structure (Figure 1B). Although free to rotate from their positions in the oxidized state, the S10 and C13 side chains do not move

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**Figure 1. Structure of reduced C10S Spx in complex with αCTD.** (A) Spx and αCTD are shown as teal and green ribbons, respectively, and their secondary structures are labelled. Helix α4, which is observed in oxidized Spx [9] but has unraveled in the reduced form, is colored magenta. The residues mutated in this study, R60 and K62, are labelled and shown as sticks with carbon atoms colored white and nitrogen atoms either blue or magenta. Residues S10 and C13 are labelled and shown as sticks with carbon and sulphur atoms colored yellow and the γ-oxygen of S10, red. (B) Close up of the region surrounding helix α4 and residues C10/S10 and C13 after the superposition of the oxidized and reduced αCTD-Spx complex structures. Reduced Spx is shown as a magenta ribbon and oxidized Spx as a teal ribbon. The C10-C13 disulfide bond is shown in orange sticks and S10 and C13 from the reduced structure are shown as yellow sticks. In the reduced form residue R92 has moved 2.8 Å away from its position in the ammonium sulphate-containing oxidized form [9]. The side chain of residue R60 beyond the Cβ atom is disordered in the sulphate-containing crystal form of oxidized Spx, which was used in the superposition visualized here [9].

doi:10.1371/journal.pone.0008664.g001
because their positions are buttressed by numerous interactions. Indeed the S10 Oγ side chain engages in hydrogen bonds to the backbone amide (NH) group of C13 and Oγ atom of residue S12. The C13 Sγ sulphydryl group hydrogen bonds to hydroxyl side chain of residue S10 and makes van der Waals contacts with residues R92 and P93. Also, due to two alternative backbone conformations around residues S7 and P8, the S7 backbone carbonyl oxygen can engage in a weak hydrogen bond to the C13 SHγ group.

One significant conformational difference is found between oxidized and reduced Spx, however, whereby helix α4 of the reduced mutant Spx structure (residues S61 to N68 in oxidized Spx) unfolds and rotates (Figure 1). As a consequence of the unravelling of helix α4, several basic residues are repositioned. Specifically, the side chain of residues K62, which points into the solvent in oxidized Spx and is disordered, turns inward and now makes hydrogen bonds to the carbonyl oxygen (CO) atom of residue G88 and the Oγ atom of residue Ser58. Residue K66, which is also solvent exposed in oxidized Spx, is now pointing into the core of the protein. Hence, the exposed electrostatic surface of α4 is decreased significantly in reduced Spx. Other helix α4-related conformational changes include the repositioning of residue R60, whereby its Cα carbon has moved 1.3 Å from its oxidized position. The side chain of the R60 residue, which points directly into the solvent and is disordered in one crystal structure of oxidized Spx but not in the second [36], has moved 5.8 Å (Cζ–Cγ) from its position in the oxidized protein and has shifted towards R92 by 1.3 Å (Figure 1B). The potential importance of this altered location is tied to residue R92. In reduced mutant Spx, the side chain of residue R92 has rotated outward by ~2.8 Å from its location in oxidized Spx. In the oxidized protein, the guanidinium side chain is engaged in an electrostatic interaction with a bound sulfate ion [9]. In the reduced protein, the R92 side chain rotates inward and makes hydrogen bonds to the peptide backbone carbonyl oxygen atoms of residues G88 and L90 (Figure 1B). This location of the R92 side chain of reduced Spx is very similar to its location in oxidized Spx crystallized from solutions that do not contain sulfate or phosphate anions [36]. Perhaps, the R92-bound sulfate ion described in the first reported oxidized Spx structure is a surrogate for one of the phosphate groups of (αCTD-Spx)-bound DNA. If so, this also places the solvent exposed guanidinium group of residue R60 near the DNA phosphate backbone and suggests its possible role in DNA binding, either to the backbone or to a guanine. Thus, loss of the Spx C10-C13 disulfide bond results only in small conformational changes that are confined primarily to helix α4. However, the resulting helix-to-coil transition repositions the side chains of several basic residues that could have functional consequences with respect to DNA binding.

Residues in or Near Helix α4 of Spx Are Important for trxβ Activation but Not for srfA Repression by ComA Activator Interference

To determine whether the structural change in helix α4 is crucial for the function of Spx in transcription activation, we next introduced single amino acid substitutions around the helix α4 region. Interestingly, there are some basic residues adjacent to and within the helix, namely, R60, K62, and K66. Since basic amino acids are known to interact with DNA through sequence recognition and charge neutralization [37], we decided to substitute each residue with glutamate and to examine the effect of these substitutions on Spx activity. Because under nonstress conditions Spx is degraded by ClpXP protease, we expressed the ClpXP-resistant forms (Spx<sup>DD</sup>) of the wild-type and mutant proteins from an IPTG-inducible promoter, as previously described [2] so that we could examine the mutational effect on trxβ transcription under nonstress conditions. Western blot analysis showed that the three mutant Spx proteins were produced only in the presence of IPTG at a level similar to the wild-type protein (Figure 2). trxβ-lacZ<sup>−</sup> expression in cells producing Spx<sup>K62E</sup> was equal to or greater than that observed in cells producing the wild-type Spx (Figure 3A). In contrast, the K62E mutation reduced transcription and the R60E mutation nearly abolished transcription, indicating that R60, and to a lesser extent K62, are important for transcriptional activation of trxβ.

Although Spx<sup>K62E</sup> and Spx<sup>K60E</sup> are produced at a level similar to the wild-type Spx in <i>B. subtilis</i> cells, we could not completely eliminate the possibility that the mutant proteins were misfolded, and thus, inactive. As described earlier, Spx plays both a positive and negative role in transcription regulation. To examine whether the mutant proteins retain the ability to exert negative transcriptional control, we determined the effect of the mutations on ComA-dependent srfA transcription. As shown in Figure 3B, srfA
transcription was severely reduced in a strain that produced the wild-type Spx protein and in all of the strains that produced the mutant proteins. These results clearly demonstrated that R60 and K62 of Spx play pivotal roles in positive control but are dispensable for its negative role in transcription.

Identification of trxB Sequences Required for Spx-Dependent Transcription Activation

One possible hypothesis for why R60 (and to a lesser extent K62) is required for trxB transcription and not for inhibition of activator-dependent spx transcription is that R60 is involved in the interaction of Spx with the trxB promoter DNA for establishing the transcription initiation complex. Although Spx itself did not bind trxB DNA [10], this result does not necessarily eliminate the possibility that Spx, by interacting with αCTD, can contribute part of a DNA-binding surface. The intracellular disulfide bond formation might facilitate the recognition and/or binding of the side-chain of R60 with a specific nucleotide in the trxB promoter region, as suggested above. Alternatively, Spx residue R60 might function indirectly in DNA sequence recognition by changing the conformation of αCTD so that it recognizes specific sequences associated with Spx-activated promoters. We think that this is unlikely as explained in the Discussion.

Our previous work demonstrated that the trxB promoter region between −50 and −36 is required for Spx-dependent transcription activation and that the nucleotides at positions −43 and −44 are essential for transcription [10,11]. The identified region corresponds well with the sequence protected from DNase I digestion in the presence of the wild-type Spx-RNAP complex [10]. The DNase I footprinting analyses also identified a hypersensitive site between −34 and −35 of the trxB template strand in the presence of Spx-RNAP [10]. Interestingly, Spx-RNAP generated a hypersensitive site at a similar position (between −35 and −36) of trxA, a gene that is also activated directly by Spx. Furthermore, the nucleotide sequences (AGCGT) of the trxA (−40 to −31) and trxB (−39 to −30) regions that include the hypersensitive site are identical. These observations prompted us to carry out further mutational analyses of the −39 to −30 region of trxB.

As in the previous study [11], we used the trxB promoter carrying −115 to +47 for base substitution experiments, except that we expressed the IPTG-inducible spxD from the strain lacking the native spx gene. The results showed that three segments of trxB are important for Spx-dependent transcription activation (Figure 4). The first segment is the AGCA sequence positioned from −45 to −42. Our previous study showed that the G at −44 and C at −43 are indispensable for transcription [10,11]. The second segment is a poly-A stretch between −41 and −36. In our previous study, base substitution of two of the A residues showed a moderate effect on trxB transcription in the spxR background [11], and here, these substitutions showed more adverse effects in cells lacking the native spx gene (Figure 4). It has been known that αCTD binds to AT-rich sequences; for example, the sequence AAAAAARNR at positions −46 to −38 of the E. coli rnb promoter P1 serves as the proximal αCTD-binding site [38]. We, therefore, propose that the sequence between −41 and −35 is a site for αCTD interaction. The last segment important for trxB transcriptional control by Spx is the GC sequence positioned at −33 and −32. Our previous work showed that Spx-dependent activation of a trxB/srfA hybrid promoter transcription was enhanced further by extending the trxB control region to −31 including the GC at −32/33 [11]. The dinucleotide resides in the center of the AGCG sequence that is similar to the upstream essential AGGA (−45 to −42) sequence. The G residue in the second position is the most critical among the tetranucleotides AGGA and AGCG. Because the two tetranucleotides are similar, with the exception that the most 3′-end of the upstream sequence is A (−42) the corresponding position of the downstream sequence is G (−31), we examined the effect of base substitution of the G at position −31. We found that substitution of G with either C or T had no significant effect, whereas the substitution with A led to a more than two-fold increase in trxB expression. One interpretation of this result is that the Spx/αCTD complex binds to trxB and that Spx contacts the AGGA sequence and αCTD contacts the downstream A-rich sequence. If this is true, then the question is which protein, if any, binds to the downstream AGCG sequence. The previous crosslinking study did not show contact of any protein to the −35 region [11], and was, therefore, inconclusive. One could envision three alternative scenarios for a protein/−35

Figure 4. Effect of base substitutions of the trxB promoter on trxB expression. Single base pair substitutions were generated in the trxB promoter (−115 to +47). The mutated promoters fused to lacZ were introduced in spx mutant strains expressing spxD from the IPTG-inducible Pspank-hy promoter. Expression of trxB-lacZ was determined in at least two independent isolates as described in Fig. 3. The effect of each base substitution is shown as a percentage of the peak trxB transcribed from the wild-type promoter, which was used as a control in each experiment. The peak expression was generally seen around 1.5 hr after the addition of IPTG.

doi:10.1371 journal.pone.0008664.g004
producing each mutant Spx DD protein as compared with those from the wild-type trxB element recognition will be discussed below (see Discussion).

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that the second AGCG sequence is a site where Spx binds. A third possibility is that the downstream transcription, but the requirement of residues R60 and C10 is completely impaired in activating transcription from the mutant promoter. This result indicates that the G52 residue, and hence, the G52R mutation on Spx R60E mutation on SpxR60E reproducibly activated transcription from the trxB(A-34T) promoter to a higher level than from the wild-type promoter. Based on the in vitro transcription assays, we conclude that the adverse effect of the R60E mutation on trxB transcription is likely caused by either a weaker interaction of the mutant Spx with the trxB promoter, RNAP, or both.

Activation-Defective Spx Mutants Other Than G52R Are Able to Activate a Mutant trxB Promoter Bearing an A-34T Mutation

The mutational analysis showed that the A-34T substitution resulted in highly elevated trxB transcription, which remained largely dependent on Spx because IPTG was still required for promoter activity (Figure 4). We examined expression of trxB(A-34T)-lacZ in cells expressing spxR60E, spxG52R and spxC10A mutants, as well as the wild-type spx. As shown in Figure 5A, transcription from the wild-type trxB promoter was severely reduced in cells producing each mutant SpxD protein as compared with those producing wild-type SpxD. In contrast, the R60E and C10A Spx mutants were able to activate the A-34T promoter, although the activity of the mutant proteins was approximately 50% of the activity of the wild-type protein (Figure 5B). No expression was observed in the absence of IPTG, indicating that the observed expression is dependent on mutant Spx protein. Unlike the two Spx activation mutants, the SpxC10A mutant protein was completely impaired in activating transcription from the mutant promoter. This result indicates that the G52 residue, and hence, the SpxC-CTD interaction is absolutely required for Spx-activated trxB transcription, but the requirement of residues R60 and C10 is conditional, given that the spx mutations R60E and C10A are partially suppressed by the A-34T mutation in the trxB promoter.

SpxR60E Activates trxB(A-34T) Transcription In Vitro

The in vivo results described above showed that SpxR60E and SpxC10A are unable to activate transcription from the wild-type trxB promoter, but are able to significantly activate trxB(A-34T) transcription. We next carried out in vitro run-off transcription experiments to determine whether the effect of the A-34T substitution can be solely attributed to interactions involving the trxB(A-34T) promoter, Spx, and RNAP. Figure 6 shows that the basal level of trxB transcription was markedly elevated by adding wild-type Spx, but only a slight increase in the transcript was detected in reactions containing SpxC10A. The trxB(A-34T) transcript accumulated slightly more than the wild-type trxB transcript at a longer reaction time (10 min) in the absence of Spx, and the transcript levels were further increased when the wild-type Spx was present. Unlike the wild-type transcript levels, those of the mutant trxB were markedly elevated by SpxC10A. We repeated these experiments almost ten times and in some experiments we did not see any difference between the wild-type and the mutant trxB transcript levels activated by the wild-type Spx as shown in Figure 6; however, in other experiments, we observed that the mutant trxB transcript level was significantly higher than the wild-type transcript level. Although we do not understand the variability in the in vitro transcription results, SpxR60E reproducibly activated transcription from the trxB(A-34T) promoter to a higher level than from the wild-type promoter.

The Spx-αCTD Complex Binds the trxB Regulatory Region

The mutational studies of the trxB promoter uncovered a region of trxA required for Spx-dependent activation [10,11], and this putative αS-acting component of Spx control was further investigated. Given that the A-rich sequence and the flanking AGCA and AGCG sequences are conserved between the trxB and trxA promoters and that the upstream AGCA sequence is also present in other Spx-controlled genes (see Discussion), one could envisage that the AGCA sequence and possibly AGCG in trxA and trxB is the site where Spx binds. To test this possibility, we next carried out EMSA analysis using a DNA fragment carrying the trxB promoter (−56 to −21). This fragment covers the putative Spx-αCTD-binding sites, but lacks the −10 region. We first examined whether either αCTD or Spx alone binds DNA. αCTD at 5 μM bound DNA, but Spx at the same concentration did not (Figure 7A, lanes 2 and 6). Although Spx itself was unable to bind DNA at the concentration tested, Spx addition resulted in a supershifted complex with promoter DNA and αCTD, indicating that a DNA-αCTD-Spx ternary complex was formed (Figure 7A, lane 3). In contrast, the supershifted band was not detected with
Figure 7. Interaction of αCTD and Spx variants with the regulatory region of the trxB promoter. The trxB probe (−56 to −21) was generated by annealing of oligonucleotides followed by labeling of the 3'-end of the template strand using Klenow fragment and [32P]dATP. Bands corresponding to the trxB/αCTD and trxB/Spx/αCTD complexes are marked with arrows. (A) EMSA analysis of αCTD and Spx binding to the trxB probe in reactions containing Spx variant or mixtures of mutant Spx proteins or mutant with the wild-type Spx (each at 5 μM). Abbreviations: W, wild-type Spx; G, SpxG52R; R, SpxR60E. (B) Redox-sensitive interaction was examined in the presence of DTT.

doi:10.1371/journal.pone.0008664.g007

Figure 8. Effect of trxB base substitutions on interaction with αCTD and Spx. The wild-type (W) and the trxB (G-44A/G-33A) mutant (M) probes were incubated with different concentrations of αCTD and Spx as described in Figure 7.

doi:10.1371/journal.pone.0008664.g008

SpxG52R (Figure 7A, lane 5), arguing that formation of the ternary complex is dependent on the interaction of Spx and αCTD. The effect of R60E. Spx on the DNA-αCTD complex was completely different from that of either the wild-type Spx or SpxG52R; SpxR60E prevented DNA and αCTD from forming a complex (Figure 7A, lane 4). Furthermore, addition of SpxG52R did not affect the ternary complex formed by the wild-type Spx (Figure 7A, lane 15). In contrast, addition of SpxR60E negatively affected the DNA-αCTD-wild-type Spx complex (Figure 7A, lane 13), and completely abolished the DNA-αCTD binary complex (Figure 7A, lane 14) when it was included in the binding reaction with the G52R protein (compare with Figure 7A, lane 11, a reaction containing the G52R mutant protein alone with αCTD). The ternary complex, but not the binary complex, was only formed in the absence of DTT (Figure 7B), which is in good agreement with the hypothesis that the oxidized form of Spx is required for DNA binding.

We next examined whether the trxB mutations that affect Spx-dependent activation had lower binding affinities for the Spx-αCTD complex. The trxB promoter fragment bearing the G-44A and G-33A mutations was used for a probe in EMSA to compare the binding affinity for αCTD and Spx-αCTD (Figure 8). When Spx was added in equal concentrations in reactions containing trxB(trxB)-αCTD or trxB(G-44A G-33A)-αCTD, the ternary complex was more abundant when the wild-type promoter was present than when the mutant promoter was present (Figure 8, lanes 4 and 5, and 8 and 9). Even in the reaction in which more αCTD bound to the mutant promoter than the wild-type promoter, Spx was unable to supershift the mutant promoter complex as efficiently as the wild-type promoter complex (Figure 8, lanes 11 to 16). A mutant trxB-lacZ fusion bearing the two nucleotide substitutions produced 0.7 units of β-galactosidase activity (data not shown). These results support the assumption that G-44A (and G-33A) is important for the binding of Spx to the trxB promoter. We carried out a similar experiment with the trxB promoter carrying either the G-44A or G-33A mutation to determine which residue is critical for Spx binding; however, the trxB promoter carrying the single mutation did not show a significant difference from the wild-type promoter in EMSA analysis (data not shown).

We examined whether the DNA-αCTD-Spx complex is specific to promoters of Spx-activated genes by using the spoVG promoter in a similar EMSA analysis. The spoVG promoter has an AT-rich upstream sequence that was shown to have properties of an UP element, and was required for transcription [39,40]. Spx did not activate transcription of spoVG; conversely, the transcription was shown to be inhibited by overproduction of Spx through an as yet undiscovered mechanism [41,42]. The spoVG promoter exhibited a much higher affinity for αCTD than the trxB promoter, yet addition of Spx did not result in a supershifted DNA-αCTD complex even when an excess of Spx over αCTD was added (Figure 9A). In addition, when the cold spoVG fragment was added, it was able to disrupt both αCTD- and αCTD-Spx-binding to the trxB promoter (Figure 9B). These results suggest that spoVG has a higher affinity for free αCTD than αCTD complexed with Spx, and once αCTD binds the spoVG promoter, Spx is unable to establish an interaction with αCTD.

Discussion

This study was aimed at elucidating how the oxidized form of Spx activates trxB transcription. The questions to be answered were: 1) how disulfide-bond formation at the redox CXXC center of Spx affects its activity as a transcriptional activator; 2) whether oxidized Spx in the Spx-αCTD complex binds to the trxB promoter region and enhances the binding of αCTD or other
subunits of RNAP to DNA; 3) Spx binds to DNA, what is the consensus Spx-binding site in trxB and other Spx-activated promoters. A comparison of the crystal structures of the oxidized (wild-type) and reduced (C10S) forms of Spx revealed that in the reduced form helix α4 partially unfolds and rotates, suggesting that helix α4 could be important for the positive role of Spx in transcription. Consistent with this assumption, mutations of Spx residues R60 and K62, which are adjacent to and within helix α4, respectively, reduced Spx-dependent trxB transcription but did not show any effect on its repression of ComA-dependent srfA expression. We found that the R60 and K62 residues are well conserved among Spx orthologs, whereas residue 66 is a glutamate instead of lysine in Spx from some Bacillus species such as Bacillus cereus, Bacillus thuringiensis, Bacillus clausii, Bacillus halodurans, and Bacillus weihenstephanensis. The observation supports the important role of residues R60 and K62 in the function of Spx as a transcriptional activator.

The current mutational analysis of the trxB promoter not only confirmed the results from our previous work, but also further defined the cis-sequences required for Spx-dependent activation. Based on the mutational analyses and the EMSA experiments, we now propose that the AGCA sequence at −45 to −42 is the site with which the complex of αCTD with oxidized Spx directly interacts. This hypothesis is further supported by the following observations. Our previous microarray analysis [2] identified nfxA, coding for nitro/flavin reductase [43,44], as one of the genes activated by Spx. nfxA was also shown to be activated in response to a number of stress condition, including oxidative stress [43,45] and the nfxA promoter contains the AGCA sequence at the same position (−45 to −42) as in the trxB promoter. Base substitutions of the first three nucleotides in this sequence, particularly G and C, resulted in a substantial reduction in the promoter activity, indicating the essential role of the AGCA sequence in nfxA transcription [43]. Our studies further showed that Spx activates nfxA-lacZ in vivo and the R60E mutation in Spx severely affects nfxA expression (A. L. and P. Z., unpublished results). In addition, an in vitro transcription assay showed that Spx directly activates nfxA transcription (A.L. and P.Z., unpublished results). The results are in good agreement with the hypothesis that the helix α4 of Spx functions in the interaction of αCTD/Spx complex with the AGCA sequence. We feel that it is unlikely that a conformational change of αCTD caused by Spx is responsible for the recognition of promoter DNA solely by the α polypeptide, as no uncharacteristic change in a conformation is observed when αCTD is bound to Spx [9,36].

In contrast to the AGCA sequence, the downstream AGCG sequence at −34 to −31, which is conserved in trxA and trxB, is absent in the srfA promoter; hence, its role in transcriptional activation is unclear. Interestingly, the substitution of A at −34 with T resulted in a slightly higher basal level of trxB expression and a five-fold increase in Spx-dependent activation as compared with transcription from the wild-type promoter (Figures 4 and 5). Furthermore, trxB transcription from the mutant promoter was significantly stimulated by Spx<sub>C10S</sub> and Spx<sub>C1LA</sub> (Figures 5 and 6), whereas the G52 residue was absolutely required for transcription. The A-34T change leads to a 3/6 match to the consensus −35 hexamer recognized by σ<sup>70</sup>RNAP [46]. One possible scenario is that σ<sup>70</sup> binds to the −35 region of the mutant promoter and the interaction between Spx and αCTD at the −44 element, as well as interaction of αCTD with σ<sup>70</sup>, stabilizes the three proteins at the mutant promoter, which partially compensates for the defect in trxB interaction conferred by the R60E substitution. In contrast, the G52R mutation, by disrupting the interaction of Spx with αCTD, destabilizes αCTD binding to trxB, resulting in decreased engagement of σ<sup>70</sup> with the −35 region. Does this possibility suggest that σ<sup>70</sup> also interacts with the −35 region of the wild-type trxB promoter? Another mutation, C at −32 to A, which results in a 3/6 match to the consensus −35 hexamer did not increase trxB transcription, and instead, showed a severe adverse effect, suggesting that σ<sup>70</sup> does not bind to the −35 region of the wild-type promoter and/or that the A-34T mutation increases the affinity of σ<sup>70</sup> binding more than C-32A. The A-34T change leads to the sequence TTGCGT. The TTG in the −35 region seem to be the most important nucleotides of the −35 hexamer as these are the most highly conserved [46], which could explain the opposite phenotypes conferred by the C-32A and A-34T mutations.

Our previous study [11] showed that σ<sup>34</sup> crosslinks to trxB at position −11 but not at −34. Furthermore, Spx did not crosslink to any nucleotide tested. One possible reason for this negative result may be the limited positions tested in the crosslinking experiments. Within the three important regions found in this work, the nucleotide positions tested for crosslinking were −46 and −34 of trxB and −47, −35, and −30 of trxA. Only −34A of trxB and −35A of trxA reside within the AGCG sequence. Another possible reason for the negative result is that the modification of a nucleotide with azidophenacyl bromide might have interfered with the binding of either Spx or σ<sup>34</sup> to DNA. It would be worth revisiting the nucleotide-specific crosslinking study by focusing on the AGCA and AGCG sites, as well as the A-rich sequence, and
by confirming that the modified templates are transcriptionally active.

Direct interaction between protein and DNA can also be verified genetically by site-specific suppressor analysis. Earlier studies of the sigma subunit-DNA interaction [47,48] demonstrated that mutations of the third G of the −35 hexamer to either A or C, but not to T, were suppressed by the substitution of Arg58 in region 4 of σ70 with His. Similarly, the defect caused by substitution of the fifth C of the −33 hexamer with either T or G was compensated for by the substitution of Arg584 of σ70 with Cys or His. We have investigated whether the various single base substitutions of G at −44, C at −43, G at −33, or C at −32, as well as double mutation of either G at −44/−43 or C at −43/−32, were restored by introducing the R60H or R60C mutations in Spx; however, we could not detect any significant suppressing effect in any of the mutant combinations (M.M.N. and P.Z., unpublished results).

The σCTD of RNASp can make specific contact with the UP element sequence of certain promoters such as those of rRNA operons [49] and the spoVG gene of B. subtilis (Figure 9). Evidently, the σCTD in the Spx-σCTD complex engages the trnB DNA in a manner that is different from its interaction with the UP element, as Spx is unable to contact σCTD on the spoVG promoter fragment. Some of the σCTD residues required for UP element contact might also function in its interaction with Spx, and thus, may not be available for Spx-σCTD complex formation. The σ1 helix of σCTD contains part of the “261 element” that is required for DNA binding, and this helix also contains the essential Tyr residue for Spx interaction. Given that part of the σ1 helix interacts with Spx further suggests that only part of the DNA-binding surface in σCTD might be exposed for DNA recognition, and that interaction with Spx is required to complete the DNA-binding surface of the σCTD/Spx promoter recognition complex. Ye, and others, have successfully cocrystallized σCTD and oxidized Spx as previously reported [9,36]. The EMSA study presented here now opens a powerful approach for cocrystallization of the σCTD-Spx complex with DNA to study the ternary interaction.

Acknowledgments

We thank Shunji Nakano for providing σCTD.

Author Contributions

Conceived and designed the experiments: MN KJN RGB PZ. Performed the experiments: MN AAL CSZ KJN. Analyzed the data: MN AAL CSZ KJN. Wrote the paper: MN RGB PZ.

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