Requirement of the Zinc-Binding Domain of ClpX for Spx Proteolysis in *Bacillus subtilis* and Effects of Disulfide Stress on ClpXP Activity[▽]

Ying Zhang and Peter Zuber*

Environmental and Biomolecular Systems, OGI School of Science and Engineering,
Oregon Health and Science University, Beaverton, Oregon

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Spx, a transcriptional regulator of the disulfide stress response in *Bacillus subtilis*, is under the proteolytic control of the ATP-dependent protease ClpXP. Previous studies suggested that ClpXP activity is down-regulated in response to disulfide stress, resulting in elevated concentrations of Spx. The effect of disulfide stress on ClpXP activity was examined using the thiol-specific oxidant diamide. ClpXP-catalyzed degradation of either Spx or a green fluorescent protein derivative bearing an SsrA tag recognized by ClpXP was inhibited by diamide treatment in vitro. Spx is also a substrate for MecA/ClpCP-catalyzed proteolysis in vitro, but diamide used at the concentrations that inhibited ClpXP had little observable effect on MecA/ClpCP activity. ClpX bears a Cys4 Zn-binding domain (ZBD), which in other Zn-binding proteins is vulnerable to thiol-reactive electrophiles. Diamide treatment caused partial release of Zn from ClpX and the formation of high-molecular-weight species, as observed by electrophoresis through nonreducing gels. Reduced Spx proteolysis in vitro and elevated Spx concentration in vivo resulted when two of the Zn-coordinating Cys residues of the ClpX ZBD were changed to Ser. This was reflected in enhanced Spx activity in both transcription activation and repression in cells expressing the Cys-to-Ser mutants. ClpXP activity in vivo is reduced when cells are exposed to diamide, as shown by the enhanced stability of an SsrA-tagged protein after treatment with the oxidant. The results are consistent with the hypothesis that inhibition of ClpXP by disulfide stress is due to structural changes to the N-terminal ZBD of ClpX.

The ATP-dependent protease ClpXP plays an important role in protein quality control during developmental processes and in the cell's response to harmful physical and chemical agents (14, 18, 19). ClpX is one of several AAA+ Clp/Hsp100 family members in prokaryotes that function as molecular chaperones or as the substrate-binding ATPase subunits of multicomponent Clp proteases. ClpX functions as an “unfoldase” that can either disassemble stable macromolecular complexes or denature substrates for delivery to the proteolytic chamber formed by its Clp protease partner subunit, ClpP (57). ClpX can recognize one or more substrate amino acid sequences, which serve as tethering or degradation tags that interact with the ATPase protease subunit (2, 13). ClpX, as well as other Clp protease ATPase subunits, sometimes requires an adaptor to offer substrates to the ATP-dependent unfoldase (5, 9, 36, 48, 63). The affinity range for recognition tags and the partnering with adaptor proteins provide broad flexibility for substrate interaction that allows the Clp proteases to respond appropriately to the regulatory signals generated by changing environmental conditions and metabolic states that require turnover of specific protein substrates (57). ClpXP can target specific proteins for degradation, including the SsrA-tagged products of interrupted translation (20), the stationary-phase RNA polymerase sigma subunit of *Escherichia coli* (σ^S) (4, 68), proteins whose production is induced by

the SOS response (49), and proteins that function in phage development (6, 17, 29).

In the sporeforming bacterium *Bacillus subtilis*, there are several AAA+ unfoldases that function as subunits of Clp proteases, such as ClpX, ClpC, and ClpE (8, 31, 38, 39). ClpX is necessary for many of the late growth processes for which the bacterium is known, such as sporulation and competence development, and is also necessary for optimal growth in minimal medium and resistance to elevated temperature (14, 38, 40, 43). Aside from SsrA-tagged proteins (64), only a few specific protein substrates of ClpXP have been identified in *B. subtilis*. Recently, the Sda peptide, which controls sporulation in response to replication stress in *B. subtilis* (54, 56), was found to be a substrate for ClpXP (55). ClpXP has also been implicated in activation of the SigW regulon in *B. subtilis* on the basis of its requirement for complete degradation of the antisigma protein RsiW (66), as part of the cell's envelope stress response.

The transcriptional regulator Spx (69) is another ClpXP substrate that is under tight proteolytic control in cells of cultures undergoing unperturbed, exponential growth. The product of *spx* is a transcriptional regulator that functions in the disulfide stress response in *B. subtilis* by interacting with RNA polymerase to repress a variety of cellular process while activating the transcription of genes whose products function in alleviating the damage caused by thiol oxidation (45, 46, 69). Spx has also been implicated as a regulatory factor for virulence-related functions in *Staphylococcus aureus* (51) and *Listeria monocytogenes* (7). Expression of *spx* is controlled at several levels. Transcription from the *spx* P3 promoter is under the negative control of two oxidant-sensitive repressors, PerR and YodB (33, 34), and is also controlled transcriptionally by

* Corresponding author. Mailing address: Environmental and Biomolecular Systems, OGI School of Science and Engineering, Oregon Health and Science University, 20000 NW Walker Rd., Beaverton, OR 97006. Phone: (503) 748-7335. Fax: (503) 748-1464. E-mail: pzuber@ebs.ogi.edu.

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TABLE 1. *Bacillus subtilis* strains and plasmids

Strain or plasmid	Relevant genotype or properties	Source or reference
Strains		
LAB2876	<i>trpC2 pheA clpX::spc</i>	40
ORB6624	<i>trpC2 pheA thrC::pZY30 (pDG795-ClpX⁺)</i>	This study
ORB6648	<i>trpC2 pheA thrC::pZY31(pDG795-ClpX[C16S])</i>	This study
ORB6649	<i>trpC2 pheA thrC::pZY32(pDG795-ClpX[C35S])</i>	This study
ORB6628	<i>trpC2 pheA thrC::pZY30 (pDG795-ClpX⁺) clpX::spc</i>	This study
ORB6650	<i>trpC2 pheA thrC::pZY31(pDG795-ClpX[C16S]) clpX::spc</i>	This study
ORB6651	<i>trpC2 pheA thrC::pZY32(pDG795-ClpX[C35S]) clpX::spc</i>	This study
LAB545	<i>trpC2 pheA SPβc2del2::Tn917::pMMN92(srfA-lacZ)</i>	40
ORB6763	<i>trpC2 pheA SPβc2del2::Tn917::pMMN92(srfA-lacZ) clpX::spc</i>	This study
ORB6681	<i>trpC2 pheA SPβc2del2::Tn917::pMMN92(srfA-lacZ) thrC::pZY30(pDG795-clpX⁺) clpX::spc</i>	This study
ORB6682	<i>trpC2 pheA SPβc2del2::Tn917::pMMN92(srfA-lacZ) thrC::pZY32(pDG795-clpX[C16S]) clpX::spc</i>	This study
ORB6683	<i>trpC2 pheA SPβc2del2::Tn917::pMMN92(srfA-lacZ) thrC::pZY32(pDG795-clpX[C35S]) clpX::spc</i>	This study
ORB6701	<i>trpC2 pheA SPβc2del2::Tn917::pSN67(trxB-lacZ)</i>	This study
ORB6702	<i>trpC2 pheA SPβc2del2::Tn917::pSN67(trxB-lacZ) clpX::spc</i>	This study
ORB6703	<i>trpC2 pheA SPβc2del2::Tn917::pSN67(trxB-lacZ) thrC::pZY30(pDG795-clpX⁺) clpX::spc</i>	This study
ORB6704	<i>trpC2 pheA SPβc2del2::Tn917::pSN67(trxB-lacZ) thrC::pZY32(pDG795-clpX[C16S]) clpX::spc</i>	This study
ORB6705	<i>trpC2 pheA SPβc2del2::Tn917::pSN67(trxB-lacZ) thrC::pZY32(pDG795-clpX[C35S]) clpX::spc</i>	This study
Plasmids		
pLysS	Plasmid for production of T7 lysozyme	Stratagene
pPROEX-1	Plasmid for construction of His ₆ -tagged fusion	Life Technologies, Rockville, MD
pTKlac	Plasmid for construction of <i>lacZ</i> transcriptional fusion	44
pDG795	Plasmid for construction of transcriptional fusion into <i>thrC</i> locus	21
pTYB1~4	Cloning vector for IMPACT T7 system	New England BioLabs
pSN17	pPROEX-1 with <i>spx</i>	42
pMMN470	pTYB4 with <i>spx</i>	40
pSN3	pTYB1 with <i>mecA</i>	42
pClpC	pTYB2 with <i>clpC</i>	42
pClpP	pTYB1 with <i>clpP</i>	42
pGFP-ssrA	pPROEX-1 with GFP-ssrA	
pMMN92	pTKlacZ with <i>srfA-lacZ</i>	67
pSN67	pTKlacZ with <i>trxB-lacZ</i>	This study
pZY23	pUC18 with <i>clpX⁺</i> (positions -786 to +1856)	This study
pZY24	pUC18 with <i>clpX(C16S)</i> (positions -786 to +1856)	This study
pZY25	pUC18 with <i>clpX(C35S)</i> (positions -786 to +1856)	This study
pZY30	pUC18 with <i>clpX⁺</i> (positions -786 to +1856)	This study
pZY31	pDG795 with <i>clpX(C16S)</i> (positions -786 to +1856)	This study
pZY32	pDG795 with <i>clpX(C35S)</i> (positions -786 to +1856)	This study
pMMN509	pTYB1 with <i>clpX⁺</i>	45
pZY29	pTYB1 with <i>clpX(C16S)</i>	This study
pZY27	pTYB1 with <i>clpX(C35S)</i>	This study

other promoters of the *yjbC spx* dicistronic operon (1, 34, 60). The activity of the Spx protein is under the redox control of a thiol/disulfide switch involving its N-terminal CXXC motif that controls productive RNA polymerase interaction (44). Spx is also the substrate for ClpXP proteolysis (45), and in a *clpX* or *clpP* mutant, Spx protein accumulates to a high concentration, which is largely responsible for the severe detrimental effects conferred by a *clpX* or *clpP* mutation (16, 38, 40, 43). Spx is also a substrate for MecA/ClpCP in vitro (42, 47), but mutations in vivo in *mecA* or *clpC* do not significantly affect Spx protein levels. In the case of Spx, a recognition tag residing at the extreme C terminus of the Spx protein is required for ClpXP-dependent proteolysis (45).

Previously reported evidence suggested that higher Spx con-

centrations in cells undergoing disulfide stress might result from down-regulation of ClpXP-catalyzed Spx turnover (45). That ClpXP might be under redox control is suggested by the presence of an essential zinc-binding domain (ZBD) of the Cys4 variety (3). The N-terminal ZBD functions in dimerization, substrate recognition, and adaptor binding (52, 62, 65). It is also thought to function in directing the substrate to the proteolytic cavity formed by the heptameric rings of ClpP through an ATP-dependent ClpX conformational change. The Cys4 clusters, like those coordinating the Zn atom of ClpX, are sensitive to oxidizing agents, exposure to which results in release of Zn or a change in the conformation of the ZBD (28).

In this report, we show that ClpXP in vitro is hypersensitive to the thiol-specific oxidant diamide, while MecA/ClpCP shows

TABLE 2. Oligonucleotides

Oligonucleotide	Oligonucleotide sequence	Nucleotide positions ^a	Note ^b
oGL03-1	5'-TGCTCGTTCTCTGGAAAAACACAA-3'	Fw, <i>clpX</i> 36–59	<i>clpX</i> (C16S)
oGL03-2	5'-TTGTGTTTTTCCAGAGAACGAGCA-3'	Rv, <i>clpX</i> 59–36	<i>clpX</i> (C16S)
oGL03-3	5'-GGTGTATATATATCTGACGAATGTATC-3'	Fw, <i>clpX</i> 90–116	<i>clpX</i> (C35S)
oGL03-4	5'-ACATTTCGTACAGATATATACACC-3'	Rv, <i>clpX</i> 113–90	<i>clpX</i> (C35S)
oGL03-7	5'-ATGAGCGGATCCGCAATTCCTCTTTCA-3'	Rv, <i>clpX</i> 1856–1830	BamHI
oGL03-8	5'-CGCAAAAGGTACCGATGAAGAAGTGGAAAC-3'	Fw, <i>clpX</i> 786–757	KpnI
oGL03-11	5'-GCTACATCTTTGACTGAAGCTGGATA-3'	Fw, <i>clpX</i> 423–448	Sequence
oZY06-1	5'-GGGAATTCATATGTTTAAATTTAACGAGGAAA AAGGAC-3'	Fw, <i>clpX</i> 1–28	NdeI
oMN02-200	5'-TAATAAGCTCTTCCGCATGCAGATGTTTTATC-3'	Rv, <i>clpX</i> 1260–1236	SapI
oSN03-48	5'-GAATTCAGCGTTGGTTCAAGCATTGTAGGAC-3'	Fw, <i>trxB</i> –510–484	EcoRI
oSN03-49	5'-GCGGATCCTCTTTCAATCATTAATGTCG-3'	Rv, <i>trxB</i> +112–+92	BamHI

^a Fw, forward primer; Rv, reverse primer.

^b Mutation or restriction site created by the primer. oGL03-11 was used as a sequencing primer.

little diamide sensitivity. Diamide treatment causes a 45% loss in Zn content and causes ClpX protein to aggregate, while little diamide-induced aggregation is observed in the case of ClpC. Mutations that change two of the Zn-coordinating Cys residues to Ser reduce Spx proteolysis in vitro and confer high Spx concentration and activity in vivo. A model in which the N-terminal ZBD of ClpX is required for Spx proteolysis and is the site of oxidant-induced protease inactivation is proposed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Bacillus subtilis* strains used in this study are derivatives of JH642 and are listed in Table 1. *B. subtilis* cells were cultured in a shaking water bath at 37°C in Difco sporulation medium (DSM) (58) for β -galactosidase assays or TSS minimal medium (53) for diamide treatment experiments and genotype verification. Diamide was purchased from Sigma.

For complementation experiments, ectopically expressed *clpX* alleles were constructed as follows. Primers oGL03-7 and oGL03-8 (Table 2) were used to amplify the *clpX* gene from *B. subtilis* strain JH642 chromosomal DNA. To generate pZY23, the PCR fragment (positions –786 to +1856, about 2,642 bp, including 1,260 bp of the coding region of *clpX* as well as 786 bp of upstream sequence and 596 bp of downstream sequence) was digested with KpnI and BamHI and then ligated with pUC18 that had been digested with the same enzymes. The *clpX* sequence in plasmid pZY23 was verified by DNA sequencing. Plasmid pZY23 was cleaved with KpnI before treatment with T4 DNA polymerase (New England BioLabs) to create a blunt end and then further digested with BamHI to release the *clpX* fragment. To generate pZY30, the fragment was ligated with pDG795 (21) that was digested with EcoRI before treatment with T4 DNA polymerase (New England BioLabs) to create blunt ends and then further digested with BamHI. Plasmid pZY30 was introduced by transformation, with selection for erythromycin/lincomycin and screening for threonine auxotrophy, into *B. subtilis* strain JH642, where the *clpX* fragment was integrated into the *thrC* locus. The resulting strain was designated ORB6624 (*thrC::clpX*). Chromosomal DNA of LAB2876 (*clpX::spe*) was used to transform ORB6624 to generate ORB6628 (*clpX::spe thrC::clpX*⁺). Mutant *clpX*(C16S) and *clpX*(C35S) alleles were constructed by PCR-based site-directed mutagenesis. The first round of PCR was performed by using pZY23 (pUC18-*clpX*⁺) plasmid DNA as the template, with primers oGL03-2 (oGL03-4) and oGL03-8 for the upstream fragment of *clpX*(C16S) [*clpX*(C35S)] and primers oGL03-1 (oGL03-3) and oGL03-7 for the downstream fragment of *clpX*(C16S) [*clpX*(C35S)]. Two PCR fragments, purified on low-melting-point agarose gels, were mixed and used as templates for the second PCR, with primers oGL03-7 and oGL03-8, to generate the full-length fragment (positions –786 to +1856) bearing the desired mutant allele. The same second-stage PCR procedure was used to create pZY24 [pUC18 with *clpX*(C16S)], pZY25 [pUC18 with the *clpX*(C35S) allele], pZY31 [pDG795 with the *clpX*(C16S) allele], and pZY32 [pDG795 with the *clpX*(C35S) allele]. The sequences of the *clpX* mutant alleles in pZY24 and pZY25 were verified by DNA nucleotide sequencing. The plasmids were introduced by transformation into JH642 to create the *thrC::pZY31*- and *thrC::pZY32*-bearing strains. These strains were then transformed with DNA from the *clpX::spe*-null mutant, strain

LAB2876, yielding the mutant complementation strains ORB6650 (*clpX::spe thrC::pZY31*) and ORB6651 (*clpX::spe thrC::pZY32*).

The plasmids used for mutant ClpX protein production and purification were constructed by PCR with primers oZY06-1 and oMN02-200 (Table 2), using either pZY24 or pZY25 as the template. To create pZY29 [pTYB1 carrying the *clpX*(C16S) allele] and pZY27 [pTYB1 with *clpX*(C35S)], the PCR fragment was digested with NdeI and SapI and then ligated with pTYB1 that was digested with the same restriction enzymes. The mutant *clpX* allele sequences in plasmids pZY27 and pZY29 were verified by DNA sequencing.

The promoter region of *trxB* was amplified by PCR with primers oSN03-48 and oSN03-49 from JH642 chromosomal DNA. To generate pSN67, the resulting PCR fragment was digested with BamHI and EcoRI and then ligated with plasmid pTKlac that was digested with the same restriction enzymes. Plasmid pSN67 (*trxB-lacZ* fusion) was used to transform cells of strain ZB307A (70) with selection for chloramphenicol resistance. An SP β -transducing lysate was produced by heat induction and was then used to transduce cells of strain ZB278 (70). Phage generated from lysogens of this strain was used to transfer the *trxB-lacZ* fusion into a wild-type background by transduction into JH642 with selection for chloramphenicol resistance to generate strain ORB6701.

Production and purification of proteins. For production of proteins used in this study, the IMPACT system (New England BioLabs), which utilizes the inducible self-cleaving intein tag, was used. Intein-tagged ClpX, ClpP, MecA, and ClpC were purified using a previously reported procedure (42, 45–47). Intein-tagged Spx was purified by using a procedure described previously (42). His₆-tagged wild-type, C10A Spx, and His₆-green fluorescent protein (GFP)-SsrA proteins were purified using a previously published procedure (44).

Transformation and transduction. Preparation of competent cells of *B. subtilis* and DNA-mediated transformation were carried out as described previously (11, 25, 50). Specialized transduction using SP β phage constructs was carried out as described previously (70).

Spx protein stability. Total-protein extracts were prepared from cultures of wild-type *B. subtilis* JH642 grown in TSS liquid media. When the optical density at 600 nm (OD₆₀₀) reached 0.5, the culture was split, and one subculture was treated with 1 mM (final concentration) diamide, while the other was left untreated. After 10 min, each subculture was split again, and to one, 0.1 mg/ml (final concentration) chloramphenicol was added. Samples (3 ml) were taken at the indicated time points and centrifuged. Cells were then treated with protoplast buffer (20 mM potassium phosphate, pH 7.5; 15 mM MgCl₂; 20% sucrose; 1 mg/ml lysozyme) for 30 min and centrifuged. The protoplasts were then suspended in lysis buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total protein (30 μ g) from each sample was applied to a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and electrophoresis was performed. The protein levels of Spx were examined by Western blot analysis using anti-Spx antiserum (40), followed by incubation with the secondary antibody conjugated to alkaline phosphatase.

Assay of β -galactosidase activity. Cells were grown in DSM medium until the OD₆₀₀ was $\approx$ 0.4 to 0.5. The cells were incubated further for 3 h, during which time samples were collected every 30 min and prepared for β -galactosidase assays. β -Galactosidase activity was determined as previously described (41) and is presented in Miller units (37).

Western blot analysis. The total-protein extracts were prepared from cells of *B. subtilis* cultures grown in DSM. Samples (1 ml) were taken at the indicated time points and centrifuged. Cells were then treated with protoplast buffer (20

FIG. 1. Effect of diamide on protein level of ClpX and Spx in wild-type and *clpX* cells. Cells were grown in DSM until mid-exponential phase and then were treated with 1 mM diamide or left untreated. Samples were taken at 0 (T₀), 10, 20, and 50 min (A) or 0 and 30 min (B) after treatments. Cells were lysed with protoplast buffer and were suspended in lysis buffer (see Materials and Methods). Thirty micrograms of protein from each sample was applied to an SDS-polyacrylamide gel for electrophoresis. The protein levels of Spx or ClpX were examined by Western blot analysis using rabbit anti-Spx or anti-ClpX antiserum. (A) Western blot analysis of ClpX and Spx levels. The wild-type strain JH642, which was grown in DSM until mid-exponential phase, was treated with or without 1 mM diamide. Samples were taken at 0, 10, 20, or 50 min after treatments. (B) Western blot analysis of ClpX and Spx levels in wild-type (wt) and *clpX* strains. Results for strains JH642 (wild type) and ORB 2876 (*clpX*) are shown. Samples were collected 0 and 30 min after diamide treatment.

mM K-phosphate, pH 7.5; 15 mM MgCl₂; 20% sucrose; 1 mg/ml lysozyme) for 30 min and centrifuged. The protoplasts were then suspended in lysis buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total protein (30 μg) from each sample was applied to an 8% (for ClpX and ClpC), 15% (for Spx), or 12% (for HrcA) SDS-polyacrylamide gel, and electrophoresis was performed. The protein levels of Spx, ClpX, and ClpC were examined by Western blot analysis using anti-Spx, anti-ClpX, or anti-HrcA (64) antiserum, followed by incubation with the secondary antibody conjugated to alkaline phosphatase.

In vitro ClpXP-catalyzed proteolysis reaction. In vitro proteolysis reaction mixtures were assembled under conditions described previously (45), with some modifications. The reactions were carried out in 50 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM Mg acetate, 5 mM dithiothreitol (DTT) (unless diamide or H₂O₂ was added as indicated), 5 mM ATP, 5 mM creatine phosphate, 0.05 U/ml creatine kinase (Sigma), and Spx (6 μM) or GFP-SsrA (3 μM). Fifty-microliter reaction mixtures were incubated at 37°C in the presence of ClpP (12 μM) and ClpX (6 μM) or ClpC (2.5 μM), ClpP (4 μM), and MecA (2.5 μM). At time intervals, a 10-μl sample from each reaction mixture was collected and treated with 2 μl stop buffer (SDS-loading dye in 0.1 M DTT). The proteins were then resolved on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel, followed by staining with Coomassie blue. Levels of Spx were defined as ratios of Spx band intensity to ClpP band intensity, since ClpP concentrations in all reactions were equal. The Spx/ClpP ratio in a reaction mixture containing no ClpX was given the value 100%.

RESULTS

The Spx protein of *B. subtilis* is a substrate for the ATP-dependent protease ClpXP in vivo and in vitro, and it accumulates to a high concentration in *clpX* and *clpP* mutant cells (40, 45, 46). Spx concentration also increases in response to disulfide stress brought about by treatment of cells with the thiol-specific oxidant diamide (45). Figure 1A shows the result of a Western blot experiment in which Spx protein levels were examined in cells of a culture treated with diamide and in cells of an untreated culture. As observed previously (45), Spx concentration is higher in the diamide-treated cells than in the

FIG. 2. Western blot analysis of Spx protein stability in cells of cultures treated with diamide and chloramphenicol (ch). The wild-type strain JH642, which was grown in TSS media until the OD₆₀₀ was 0.3, was treated with or without 1 mM diamide, and after 10 min, the culture was split into two subcultures of equal volumes. Chloramphenicol (0.1 mg/ml) was added one of the subcultures. Samples were taken at 10, 30, 60, and 90 min after diamide treatment. Cells were harvested by centrifugation and lysed by the protoplast method. The protein extracts were applied to an SDS-polyacrylamide gel for electrophoresis and then blotted for Western analysis using anti-Spx antiserum. A sample of strain ORB 3834 (*spx::neo*) was taken at an OD₆₀₀ of 0.5 from TSS media. The lower panel is a plot of Spx band intensity versus time. The Spx amount at 10 min without diamide treatment is denoted as 1. Standard deviations on the plot were obtained from three independent experiments.

untreated cells. Spx concentration is also high in a *clpX* mutant (Fig. 1B), suggesting that the increase in Spx concentration observed in diamide-treated cells could be due to a reduction in ClpXP activity, thus creating a condition resembling the phenotype of a *clpX*-null mutant.

The stability of Spx protein in diamide-treated cells was examined to determine if the elevated concentration of Spx during oxidative stress was attributable to posttranslational control. *B. subtilis* cells of strain JH642 were grown in TSS medium until mid-log phase. One culture was treated with diamide, and the other was left untreated. The two cultures were split, and one was treated with chloramphenicol. Protein extracts were obtained by lysozyme treatment in protoplast buffer and were applied to an SDS-polyacrylamide gel for electrophoresis. Western blot analysis was performed using anti-Spx antiserum. In the culture that was not treated with diamide, the level of Spx protein remained constant in untreated cells, but the protein disappeared after chloramphenicol treatment (Fig. 2), indicating low Spx stability. However, in the diamide-treated culture, subsequent treatment with chloramphenicol did not result in a significant reduction in Spx concentration (Fig. 2). These results suggest that there is a down-regulation of Spx proteolysis during oxidative stress, possibly directed at ClpXP.

FIG. 3. Strains JH642 (wild type [wt]), ORB 4381 (*lacA::hrcA-ssrA* [HrcA-AA]), ORB 4382 (*lacA::hrcA-ssrA*ΔDD [HrcA-DD]), and ORB 4383 (*lacA::hrcA-ssrA* [HrcA-AA] *clpX*::Spc) were grown in DSM at 37°C with shaking. Samples were taken when the OD₆₀₀ was 0.5, and each culture was treated with 1 mM diamide. Samples were taken 30 min after diamide treatment. Cells were lysed with protoplast buffer and were suspended in lysis buffer (see Materials and Methods). Thirty micrograms of protein from each sample was applied to an SDS-polyacrylamide gel for electrophoresis. The protein levels of HrcA were examined by Western blot analysis using anti-HrcA antibody.

Diamide treatment causes increase in SsrA-tagged protein concentration. The *in vivo* activity of ClpXP in diamide-treated *B. subtilis* cells was next examined using an artificial ClpXP substrate, HrcA-SsrA, constructed as previously reported (64). The *hrcA-ssrA* allele is transcribed from a constitutive promoter (promoter of the *hrcA-dnaK* operon without the CIRCE [controlling inverted repeat of chaperone expression] operator

FIG. 4. Effect of diamide and H₂O₂ on ClpXP-catalyzed proteolysis of Spx in vitro. Spx (6 μM), ClpX (6 μM), and ClpP (12 μM) were incubated at 37°C in the presence of ATP and an ATP-regenerating system (creatine kinase) with 5 mM DTT, diamide, or H₂O₂ in a proteolysis reaction buffer containing 50 mM HEPES-KOH (pH 7.6), 50 mM KCl, and 10 mM Mg acetate as described in Materials and Methods. Ten-microliter samples were taken at 0, 10, 20, and 30 min, and the reactions were stopped by mixing with 2 μl SDS-loading dye containing 0.1 M DTT. Samples were analyzed by SDS-PAGE, followed by staining with Coomassie blue. Creatine phosphate kinase (CrPK; 0.05 U/μl), 5 mM ATP, and 5 mM creatine phosphate were used as an ATP-regenerating system. The plot of Spx band intensities against time of reaction was derived from two repeats of the experiment. The intensities of ClpP protein in each reaction were used as internal controls. The Spx/ClpP ratio in the reaction without ClpX was referred to as 100%.

FIG. 5. Effect of diamide and H₂O₂ on ClpXP-catalyzed proteolysis of GFP-SsrA in vitro. GFP-SsrA (6 μM), ClpX (6 μM), and ClpP (12 μM) were incubated at 37°C in the presence of ATP and an ATP-regenerating system with 5 mM DTT, diamide, or H₂O₂ as described in Materials and Methods. Ten-microliter samples were taken at 0-, 15-, 30-, and 60-min time points. The plot of GFP-SsrA/ClpP band intensity ratios against time of reaction was derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as an internal control. The GFP-SsrA/ClpP ratio in the reaction without ClpX was referred to as 100%.

elements [64]) and integrated in the *lacA* gene. Removal of the CIRCE elements eliminates the transcriptional and posttranscriptional control of the transcript synthesized from the *hrcA-dnaK* operon promoter (26, 59). Cells of wild-type (JH642) and *lacA::hrcA-ssrA* cells were grown in DSM to mid-log phase and then split into two cultures, one treated with diamide and the other untreated. After 30 min of incubation, a sample was taken for western analysis. In JH642, HrcA protein increases in concentration after diamide treatment (Fig. 3), which is expected because the *hrcA* gene transcription is induced eightfold by diamide treatment (35). HrcA-SsrA (HrcA-AA) protein is undetectable in a *clpX*⁺ background but is observed to increase in concentration after diamide treatment. A protease-resistant form of the HrcA-SsrA (HrcA-DD) protein, bearing two aspartyl residues at the extreme C terminus instead of the alanine residues normally found in the SsrA peptide, is observed to be present in both diamide-treated and untreated cells. Likewise, in *clpX* mutant cells, the HrcA-SsrA-tagged product (HrcA-AA) is observed in both untreated and diamide-treated cells. The increased levels of the product encoded by the *hrcA-ssrA* allele after diamide treatment strongly suggest that ClpXP activity is reduced in *B. subtilis* cells exposed to a toxic oxidant.

ClpXP activity in vitro is reduced in the presence of oxidant. Spx is degraded by ClpXP in vitro (Fig. 4A) (45), but ClpXP-catalyzed proteolysis of Spx is reduced in the presence of diamide or hydrogen peroxide (Fig. 4A). While ClpXP-catalyzed proteolysis reduces Spx concentration 90%, there was little reduction in Spx protein levels in reaction mixtures containing diamide, and there was less than 20% reduction of Spx in reaction mixtures treated with H₂O₂ (Fig. 4B). This effect of diamide on ClpXP-catalyzed proteolysis is not substrate spe-

FIG. 6. Effect of diamide on ClpCP proteolysis of Spx in vitro. Spx (8 μ M), ClpC (2.5 μ M), ClpP (4 μ M), and MecA (2.5 μ M) were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT, diamide, or H_2O_2 as described in Materials and Methods. Samples (10 μ l) were taken at 0-, 2-, 5-, and 10-min time intervals. The plot of Spx/ClpP band intensity ratios versus time was derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as an internal control. The Spx/ClpP ratio in the reaction without ClpX was referred to as 1.00.

cific, since an SsrA-tagged derivative of GFP (His₆-Gfp-SsrA), a substrate for ClpXP in vitro (Fig. 5), is degraded at a reduced rate when the reaction mixture contains diamide. Hydrogen peroxide addition also reduces ClpXP-catalyzed proteolysis of His₆-Gfp-SsrA (Fig. 5).

The ATP-dependent protease ClpCP/MecA can utilize Spx as a substrate in vitro (42, 47), but mutations in *clpC* or *mecA* have little effect on the in vivo concentration of Spx. Figure 6 shows that Spx concentration is sharply reduced in proteolytic reaction mixtures containing ClpCP/MecA. Addition of diamide at concentrations that inhibit proteolysis of Spx by ClpXP has little effect on ClpCP/MecA-catalyzed proteolysis, showing only an initial reduction in the reaction when the oxidant is present. While ClpC is structurally similar to ClpX, being a member of the HSP100/Clp family of proteins, ClpC does not possess a ZBD.

The addition of diamide to a reaction mixture containing Spx will likely create a disulfide linkage between Cys10 and Cys13 at the N-terminal redox disulfide center of Spx (44). It was possible that the formation of the disulfide bond could render Spx resistant to ClpXP-catalyzed proteolysis. The SpxC10A mutant protein (44) was tested to see if it could be degraded in the presence or absence of diamide. Figure 7 shows that SpxC10A was degraded by ClpXP but not when the reactions contained diamide, showing that SpxC10A exhibited the same resistance to proteolysis as wild-type Spx. This result,

FIG. 7. Effect of diamide on ClpXP proteolysis of wild-type (WT) Spx and C10A Spx in vitro. Wild-type Spx or 6 μ M C10A Spx, 6 μ M ClpX, and 12 μ M ClpP were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT or diamide in a proteolysis reaction buffer described in Materials and Methods. Samples (10 μ l) were taken at 0-, 10-, 20-, and 30-min time points. The plot of Spx/ClpP band intensity ratios against time of reaction was derived from triplicate experiments. The intensities of ClpP protein in each reaction were used as an internal control. The Spx/ClpP ratio in the reaction without ClpX was referred to as 100%.

along with the results shown in Fig. 5, indicated that diamide was affecting protease activity rather than changing the structure of the substrate, thereby rendering Spx protease resistant.

Amino acid substitutions in the ZBD of ClpX reduce Spx proteolysis by ClpXP. The ZBD of ClpX is a likely target for direct oxidant-dependent inactivation of ClpXP protease. To determine if the ZBD is required for Spx proteolysis, amino acid substitutions in the Cys4 Zn-binding cluster were created by in vitro PCR mutagenesis and the products of the resulting alleles were tested for activity in vitro and in vivo. Cysteine-to-serine substitutions were created at positions 16 and 35 of the ClpX N-terminal ZBD (Fig. 8A).

The alleles encoding the mutant ClpX proteins were introduced into the *thrC* locus of the *clpX* null mutant, bearing either a *trxB-lacZ* fusion or a *srfA-lacZ* fusion, to determine if they could complement *clpX* with respect to Spx activity. The *trxB* gene (encoding thioredoxin reductase) is positively controlled by Spx (44, 45) and is expressed at a high level in a *clpX* mutant due to the accumulation of Spx protein. The introduction of a wild-type allele to an ectopic location (the *thrC* locus) resulted in reduced *trxB-lacZ* expression relative to that of the *clpX* mutant (Fig. 8B). The introduction of either the C16S or the C35S allele of *clpX* into the *thrC* locus of the *clpX trxB-lacZ* strain resulted in high levels of expression, similar to that observed in the *clpX* mutant, indicating a failure of either ZBD mutant allele to complement *clpX*.

The *srf* operon is repressed in a *clpX* mutant due to the

FIG. 8. Effect of ZBD mutations of *clpX* on Spx-dependent regulation of *srf* and *trxB* transcription. (A) Diagram of C4 type ZBD sequence showing the CXXC and CXXCXXXC motifs. Also shown are the Cys16 and Cys35 positions that were changed to Ser. (B) The left panel shows measurement of β -galactosidase activity in a time course experiment of cultures of *trxB-lacZ*-bearing cells in either a wild-type (WT; $\blacklozenge$), *clpX* ($\blacksquare$), *clpX thrC::clpX⁺* ($\diamond$), *clpX thrC::clpX(C16S)* ($\square$), or *clpX thrC::clpX(C35S)* ($\triangle$) background. Data were from three independent experiments. The right panel shows measurement of β -galactosidase activity in a time course experiment of cultures of *srf-lacZ*-bearing cells in either wild-type ($\blacklozenge$), *clpX* ($\blacksquare$), *clpX thrC::clpX⁺* ($\diamond$), *clpX thrC::clpX(C16S)* ($\square$), or *clpX thrC::clpX(C35S)* ($\triangle$) backgrounds. Data shown are from three independent experiments. (C) Examination of ClpX and Spx protein levels in JH642 (wild type), LAB 2876 (*clpX*), ORB 6624 (*clpX thrC::clpX⁺*), ORB 6648 [*clpX thrC::clpX(C16S)*], or ORB 6649 [*clpX thrC::clpX(C35S)*] by Western blot analysis. Cultures grown in DSM until mid-exponential phase were treated with or without 1 mM diamide or H₂O₂. Samples were collected after 30 min and were suspended in protoblast buffer followed by resuspension in lysis buffer (see Materials and Methods). Protein (30 μ g) from each sample was applied to SDS-polyacrylamide gel for electrophoresis. The levels of Spx or ClpX protein were determined by Western blot analysis using rabbit anti-Spx or anti-ClpX antiserum.

accumulation of Spx, which blocks ComA-dependent activation of the *srf* operon (45, 46, 67). Expression of the *srf-lacZ* fusion is repressed in a *clpX* mutant (Fig. 8B), but the *clpX*-null mutation can be complemented by the wild-type copy of the *clpX* gene, as shown by the increase in *srf-lacZ* expression (Fig. 8B). The introduction of either the C16S or C35S allele of *clpX* into an ectopic position (the *thrC* locus) within the *clpX* mutant

genome fails to increase *srf-lacZ* expression, indicative of a defect in Spx proteolysis.

The Western blot in Fig. 8C shows that SpX protein levels are low in wild-type cells and in cells of the *clpX⁺/clpX* merodiploid strain but are high in the *clpX* mutant and in cells of the *clpX* (*C16S*)/*clpX* and *clpX* (*C35S*)/*clpX* strains, confirming that the ZBD mutants of ClpX are unable to participate in proteolytic

FIG. 9. Effect of ZBD mutations of *clpX* on ClpXP-catalyzed proteolysis of Spx in vitro. (A and B) Spx (6 μ M), ClpX (6 μ M; wild type [WT], C16S, or C35S), and ClpP (12 μ M) were incubated at 37°C in the presence of ATP and 5 mM DTT (A) or 5 mM diamide (B) in proteolysis reaction buffer as described in Materials and Methods. Samples (10 μ l) were collected at 0, 10, 20, and 30 min, and the reactions were stopped by mixing with 2 μ l SDS-loading dye containing 0.1 M DTT. Samples were analyzed by SDS-PAGE, followed by staining with Coomassie blue. Creatine phosphate kinase (CrPK; 0.05 U/ μ l) 5 mM ATP, and 5 mM creatine phosphate were used as an ATP-regenerating system. (C) Plot of Spx band intensities against time derived from triplicate experiments. Values of Spx levels were determined as described for Fig. 4 to 7.

turnover of Spx. ClpX protein was detected in the wild-type and mutant merodiploid strains, although somewhat lower levels were produced in the C-to-S-mutant-producing cells (Fig. 8C).

Proteolysis reaction mixtures containing either wild-type ClpXP or protease-bearing C-to-S mutant versions of ClpX showed that the mutant enzymes were defective in utilizing Spx protein as a substrate in vitro. The addition of diamide to the reaction reduced proteolysis of Spx in reaction mixtures containing wild-type ClpXP enzyme, but little effect of diamide treatment was detected in the reactions of ZBD mutant ClpXP, the activity of which was already compromised (Fig. 9B and C).

Diamide treatment results in aggregation of ClpX protein.

The Zn content of wild-type and mutant proteins was examined by pyridyl azo resorcinol (PAR) staining of ClpX protein resolved on polyacrylamide gels (data not shown). The wild-type ClpX protein showed reduced Zn content after diamide treatment, as judged from the band intensity after PAR staining. No detectable PAR staining was observed in lanes

containing mutant ClpX(C35S) protein. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis of untreated and diamide-treated ClpX showed a 45% reduction in Zn content after diamide treatment. The mutant ClpX(C35S) and ClpX(C16S) proteins had only 33% of the Zn content found in wild-type ClpX, as determined by ICP-OES. The modest reduction in Zn content after diamide treatment suggested that Zn release upon Cys oxidation provides a partial explanation for the reduction in ClpXP activity after treatment with the thiol-specific oxidant.

The ClpX protein was again examined in proteolysis reaction mixtures containing the Spx substrate and treated with various concentrations of diamide (Fig. 10). A concentration of 8 μ M diamide results in a 50% reduction in Spx proteolysis after 10 min (Fig. 10A). ClpX protein treated with diamide at the concentrations indicated caused a reduction in the intensity of the ClpX band in nonreducing SDS-polyacrylamide gels, with the appearance of slower-migrating bands (Fig. 10B). Western analysis using anti-ClpX antibody shows that ClpX protein aggregates upon diamide treatment, as shown by the appearance of anti-ClpX-reacting proteins migrating slowly on the nonreducing SDS-polyacrylamide gel (Fig. 10B, lower panel).

The effects of the oxidants on ClpX protein were examined, this time treating *B. subtilis* log phase cells with the same concentrations of diamide and H₂O₂ as were used in the proteolysis reactions. Western analysis of the protein extracts was performed using either anti-ClpX or anti-ClpC antisera. Treatment with increasing concentrations of diamide resulted in a reduction in the intensity of the ClpX band and the appearance of higher-molecular-weight material reacting with anti-ClpX antibody (Fig. 10C, upper panel). Some decrease in the ClpX band intensity is observed with H₂O₂, but with less higher-molecular-weight material observed. ClpC protein is affected only slightly by treatment with diamide and H₂O₂ (Fig. 10C, lower panel), with some higher-molecular-weight material reacting with anti-ClpC antibody at higher concentrations of diamide. The data suggest that ClpX protein is induced to aggregate in vivo after treatment with oxidants. This and the reduction in Zn content observed in vitro could contribute to reduced ClpX activity.

DISCUSSION

In previously reported work, we had shown that Spx protein concentration increases 30 min after diamide treatment of *B. subtilis* cells (45). This can be explained in part by the increase in transcription of the *spx* gene resulting from inactivation of the YodB and PerR (32) repressors upon oxidative stress (33, 34). However, replacement of the promoter region of *spx* with an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter did not render Spx production constitutive, as shown by a dramatic increase in Spx protein concentration after diamide treatment (Fig. 2) (45). A posttranslational mechanism of Spx control involving the ClpXP protease was proposed (69).

No adaptor protein for recognition of Spx for ClpXP-catalyzed proteolysis has been reported. However, von Wachenfeldt and coworkers have reported that a mutation in the gene *yjbH* of *B. subtilis* results in an increase in Spx concentration without affecting *spx* transcript levels, suggestive of a role in

proteolytic control of Spx (31a). We note that the rate of proteolysis as catalyzed by ClpXP in vitro is much less than that of ClpCP/MecA, while ClpXP appears to be a primary determinant of Spx stability in vivo. Hence, it is reasonable to propose that ClpXP might require a cofactor/adaptor for degradation of Spx and perhaps other substrates in *B. subtilis*. YjbH or a factor under its control could serve as an adaptor for Spx degradation by ClpXP.

The ZBD of ClpX has been implicated in substrate and adaptor interaction as well as ClpX multimerization (52, 62, 65). Recent studies of *E. coli* ClpX indicate that the N-terminal section of ClpX, which includes the ZBD, undergoes dramatic, ATP-dependent changes in its position within the ClpXP complex (61). These studies suggest a model in which the N-terminal domain of ClpX functions in the introduction of bound substrate into the proteolytic cavity formed by the ClpP heptameric rings. The ZBD contains a Cys-4 type Zn-binding motif that is sensitive to thiol-reactive compounds (3). Zn release has been reported to affect ATP binding, interaction with ClpP, and oligomerization, which are also observed when the four Cys residues that coordinate Zn are changed to Ser (3).

In the work reported here, treatment with the thiol-specific oxidant diamide resulted in a severe reduction in ClpXP-cat-

FIG. 10. Diamide dose-dependent inhibition of ClpXP proteolysis of Spx in vitro. (A) Spx (6 μ M), ClpX (6 μ M), and ClpP (12 μ M) were incubated at 37°C in the presence of ATP and an ATP-generating system with 5 mM DTT or various concentrations of diamide in 10 μ l proteolysis reaction buffer as described in Materials and Methods. Two microliters of SDS-loading dye containing 0.1 M DTT was mixed with the reaction mixture after 10 min. Samples were analyzed by SDS-PAGE, followed by staining with Coomassie blue. The plot of Spx band intensities against times of reaction was derived from triplicate experiments, and the values were determined as described for Fig. 4 to 7. (B) ClpX protein (10 μ l, 6 μ M) treated with DTT or various concentrations of diamide (lanes 1 and 2, 5 mM DTT; lane 3, H₂O; lanes 4 to 10, 0.32, 1.6, 8, 40, 200, 1,000, and 5,000 μ M diamide, respectively) was applied to nonreducing SDS-polyacrylamide gels. The 5 mM DTT-treated sample (lane 1) was mixed with SDS-loading dye containing 0.1 M DTT. The samples in lanes 2 to 10 were mixed with SDS-loading dye without DTT. Each sample was heated at 90°C for 2 min before loading. The 8% SDS-polyacrylamide gel was stained with Coomassie blue. Diamide treatment of ClpX at the indicated concentrations reduced the ClpX band intensities in nonreducing SDS-polyacrylamide gels, causing slower migration of bands (*). The ClpX protein in panel B was detected by Western blot analysis of the SDS-polyacrylamide gel with anti-ClpX antiserum (lower panel). (C) Western blot analysis of ClpX and ClpC in wild-type cells treated with oxidants. Cells of strain JH642 (wild type) were grown in DSM until mid-exponential phase and then treated with various concentrations of DTT, diamide, or H₂O₂ (lanes 1 and 2, 5 mM DTT; lane 3, H₂O; lanes 4 to 8, 0.5, 5, 50, 500, and 5,000 μ M diamide, respectively; lanes 9 to 13, 0.5, 5, 50, 500, and 5,000 μ M H₂O₂, respectively). Cells were lysed with protoplast buffer, followed by treatment with lysis buffer as described in Materials and Methods. Protein (30 μ g) was applied to nonreducing SDS-polyacrylamide gels. The sample in lane 1 was treated with 5 mM DTT and mixed with SDS-loading dye containing 0.1 M DTT. All the samples applied to lanes 2 to 13 were mixed with SDS-loading dye without DTT. Each sample was heated at 90°C for 2 min before loading. The ClpX and ClpC protein on an 8% SDS-polyacrylamide gel were detected by Western blot analysis with rabbit anti-ClpX or anti-ClpC antiserum. Molecular mass markers are labeled in kDa.

alyzed Spx degradation in vitro and accumulation of Spx as well as an SsrA-tagged protein in vivo. The concentrations of diamide used did not affect the activity of ClpCP/MecA, which can also utilize Spx as a substrate. Hydrogen peroxide inhibited ClpXP activity in vitro, but little effect was observed for Spx concentration in vivo, perhaps because of the multiple mechanisms possessed by the cell for removing H₂O₂ (10, 12, 15). The diamide effect was not due to enhanced resistance of oxidized Spx to proteolysis, as a C10A mutant, defective in disulfide bond formation at the redox disulfide center of Spx, also shows reduced proteolysis by ClpXP in the presence of diamide. Furthermore, His₆-GFP-SsrA, another substrate of ClpXP, is not degraded in ClpXP reactions containing diamide. A similar loss of proteolytic activity is observed when either of two Cys residues of the Cys-4 Zn-binding motif is changed to Ser. The replacement of either of the two Cys residues results in a significant loss of Zn, as shown by Zn-specific staining of SDS-PAGE gels with PAR (data not shown) and by ICP-OES (33% Zn content in the mutant protein compared to the wild-type level). Diamide has two discernible effects on wild-type ClpX protein. First, it causes an approximately 45% reduction in Zn content, as determined by ICP-OES. Second, diamide treatment leads to the formation of higher-molecular-weight forms of ClpX, suggestive of aggregate formation. Aggregates of ClpX protein are observed along with a disappearance of monomeric ClpX after treatment with increasing concentrations of diamide (Fig. 10B), while fewer higher-molecular-weight forms of ClpC are observed on gels of diamide-treated ClpC protein preparations. Western blot analysis of *B. subtilis* soluble protein extracts shows a similar result after diamide treatment (Fig. 10C). The results suggest that ClpX, unlike ClpC, undergoes structural changes upon exposure to thiol-reactive compounds that correlate with reduced activity. ClpX bears seven cysteine residues, five of which reside in the ZBD. ClpC and MecA proteins contain a single Cys residue each, which likely does not participate significantly in proteolytic activity or is not accessible to thiol-reactive azo-bearing compounds, such as diamide.

Exposure of Cys4 ZBDs, such as that of GATA-1, to thioester-forming electrophiles results in efficient displacement of zinc (28). This is not the case for some Cys2-His-Cys or Cys2-His2 ZBDs, which show resistance and retain the Zn atom after treatment with electrophile. Resistance is thought to be due in part to the substitution of a thiolate for a coordinating histidine and to secondary interactions involving residues surrounding the metal-binding site. Studies of the vulnerability of ZBDs to thiol-reactive agents showed that Cys4 ZBDs, such as the one occupying the N-terminal domain of ClpX, might be particularly sensitive to oxidation (28). As mentioned above, the treatment of ClpXP with diamide results in a modest reduction (45%) in Zn content. However, reaction of ZBDs with electrophiles need not result in zinc release in order to alter protein activity. The Ada protein undergoes a methylation to create a charge-neutral thioether at a Cys4 Zn-coordinating Cys residue without Zn release from the Ada N-terminal domain (23). This changes the sequence specificity of Ada's DNA-binding activity. While reaction of ClpX with diamide leads to some loss of Zn, aggregation that is likely the result of thiol oxidation and disulfide formation is also observed. The ZBDs of the ClpX hexamer have been reported to interact to

form three dimers (65), with Zn-coordinating Cys residues of adjacent monomers in position to possibly react covalently. Oxidation of the Cys residues of the ZBD might lead to both intra- and interchain disulfide cross-links that contribute to the formation of the higher-molecular-weight species observed on nonreducing gels, as shown in Fig. 10. The higher-molecular-weight forms might still contain coordinated Zn despite reduced ligand coverage due to disulfide formation.

A mobile substrate- and adaptor-binding N-terminal domain is characteristic of the AAA+ component of Clp proteases and chaperones (22, 24, 30, 61, 62). That the N-terminal domain may also possess a sensory function can be proposed based on the data reported herein. Among the questions that one could address is whether the changes to ClpX brought about by exposure to thiol-reactive electrophiles are reversible, as is the case with other redox-controlled, Zn-binding proteins (27). Disruption of the ZBD could affect the interaction of the substrate and/or the putative substrate adaptor (YjbH) with the protease.

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