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Sequence Requirements for Lon-dependent Degradation of the *Escherichia coli* Transcription Activator SoxS: Identification of the SoxS Residues Critical to Proteolysis and Specific Inhibition of *in vitro* Degradation by a Peptide Comprised of the N-terminal 21 Amino Acid Residues

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When *Escherichia coli* encounter redox-cycling compounds that endogenously generate superoxide, the cell's defense response is initiated by the *de novo* synthesis of SoxS, which then activates transcription of the genes of the SoxRS regulon. Recently, we showed that after the oxidative stress is relieved, the SoxRS system resets by an active process wherein SoxS synthesis ceases and the intrinsically unstable SoxS protein is rapidly degraded, primarily by Lon protease. Here, we use deletion mutants and a library of alanine-stretch mutants of the entire protein to identify the SoxS features responsible for Lon-dependent proteolysis *in vivo*. We found that the 17 amino acid residues at the SoxS N terminus play the primary role in protease recognition and that the addition of the N-terminal 21 residues of SoxS to the otherwise stable green fluorescent protein is sufficient to signal the chimera for Lon-dependent degradation. With a minimal *in vitro* degradation system, we confirm the intrinsic instability of SoxS and the sequence requirements for Lon-dependent degradation. Lastly, we demonstrate that the addition of a peptide comprised of the 21 N-terminal amino acid residues of SoxS is able to inhibit specifically the *in vitro* proteolysis of SoxS.

Keywords: SoxS; pre-recruitment; Lon protease; protease recognition; *in vitro* degradation assay

Introduction

In *Escherichia coli*, the defense against superoxide anion, produced by the action of redox-cycling compounds like paraquat, is carried out by the two-stage induction of the genes of the SoxRS regulon.[1–5] In the first stage, the superoxide stress leads to the conversion of constitutively expressed SoxR into an active transcription activator, which then activates transcription of a single gene, *soxS*.[6–12] In the second stage, newly synthesized SoxS activates transcription of the member genes of the SoxRS regulon whose products remove the reactive oxygen species, repair the damage caused by them, and restore the cell's normal redox potential.[9,13–16]

SoxS has been proposed to activate transcription by a novel mechanism termed "pre-recruitment".[17,18] In pre-recruitment, also known as "DNA scanning",[19] the transcription activator first forms a binary complex with RNA polymerase (RNAP) in solution and in the absence of specific DNA binding; then, the activator–RNAP binary complexes scan the chromosome for activator binding sites properly located within activator-dependent promoters. The utility of the mechanism is that by combining the DNA recognition properties of the activator and the sigma factor of RNAP it provides the means for an activator whose binding site is highly degenerate to distinguish binding sites in activator-dependent promoters from an excess of sequence-equivalent binding sites that do not reside in promoters. Thus,

Abbreviations used: RNAP, RNA polymerase; CTD, C-terminal domain; GFP, green fluorescent protein.

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the pre-recruitment mechanism was proposed as a solution to the problem of how *de novo* synthesized SoxS is able to rapidly activate transcription of the genes of the SoxRS regulon when the total number of potential SoxS binding sites per fast-growing cell (~65,000) exceeds the number of SoxS-dependent promoters per cell (~200) by ~300-fold and exceeds the number of SoxS molecules per cell (2500) by ~25-fold.[17] Our recent report of the dominance of plasmid-borne DNA binding mutations of SoxS relative to the chromosomally encoded wild-type allele has provided strong *in vivo* evidence supporting the pre-recruitment hypothesis,[18] while the previous demonstration of efficient complex formation between purified RNAP and purified MarA, a close relative of SoxS, and between purified RNAP and purified SoxS, provided strong *in vitro* evidence.[19]

The nature of the protein–protein interactions between SoxS and RNAP and between MarA and RNAP also distinguishes SoxS and MarA from most other bacterial transcription activators in that they have been shown to make protein–protein contacts with the DNA binding determinant of the α subunit of RNAP (also known as the 265 determinant),[20,21] the surface of the α subunit in its C-terminal domain (CTD) that interacts with the UP element of rRNA and other "strong" promoters.[22,23] Indeed, we showed that induction of SoxS synthesis interferes with the utilization of the UP element of the *rrnB* P1 promoter *in vivo*.[20] In so doing, SoxS functions as a co-sigma factor, diverting RNAP from UP element-containing promoters to SoxS-dependent promoters.[20] Moreover, although the ability of *de novo* synthesized SoxS to redeploy RNAP from the *rrn* promoters to SoxS-activatable promoters is beneficial to a cell undergoing oxidative stress, because it enables the cell to overcome the stress, it is obvious that once the stress is alleviated, the distribution of RNAP should return to that of the pre-stress condition.

In agreement with this prediction, we reported recently that after removal of an oxidative stress-inducing signal, SoxS-dependent transcription of the regulon's genes rapidly falls to the basal, uninduced rate, indicating that after the cessation of *de novo* SoxS synthesis, the SoxRS system resets by an active process.[24] We further showed that the active process is the degradation of SoxS, that SoxS is intrinsically unstable with a half-life of ~2 min, that Lon protease is primarily responsible for the rapid degradation, that FtsH protease plays an auxiliary role, and that SoxS is nearly completely stable in a mutant deficient in the two proteases.[24] In addition, we found that access of the proteases to the N terminus of SoxS is required for degradation, but access to the C terminus is not, since addition of a His_6 tag to the former but not to the latter substantially stabilizes the protein (half-life of ~25 min).[24]

Given that SoxS activates transcription by a newly described mechanism,[17,19] that it makes an unusual protein–protein interaction with the α subunit of RNAP,[20,21] that it is intrinsically unstable,[24] that the instability is of clear physiological importance,[24] and that a number of other genetic and biochemical resources are available,[25] we decided to undertake a more thorough characterization of the basis for the intrinsic instability of SoxS by Lon protease. Accordingly, we prepared and determined the effects on SoxS degradation of a set of deletions removing increasing numbers of amino acid residues from the N terminus of SoxS and of a comprehensive library of alanine-stretch mutants of SoxS (alanine-stretch mutagenesis substitutes four consecutive alanine residues for four native amino acid residues and has been used previously as a systemic way to identify over a fairly large region the amino acids critical to a given function[26]). This *in vivo* work culminated in the demonstration that the addition of the 21 N-terminal amino acid residues of SoxS onto the N terminus of the otherwise stable green fluorescent protein (GFP) is sufficient to target the chimera for Lon-dependent degradation. Then, we set up a minimal *in vitro* degradation system with purified SoxS and Lon protease. We found that Lon degradation of SoxS is highly processive and that the half-life *in vitro* is about the same, 2 min, as it is *in vivo*. This *in vitro* work culminated in the demonstration that the addition to the minimal degradation system of a peptide comprised of the N-terminal 21 amino acid residues of SoxS is sufficient to specifically block the Lon-dependent proteolysis. To the best of our knowledge, this is the first example of a specific peptide being able to inhibit the proteolytic activity of Lon protease on a given protein substrate.

Results

Deleting amino acids from the N terminus of SoxS increases SoxS stability *in vivo*

Access to the N terminus of SoxS is required for its degradation *in vivo* as shown by our finding that the addition of a small tag like His_6 to the N terminus of SoxS stabilizes the protein ~13-fold.[24] In fact, the addition of as few as two histidine residues to the N terminus is able to partially stabilize the protein (Figure 1). These effects suggest that the recognition signal for Lon-mediated degradation of SoxS resides within the N terminus of the protein and thus that removing amino acid residues from the N terminus should increase the stability of the protein. To test this prediction, we determined the effect of N-terminal deletions of SoxS on the *in vivo* half-life of the mutant proteins. We generated plasmid-encoded mutants of SoxS that removed N-terminal amino acid residues 2–5 (SoxSΔ5), 2–10 (SoxSΔ10), 2–15 (SoxSΔ15), and 2–20 (SoxSΔ20). The respective mutant proteins were expressed from the arabinose-inducible promoter P_{BAD} carried on plasmid pBAD18-SoxS and the half-lives were measured by Western blotting with anti-SoxS antibodies following inhibition