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The Unfolding Story of a Redox Chaperone

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Oxidative stress, especially in combination with heat stress, poses a life-threatening challenge to many organisms by causing protein misfolding and aggregation. In this issue, Reichmann et al. demonstrate how a destabilized linker region of the bacterial chaperone Hsp33 prevents aggregation of a denatured protein by stabilizing structural elements.

Coping with stressful conditions is a decisive advantage in the evolutionary struggle for existence. Oxidative stress constitutes a major challenge. Reactive oxygen species (ROS) occur as a byproduct of oxidative metabolism, but they are also generated by host cells as assault weapons against pathogenic invaders. Oxidative stress from ROS induces protein misfolding, and thus, bacteria have evolved a specialized chaperone, Hsp33, to prevent aggregation of unfolding proteins during periods of oxidative stress. Hsp33 is rapidly activated by severe oxidative stress or a combination of oxidative and heat stress (Winter et al., 2008). In this issue of *Cell*, Reichmann et al. (2012) unravel how Hsp33 uses a dynamic, flexible region to recognize and stabilize folding intermediates and then release them in a more unfolded state to more traditional chaperones (Reichmann et al., 2012).

Hsp33 is composed of three domains: an N-terminal core domain with three α helices sandwiched between two β sheets; a linker region with three α helices packed onto the core domain; and a C-terminal redox-switch domain, which contains the tetra-cysteine Zn-coordination center (Janda et al., 2004) (Figure 1A). In the reduced state, $Hsp33_{red}$ does not bind to proteins, either folded or unfolded. Upon oxidative stress, the Zn-coordinating cysteines in the C-terminal domain form two disulfide bonds, and the Zn^{2+} ion dissociates. The C-terminal domain unfolds, and the core domains dimerize, presumably with a swap of the linker regions (Graf et al., 2004; Vijayalakshmi et al., 2001) (Figure 1B).

Oxidized Hsp33 ($Hsp33_{ox}$) binds denatured proteins with high affinity. Upon return to reducing conditions, the disulfide bonds break, the C-terminal redox-switch domain refolds, and, Zn^{2+} is readily coordinated. However, the bound substrate is not released spontaneously, for this requires the bacterial Hsp70 chaperone DnaK and its cochaperones DnaJ and GrpE, which take over the misfolded protein to refold it (Hoffmann et al., 2004). This reaction cycle appears logical, but the molecular mechanism of it has been not well understood.

Now Reichmann et al. ask the simple question, how can $Hsp33_{ox}$ recognize unfolding proteins but not bind to its own disordered C-terminal domain? They use peptide arrays to analyze substrate specificity of $Hsp33_{ox}$. They find that the oxidized chaperone prefers hydrophobic and positively charged peptides, and it strongly disfavors negatively charged residues and cysteines; Hsp33 shares all of these properties with ATP-dependent (DnaK, ClpB) and ATP-independent (trigger factor) chaperones (Patzelt et al., 2001; Rüdiger et al., 1997; Schlieker et al., 2004). Interestingly, Hsp33, in contrast to other chaperones, also disfavors lysine and favors the hydrophilic residues glutamine and threonine.

Despite testing a large number of peptides in the arrays, Reichmann et al. could not identify a binding motif, suggesting that $Hsp33_{ox}$ surprisingly does not bind to a linear, unfolded peptide but rather to a structural motif. Indeed, all $Hsp33_{ox}$-binding peptides tested in the study exhibit α-helical structure in solution, whereas the nonbinding peptides are unstructured. Whether Hsp33 binds only α-helical and not β sheet elements remains to be determined. This binding preference for secondary structure explains why $Hsp33_{ox}$ does not bind to its own disordered C-terminal domain. However, these results do not explain how $Hsp33_{ox}$ recognizes stress-denatured proteins.

To rationalize these finding in the context of an unfolding protein, Reichmann and coworkers use the Arc repressor as a model substrate. The Arc repressor is typically a dimer, but it dissociates into monomers in salt-free buffers and subsequently unfolds slowly without aggregating. Reichmann et al. find that the apparent binding of $Hsp33_{ox}$ to Arc is highest immediately after Arc dissociation and then decreases with time. However, binding of DnaK increases over time, suggesting that $Hsp33_{ox}$ binds to early unfolding intermediates, whereas DnaK binds to later, more unfolded conformers. This observation is consistent with DnaK's preference for hydrophobic segments, generally found within the hydrophobic core of a protein, which are bound in an extended conformation (Rüdiger et al., 1997).

In a set of extremely challenging experiments, Reichmann and colleagues follow the conformational changes in Hsp33 upon oxidation, upon substrate binding, and after reduction in the substrate-bound state. Under these different conditions, they use partial tryptic digestion to map changes in the accessibility of lysines and arginines. They also determine local stability with equilibrium guanidinium unfolding, which they monitor by amide hydrogen exchange mass spectrometry (SUPREX) (Figure 1A). From this data, the authors conclude that, in contrast to earlier models, substrates bind to the linker region between the unfolded C-terminal domain and folded N-terminal domain. This linker region is destabilized and more dynamic upon

oxidation but then regains stability upon substrate binding.

Similarly, the authors compare local stability as well as lysine and arginine accessibility in substrates. When bound to $Hsp33_{ox}$, substrates have more regions accessible to proteolysis than when they are in the native state; after return to reducing conditions, accessibility increases further. For one substrate, citrate synthase, Reichmann and colleagues show that some of the good Hsp33-binding regions are hidden in the dimer interface in the native protein. These regions are destabilized in the $Hsp33_{ox}$-bound form and become further destabilized upon reduction.

Taking the data all together, a new picture emerges for the reaction cycle of the Hsp33 chaperone (Figure 1B). Upon oxidation, $Hsp33_{ox}$ recognizes proteins as they unfold, when previously hidden structural elements, with high-affinity for Hsp33, start becoming exposed. Hsp33 stabilizes these elements in a partially unfolded state and promotes further unfolding upon return to reducing conditions. Thus, after reduction, $Hsp33_{red}$ might present the substrates in a sufficiently unfolded conformation to expose DnaK-binding sites for substrate transfer and refolding.

The concept of mutual stabilization of structural elements of the substrate and the helices of the linker region of $Hsp33_{ox}$ seems not extremely surprising because individual α helices are rather dynamic in solution but are stabilized by side chain contacts with other structural elements. The question is, why does the linker region need to be destabilized in order to bind to unfolding substrates? In the reduced state, the linker region forms strong contacts with the core domain and thus may present a rigid surface to unfolding substrates. Upon destabilization of these contacts with the core domain, the linker region may offer a "soft" surface that is able to mold itself to the surface of the unfolding substrate and thereby execute more high-affinity interactions. Upon return to reducing conditions, a refolding of the redox-switch domain would exert strain on the linker region, and this could be transmitted to the substrate, leading to increased unfolding in certain parts of the substrate. Thereby, the energy from the change in redox potential is converted into mechanical work of unfolding the substrate. Clearly, more structural studies are needed with different substrates to verify this model and to establish its generality. Indeed, Hsp33 appears to be the ideal chaperone for such in-depth analysis.

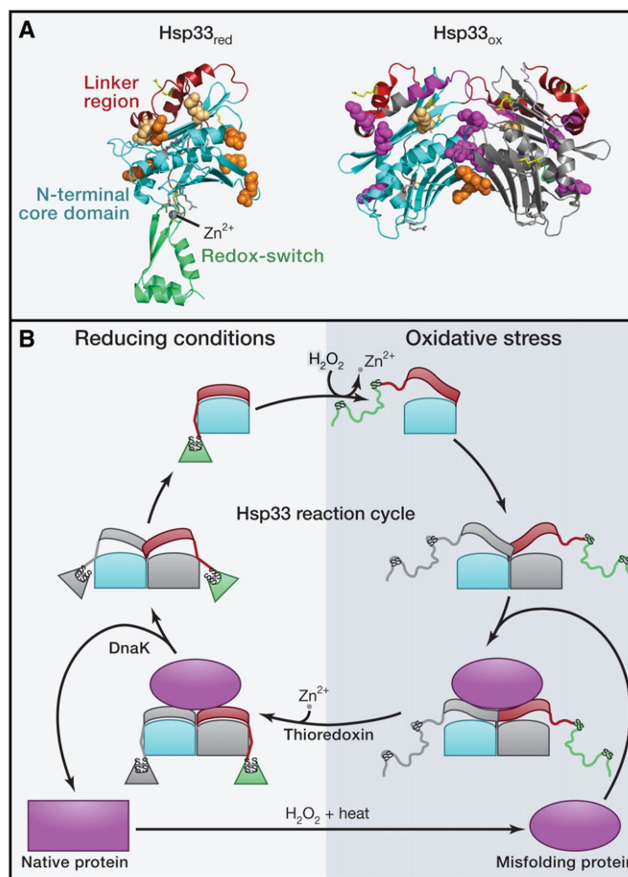

Figure 1. Structures and Reaction Cycle of Hsp33

(A) On the left, a cartoon representation of a homology model of *E. coli* Hsp33 on the structure of *Bacillus subtilis* Hsp33 (Protein Data Bank [PDB] ID 1vzy; Janda et al., 2004) shows the N-terminal domain in cyan, linker region in dark red, and C-terminal redox-switch domain in green. Lysines and arginines, which do not change in accessibility for proteolytic digestion upon oxidation or substrate binding, are shown as sticks (gray, low accessibility; yellow, high accessibility); lysines and arginines with increased proteolytic accessibility are shown as orange spheres (light orange, small changes). On the right, a truncated variant of *E. coli* Hsp33 lacking the redox-switch domain (PDB ID 1hw7; Vijayalakshmi et al., 2001) is shown. This construct is thought to represent the activated conformation of Hsp33. One protomer is colored as in the left panel, and the second protomer is in gray except for two segments: 174–192 and 203–221. The stability of these segments changes upon oxidation and substrate binding. Lysines and arginines are colored as in the left panel except that lysines and arginines that become protected upon substrate binding are shown in magenta.

(B) Upon oxidative stress, the Zn-coordinating cysteines form disulfide bridges, Zn^{2+} is released, and the C-terminal redox-switch domain (green) unfolds. Hsp33 dimerizes in the N-terminal core domain (cyan) and possibly swaps the linker regions (brown). The linker region becomes flexible and dynamic, and thus it is able to bind to early unfolding intermediates of denaturing proteins (Reichmann et al., 2012). Upon return to reducing conditions, the redox-switch refolds, but substrate release requires the action of the Hsp70 chaperone DnaK.

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