Activation of the Redox-regulated Chaperone Hsp33 by Domain Unfolding*

Paul C. F. Graff§§, Maria Martinez-Yamout¶, Stephen VanHaeren†§, Hauke Lilie¶, H. Jane Dyson†, and Ursula Jakob**

From the §Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048, the ¶Program in Cellular and Molecular Biology, ¶Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, and the **Department of Biotechnology, University of Halle, D-06120 Halle, Germany

The molecular chaperone Hsp33 in Escherichia coli responds to oxidative stress conditions with the rapid activation of its chaperone function. On its activation pathway, Hsp33 progresses through three major conformations, starting as a reduced, zinc-bound inactive monomer, proceeding through an oxidized zinc-free monomer, and ending as a fully active oxidized dimer. While it is known that Hsp33 senses oxidative stress through its C-terminal four-cysteine zinc center, the nature of the conformational changes in Hsp33 that must take place to accommodate this activation process is largely unknown. To investigate these conformational rearrangements, we constructed constitutively monomeric Hsp33 variants as well as fragments consisting of the redox regulatory C-terminal domain of Hsp33. These proteins were studied by a combination of biochemical and NMR spectroscopic techniques. We found that in the reduced, monomeric conformation, zinc binding stabilizes the C terminus of Hsp33 in a highly compact, α-helical structure. This appears to conceal both the substrate-binding site as well as the dimerization interface. Zinc release without formation of the two native disulfide bonds causes the partial unfolding of the C terminus of Hsp33. This is sufficient to unmask the substrate-binding site, but not the dimerization interface, rendering reduced zinc-free Hsp33 partially active yet monomeric. Critical for the dimerization is disulfide bond formation, which causes the further unfolding of the C terminus of Hsp33 and allows the association of two oxidized Hsp33 monomers. This then leads to the formation of active Hsp33 dimers, which are capable of protecting cells against the severe consequences of oxidative heat stress.

The molecular chaperone Hsp33 belongs to a novel class of redox-regulated proteins, whose activity is regulated by their redox state (1). In prokaryotes, Hsp33 is located in the highly reducing environment of the cytosol, where it is monomeric and largely devoid of chaperone function. Upon exposure to oxidative stress in vitro or in vivo, Hsp33 is quickly activated as a potent molecular chaperone (2). The switch that regulates the activity of Hsp33 is a novel, very high affinity zinc-binding motif (CXXC27–32CXXC) that is located in the C terminus of the protein (3). The four absolutely conserved cysteines that constitute this redox switch are kept in the reduced deprotonated thiolate anion state and together coordinate one zinc(II) ion (Kd = 10^-18 M) (3). Under oxidative stress conditions, these four cysteines release zinc and rapidly form two intramolecular disulfide bonds, connecting the two pairs of neighboring cysteines, Cys232 with Cys234 and Cys265 with Cys268 (4). Disulfide bond formation and concomitant zinc release then induces the dimerization of two oxidized Hsp33 monomers (5). Once activated, Hsp33 is a highly efficient chaperone holdase, which is able to prevent the aggregation of a variety of unfolded proteins. To prime Hsp33 for substrate protein release, reducing conditions have to be restored. This turns Hsp33 into a reduced dimer, which is still active but now able to transfer the substrate proteins to the DnaK/DnaJ/GrpE foldase system for refolding once this system becomes available (6).

Biochemical studies showed that Hsp33 senses reactive oxygen species through its C-terminal four-cysteine zinc center, with zinc playing an important role for the formation of the correct disulfide bonds and the rapid activation of the chaperone function of Hsp33 (3). The precise structural changes that accompany disulfide bond formation and permit the dimerization of Hsp33 are, however, largely unknown. This is in part because no structural information exists for the redox-active C terminus of Hsp33. Both of the crystal structures that were solved were of N-terminal fragments, which ended just after the first pair of redox-active cysteines (7, 8). Previously, Raman et al. (9) used circular dichroism to demonstrate that Hsp33 undergoes dramatic conformational changes upon oxidation. They also showed that Hsp33 exposes hydrophobic surfaces in the oxidized conformation, but not in the reduced, inactive state. Because most molecular chaperones interact with their substrate proteins through hydrophobic interactions, these exposed hydrophobic surfaces are likely to represent the substrate-binding site of Hsp33. Thus, it is conceivable that the substrate-binding site is masked in the reduced, inactive form but exposed in the oxidized, active form. At the time of these studies, however, neither the activation mechanism nor the fact that oxidized Hsp33 can form both monomers and dimers were known.

We show here that the C-terminal zinc-binding domain is fully folded in the reduced zinc-coordinated Hsp33 conformation. This appears to mask both the dimerization interface and the substrate-binding site. Upon zinc release and disulfide...
bond formation, the C-terminal domain of Hsp33 dramatically unfolds. This exposes the substrate-binding site and unmasks the dimerization interface. NMR and CD studies of the isolated C-terminal domain and the full-length Hsp33 wild type protein suggested that only the C-terminal redox switch domain unfolds, while the structure of the N-terminal chaperone domain appears largely unaltered by the oxidation and dimerization process.

EXPERIMENTAL PROCEDURES

Proteins—Wild type Hsp33 and the cysteine-free Hsp33 mutant protein were purified in the absence of reducing agents as described previously (2, 5). The Glu150 to Arg150 point mutation was introduced using the QuikChange site-directed mutagenesis kit (Stratagene) using matching primers. The forward primer that was used read 5′-GATTACC-TTATGCGTTCTAGACAGCTGCCGACGCGCC-3′/H11032

The coding sequence of the region was sequenced. BL21 strains containing an insertion mutation in hisO, the gene encoding Hsp33 (hisO-Km) (JH13) were transformed with this plasmid (5), and the mutant protein (Hsp33-E150R) was overexpressed and purified upon the purification protocol of wild type Hsp33 (2).

The coding sequences for each of the C-terminal domain fragments of Hsp33 were obtained using standard PCR methods and pUJ30 as template DNA. The sequences were re-cloned into a PET21a expression vector, and DNA sequencing was used to confirm the identity of the template DNA. The sequences were re-cloned into a pET21a expression vector, and DNA sequencing was used to confirm the identity of the template DNA. The sequences were re-cloned into a pET21a expression vector, and DNA sequencing was used to confirm the identity of the template DNA. The sequences were re-cloned into a pET21a expression vector, and DNA sequencing was used to confirm the identity of the template DNA. The sequences were re-cloned into a pET21a expression vector, and DNA sequencing was used to confirm the identity of the template DNA.

Results

The Four Hsp33 Conformations—The activation mechanism of Hsp33 is at least a two-step process (5). In the first step, reduced zinc-coordinated Hsp33 undergoes oxidation-induced disulfide bond formation and releases its bound zinc. This causes the formation of zinc-free oxidized monomeric Hsp33 intermediates. In a second step, two oxidized Hsp33 monomers dimerize to form the fully active molecular chaperone.

To dissect the roles of zinc binding and disulfide bond formation in the activation process of Hsp33, and to analyze and compare the functional and conformational changes that accompany this process, we required at least four different stable conformations of Hsp33: 1) reduced, zinc-coordinated monomeric Hsp33, 2) reduced, zinc-free monomeric Hsp33, 3) oxidized monomeric Hsp33, and 4) oxidized dimeric Hsp33. Both reduced, zinc-coordinated Hsp33 monomers and oxidized Hsp33 dimers are stable, easy to prepare, and have been characterized in detail before (2, 5). To create a stably reduced, metal-free Hsp33 variant, we decided to use a mutant of Hsp33, in which all cysteine residues had been replaced (Cys-free Hsp33) (5). This mutant protein should allow us to specifically analyze the role of zinc coordination on the activity and conformation of monomeric Hsp33 without the potential interference of disulfide bond formation. To generate oxidized monomeric Hsp33, which is presumably an intermediate in the activation pathway of Hsp33, and thereby only transiently present, we decided to introduce mutations into the highly conserved dimerization interface in an attempt to disrupt intersubunit contacts. Three residues, Ser149, Glu150, and Gin151 are located precisely at the dimerization interface and are
highly conserved (7). Ser^{49} and Gln^{151} are absolutely conserved residues found in every Hsp33 homologue identified so far, while Glu^{150} has been found in all but two Hsp33 homologues, where it is substituted with a glutamine residue. Both Glu^{150} and Gln^{151} make numerous stabilizing contacts between the two subunits. For instance, hydrogen bonds exist between the Glu^{150} carboxylate oxygen of one subunit and the backbone amide of Gln^{151} as well as the carboxylate group of Glu^{150} of the other subunit (7). Our rationale was now that the mutation of Glu^{150} to Arg resulted indeed in a redox-regulated, constitutively monomeric Hsp33 variant. This mutant protein, therefore, should represent the stable oxidized monomeric Hsp33 species, which is presumably only transiently present during the activation process of Hsp33 in vitro.

**The Oxidized Monomer Has Partial Chaperone Activity**—To analyze the activity of the oxidized monomeric variant of Hsp33, we used the same activity assays that have been previously employed to characterize the chaperone activity of wild type Hsp33 (5). We first analyzed the influence of the oxidized, constitutively monomeric Hsp33-E150R mutant protein on the aggregation of chemically unfolded luciferase. Luciferase, once completely unfolded by incubation in high concentrations of guanidinium HCl, is unable to refold back to its native state upon dilution into refolding buffer. Instead, luciferase rapidly forms large, insoluble aggregates, which can be detected by light scattering (Fig. 2A, trace a). As previously shown, a 2-fold molar excess of oxidized Hsp33 dimer to luciferase was able to almost completely suppress the aggregation of luciferase (trace b). In contrast, the same monomer concentration of oxidized monomeric Hsp33-E150R mutant protein exhibited only a slight influence on the aggregation behavior of chemically denatured luciferase (trace c). This was very similar to the light scattering signal in the presence of reduced wild type Hsp33 monomers (trace d) or reduced Hsp33-E150R monomers (data not shown), which are considered to be largely inactive Hsp33 preparations. Very similar results were obtained when chemically denatured citrate synthase was used as a substrate protein instead of luciferase, suggesting that oxidized Hsp33 monomers are either unable to interact with highly unfolded proteins at all or that they do not exert sufficiently high affinity for these extremely aggregation-sensitive folding intermediates to effectively compete with their fast aggregation process.

To distinguish between these two possibilities, we tested the activity of oxidized Hsp33-E150R monomers on the aggregation of thermally unfolding substrate proteins. Proteins that are incubated under slightly denaturing temperature condi-
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**Fig. 2.** Oxidized Hsp33 monomers have partial chaperone activity in vitro. A, only oxidized dimeric Hsp33 is able to prevent the aggregation of chemically denatured luciferase. Luciferase was denatured in 4.5 M guanidine hydrochloride and then diluted 1:160 (final concentration 48 mM) into 40 mM HEPES-KOH buffer, pH 7.5, in the absence of any chaperones (trace a) or in the presence of oxidized wild type Hsp33 dimers (trace b), reduced wild type Hsp33 monomers (trace c), or oxidized Hsp33-E150R monomers (trace d). For each Hsp33 preparation, the Hsp33 concentration was 192 nM Hsp33 protomers. Light scattering measurements were performed at 30°C. Luciferase was added at the time point indicated by the arrow. Very similar results were obtained with chemically denatured citrate synthase as a substrate protein. B, oxidation-induced disulfide bond formation is sufficient for Hsp33 to prevent aggregation of thermally unfolding luciferase. Native luciferase was diluted into prewarmed 40 mM HEPES-KOH, pH 7.5 (final concentration: 100 mM), either in the absence of any chaperones (trace a) or in the presence of oxidized wild type Hsp33 dimers (trace b), reduced wild type Hsp33 monomers (trace c), or oxidized Hsp33-E150R monomers (trace d). The Hsp33 concentration was 100 mM Hsp33 protomers. Light scattering measurements were performed as described. Luciferase was added at the time point indicated by the arrow. Very similar results were obtained when the influence of the various Hsp33 preparations was analyzed using thermally unfolded citrate synthase as substrate protein. C, oxidation-induced disulfide bond formation of Hsp33 alone should activate Hsp33 to a functional level that is sufficient to prevent the aggregation of slowly unfolding proteins such as thermally unfolding or oxidatively damaged RrmJ. Activated RrmJ was tested (data not shown). These results indicated that the influence of Hsp33 on slowly unfolding substrate proteins such as thermally unfolding luciferase or thermally unfolding citrate synthase was mainly determined by the absolute concentration of oxidized Hsp33 monomers and not by the concentration of Hsp33 dimers. This suggested that Hsp33, once oxidized, exposes a substrate-binding site, which has sufficiently high affinity for slowly unfolding proteins. To effectively compete against the fast aggregation that occurs during the refolding of completely unfolded proteins such as chemically denatured luciferase or citrate synthase, however, the substrate affinity of the chaperone must be significantly higher. This appears to be accomplished by the dimerization of two oxidized monomers, which might bring the two substrate-binding sites into close proximity and, therefore, enlarge the substrate-binding site. Oligomerization as a mechanism to increase the size and affinity of substrate binding sites is a common theme in chaperone biology and has been well studied with the 14-mer GroEL (12). This hypothesis is also supported by the structural analysis of active Hsp33 dimers, which identified two potential substrate-binding sites, both of which involve both Hsp33 subunits (7, 8).

Kinetic analysis of H$_2$O$_2$-induced reactivation of Hsp33 using chemically denatured protein as a substrate protein revealed that the activation process of Hsp33 is a slow, concentration-dependent process, which is significantly preceded by disulfide bond formation (5). This indicated that oligomerization of the oxidized Hsp33 monomers is absolutely required for Hsp33 to interact with highly aggregation-sensitive folding intermediates such as chemically denatured luciferase or citrate synthase, which quickly aggregate. Based on our previous observations, we predicted that disulfide bond formation of Hsp33 alone should activate Hsp33 to a functional level that is sufficient to prevent the aggregation of slowly unfolding proteins such as thermally unfolding or oxidatively damaged proteins. This should lead to reactivation kinetics of Hsp33 that when tested with thermally unfolded proteins as substrate proteins
should parallel the fast kinetics of disulfide bond formation and should be concentration independent. As shown in Fig. 2, we found that this was indeed the case. Reduced and zinc-reconstituted wild type Hsp33 and Hsp33-E150R mutant protein were incubated with H$_2$O$_2$ at elevated temperatures. At the time points indicated, aliquots were withdrawn and either tested for zinc release and oxidation (compare with Fig. 1) or for the ability to prevent the aggregation of thermally unfolding citrate synthase at 43 °C (Fig. 2C). The apparent $t_{1/2}$ values of Hsp33 reactivation were about 17 min for both wild type Hsp33 (Fig. 2, closed circles) and Hsp33-E150R mutant proteins (Fig. 2C, open circles) and, therefore, very similar to the obtained $t_{1/2}$ values of zinc release and oxidation (Fig. 1). Moreover, both wild type Hsp33 and Hsp33-E150R mutant reactivated with the same rate to about the same extent, confirming that disulfide bond formation of Hsp33 alone is indeed sufficient to convert Hsp33 into a molecular chaperone that can interact with slowly unfolding and aggregating protein folding intermediates.

**Hsp33 Dimerization Is Required to Protect Cells against Heat and Oxidative Stress Treatment**—To investigate the in vivo function of Hsp33 and to assess how important Hsp33 dimerization is for cell viability, we performed a phenotypical analysis of Hsp33 deletion strains. We found that the presence of the Hsp33 gene $hsoI$ is required for the high temperature growth of *E. coli* cells that are constitutively oxidative stressed due to the absence of thioredoxin reductase (ΔtxrB) (Fig. 3). ΔtxrB cells have previously been shown to have a higher resistance against oxidative stress treatment (13), which appears to be at least in part due to the accumulation of oxidized and activated Hsp33 (2). Here we show that Hsp33 also plays a major role in protecting these cells against heat treatment. While ΔtxrB cells that express functional Hsp33 (WM93) grew well at 43 °C on MacConkey agar, the double mutant ΔtxrB ΔhsoI (WM97), where both thioredoxin reductase and Hsp33 proteins are absent, was unable to form colonies under these combined conditions of intrinsic oxidative stress and exogenous heat shock (Fig. 3).

This phenotype was complemented by transforming the cells with a plasmid encoding wild type Hsp33 (UJ118), showing that the observed phenotype was due to the absence of functional Hsp33. Importantly, growth of ΔtxrB ΔhsoI cells was not impaired at 37 °C (data not shown), suggesting that the presence of Hsp33 is dispensable under normal growth conditions but becomes essential for ΔtxrB cells under heat stress conditions. This showed that the redox-regulated Hsp33 is important for high temperature growth in cells that are oxidatively stressed.

To identify whether the dimerization of oxidized Hsp33 monomers is required for the ability of Hsp33 to protect cells against this severe stress treatment, we transformed ΔtxrB ΔhsoI double mutant cells with a plasmid encoding the Hsp33-E150R monomeric mutant protein generating the strain KT3. Western blot analysis confirmed that the level of Hsp33 expression from this plasmid was comparable with the expression level of endogenous Hsp33 from the chromosome (data not shown). Growth analysis of these cells under heat shock temperatures on MacConkey plates revealed that the constitutively monomeric Hsp33 mutant was unable to protect the cells against this combination of heat and oxidative stress (Fig. 3). KT3 cells were unable to form colonies after overnight incubation at 43 °C and behaved very similarly to cells lacking the Hsp33 gene altogether. This result showed clearly that under conditions of severe oxidative heat stress, dimerization of Hsp33 is required for cell survival. It did not exclude, however, that oxidized Hsp33 monomers might play a role under milder oxidative stress conditions in vivo, where fewer and potentially less aggregation-sensitive proteins accumulate, and which are often not accompanied by an overt phenotype.

**The High Affinity Zinc Center Masks Hydrophobic Surfaces in Hsp33**—The results that were obtained with the constitutively monomeric Hsp33-E150R mutant protein were reminiscent of the results that were obtained with the cysteine-free Hsp33 mutant (5, 8). This mutant protein, which lacks all four highly conserved as well as the two non conserved cysteine residues, is unable to coordinate zinc, is unable to respond to oxidative stress by the formation of disulfide bonds, and is unable to dimerize. Comparison of the reported in vitro chaperone activity of the Cys-free Hsp33 mutant protein with the chaperone activity of the oxidized Hsp33-E150R monomer mutant suggested that it is not disulfide bond formation directly that causes the partial activation of the chaperone activity of Hsp33 but that it is the loss of zinc coordination. Like the oxidized Hsp33-E150R mutant, the cysteine-free Hsp33 mutant protein was inactive as a molecular chaperone when tested with chemically denatured luciferase (5) and citrate synthase (data not shown) but active when tested with thermally unfolded luciferase (8) and citrate synthase (data not shown). This suggested that under reducing conditions, the C-terminal zinc coordination masks the substrate-binding site of Hsp33, rendering reduced zinc-coordinated Hsp33 inactive. When zinc is released, either by disulfide bond formation, zinc chelation, or cysteine mutation, the substrate-binding site of the Hsp33 monomer is exposed.

It has previously been shown that Hsp33 undergoes significant conformational changes upon oxidation that causes the exposure of hydrophobic surfaces in the protein (9). If these hydrophobic surfaces were indeed to represent the substrate-binding site in Hsp33 as suggested, we hypothesized that loss of zinc coordination either due to cysteine substitution or due to disulfide bond formation should cause the exposure of hydrophobic surfaces in Hsp33. To compare the surfaces of our four different Hsp33 conformations, we utilized the hydrophobic probe bis-ANS. Bis-ANS shows a very low fluorescence signal...
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...with an emission maximum of ~515 nm in aqueous solution, but exerts a significant increase in signal as well as a ~30–40 nm blue shift in emission maximum when bound to a hydrophobic environment (14). In agreement with previous results, we found that oxidized Hsp33 dimers bind bis-ANS very strongly and cause a dramatic increase in the relative fluorescence of bis-ANS at ~480 nm (Fig. 4, trace a), while zinc-coordinated wild type and E150R-Hsp33 monomers bind bis-ANS only very poorly (Fig. 4, traces b and c). Analysis of the bis-ANS binding properties of the Cys-free Hsp33 mutant in comparison with the oxidized Hsp33-E150R monomer confirmed our functional analysis and revealed that it is indeed the zinc binding in reduced Hsp33 that is responsible for masking the hydrophobic surfaces of Hsp33 and keeping Hsp33 inactive (Fig. 4, traces c and d). Loss of zinc coordination either by mutation of the coordinating cysteine residues (Cys-free Hsp33) or oxidative stress induced disulfide bond formation leads to the unmasking of an approximately equal amount of hydrophobic surfaces and to the partial activation of the chaperone function of Hsp33.

**Hsp33 Undergoes Massive Conformational Changes upon Zinc Release and Oxidation**—We wondered what conformational changes occur in Hsp33 upon zinc release and oxidation that allow such significant structural rearrangements and support the dimerization of Hsp33. Currently, the only structural data that are available come from the crystal structure of the active, dimeric conformation of an Hsp33 fragment that lacks much of the redox active C terminus (7, 8). In addition, circular dichroism spectroscopy has been performed, which suggested that Hsp33 undergoes significant conformational changes upon oxidation with an overall loss of α-helical structure and a gain in random coil (9). Because these studies only compared the conformation of full-length Hsp33 protein in either the reduced, zinc-coordinated monomeric conformation or the oxidized conformation, neither the chain of conformational events nor the location of the conformational changes have been identified.

To dissect, what chemical change in Hsp33 causes what conformational change in the protein, we compared the secondary structure of all four Hsp33 conformations. CD measurements showed that reduced, zinc-coordinated wild type and Hsp33-E150R monomers have a high α-helical content, confirming previous observations (Fig. 5A, traces a and b). In the absence of zinc binding, a significant gain in random coil at the expense of α-helical content was observed (Fig. 5A, trace c). This suggested that it is zinc binding that stabilizes Hsp33 in a compact fold and that loss of zinc alone already causes significant unfolding of the polypeptide. This finding agreed with previous protease susceptibility experiments, which revealed a significantly higher susceptibility of metal-free reduced Hsp33 monomers as compared with zinc-reconstituted Hsp33 monomers (3, 5).

Interestingly, both oxidized Hsp33-E150R monomers (Fig. 5A, trace d) as well as oxidized Hsp33 dimers (Fig. 5A, trace e) showed a further loss in α-helical content and increase in random coil, suggesting that disulfide bond formation causes an even further unfolding of Hsp33. Because the oxidized Hsp33 monomer and dimer appear to be structurally quite similar, we concluded from these results that most of the secondary structure changes during the activation process of Hsp33 take place at the transition of the reduced to the oxidized monomer stage. The full activation that accompanies the dimerization process of Hsp33, and which is reflected in a significantly increased affinity for highly aggregation-sensitive
The C-terminal Redox Domain of Hsp33 Unfolds upon Oxidation and Zinc Release—The data we obtained did not define which region(s) of Hsp33 are involved in the major unfolding events that appear to accompany the activation process of Hsp33. In an effort to dissect where the unfolding occurs in Hsp33, we decided to focus on the structure and stability of the isolated C-terminal redox domain. The amino acid sequence of E. coli Hsp33, together with structural information from the two x-ray crystal structures (7, 8), was used to design various C-terminal fragments of the protein that were cloned and expressed in E. coli. The constructs Hsp33-179–287 aa and Hsp33-218–287 aa were found to be both stably expressed in medium with added zinc and could be purified under native, reducing conditions. Based on CD spectroscopy, both Hsp33 fragments appeared to be well folded into an α-helix-containing structure (Fig. 5B, trace a, and data not shown). Analysis of the zinc content and redox sensitivity revealed that even the shorter Hsp33-218–287 aa fragment has one equivalent of zinc bound, which can be reversibly released by the oxidative stress-induced formation of two disulfide bonds (data not shown). This clearly showed that the C-terminal domain of Hsp33 is an independent folding unit, which maintains its zinc binding capacity and its redox sensitivity.

When zinc was removed by chelation with TPEN followed by desalting, a significant amount of the helical structure was lost, indicating that zinc coordination maintains the helical character of the C-terminal redox domain. Comparison of the secondary structure of the zinc free, reduced redox domain with that of the zinc-free, disulfide-bonded redox domain, however, showed again that disulfide bond formation causes an additional loss in secondary structure (Fig. 5B, compare traces b and c). The disulfide-bonded Hsp33-218–287 aa fragment appeared to be almost completely unfolded. These results were surprising, given that disulfide bonds are known to stabilize protein folds and have to our knowledge not been shown to cause a further unfolding of a polypeptide chain. It might, however, be the combination of loss of structure due to loss of zinc coordination and formation of disulfide bonds between nearest neighbor cysteines that could cause these major unfolding events. Albeit surprising, these results were in excellent agreement with our CD spectra of the full-length Hsp33 protein that also showed a loss of secondary structure concomitant with disulfide bond formation and in addition to the loss of secondary structure that accompanied zinc dissociation.

To investigate whether most of the conformational changes are restricted to the C-terminal redox domain of Hsp33 or also involve the N-terminal chaperone domain, we compared the 15N-1H HSQC spectra obtained for the reduced and oxidized C-terminal Hsp33 domains (Fig. 6) with the 15N-1H HSQC spectra obtained for reduced and oxidized wild type Hsp33 (Fig. 7A). The overlay of the (green) reduced Hsp33-287 aa fragment in 10 mM Tris buffer, pH 6.6, 140 mM NaCl, 50 μM ZnSO4, 5 mM DTT with the 15N-1H HSQC spectrum (red) at 500 MHz of oxidized Hsp33-179–287 aa in 10 mM Tris buffer, pH 6.6, 100 mM NaCl shows an expanded region of NMR spectra of reduced, zinc-bound wild type Hsp33 (black), oxidized wild type Hsp33 (red), and the reduced, zinc-bound 179–287 aa fragment (green). Many of the red and black peaks are coincident. As such, these appear to represent N-terminal residues, which do not change upon oxidation. In two cases, marked with arrows, the oxidized (red) peaks are missing, but the reduced (black) cross-peak overlays with that of the reduced fragment (green), indicating that it belongs to a residue in the C-terminal region of the protein. That the oxidized cross-peak is missing (perhaps moved to a different part of the spectrum) is an indication that the structure of the C-terminal cysteine-rich domain is significantly changed upon oxidation.

DISCUSSION

The molecular chaperone Hsp33 belongs to a recently recognized class of proteins, whose activity is redox-regulated. These proteins have in common highly conserved cysteines, which are particularly sensitive to reactive oxygen species and are easily modified both in vitro and in vivo. Upon modification of the cysteine residues, proteins such as Hsp33 (2), protein kinase C (15), and OxyR (16) are activated, while proteins like PTEN (17) are inactivated. Other redox-regulated protein display altered affinity to regulatory partner proteins such as sigma.
factors (e.g. RsrA (18)) or nuclear export factors (e.g. Yap1p (19)) depending on their redox status.

In a number of redox-regulated proteins, zinc has been found to be part of the redox switch. This is, for instance, the case in Raf kinases (20), the anti-α-factor RsrA (21), as well as Hsp33 (3). Here, the highly conserved cysteines coordinate zinc under reducing conditions. Upon exposure of proteins to oxidative stress, disulfide bonds form, and zinc is released. Upon return to non-stress conditions, the cysteines are reduced, and zinc is re-coordinated.

Very little is known about the structural changes that are caused by oxidation-induced disulfide bond formation in redox-regulated proteins and which lead to the observed significant differences in protein activity. To investigate this important aspect, we decided to use the redox-regulated chaperone Hsp33 as a model protein, whose activation process involves at least three different Hsp33 conformations: the reduced inactive Hsp33 monomer, the oxidized Hsp33 monomer, and the fully active oxidized Hsp33 dimer (Fig. 8).

In reduced, monomeric Hsp33, all four cysteines are involved in the high affinity binding of zinc. We have now found that zinc serves several purposes in Hsp33; first, it primes the cysteines for a quick attack by H$_2$O$_2$ and allows the correct disulfide bonds to form (3). Probably even more important, however, is the finding that zinc coordination keeps the chaperone function of Hsp33 down-regulated under reducing non-stress conditions.

The C-terminal redox domain of Hsp33 is fully folded into a predominantly α-helical structure when zinc is coordinated. This folded zinc-binding domain appears to mask the substrate-binding site of Hsp33 presumably by shielding hydrophobic residues from interacting with unfolding intermediates. Loss of zinc, either by the use of zinc chelators or by the introduction of point mutations that substitute zinc-coordinating residues (Cys-free Hsp33), destabilizes the C-terminal redox domain of Hsp33 dramatically and causes the C-terminal domain to partly unfold. This exposes hydrophobic surfaces and activates the chaperone function of Hsp33 to a degree that allows Hsp33 to bind slowly unfolding proteins and to out-compete the otherwise irreversible aggregation reaction (Fig. 8).

Interestingly, this zinc-free, partially unfolded conformation of Hsp33 appears to be thermodynamically stable. However, upon formation of the two disulfide bonds, Hsp33 further unfolds. This further unfolding seems critical for the subsequent dimerization of oxidized Hsp33 monomers and for the full activation of the chaperone function of Hsp33. Upon disulfide bond formation, very little secondary structure appears to be left in the C-terminal domain of Hsp33 as seen in the isolated C-terminal fragment (179–287 aa) as well as in full-length Hsp33. Noteworthy, in the crystal structure of the truncated Hsp33 dimer (1–235 aa), the first 57 aa (179–235 aa) residues of the C-terminal domain are domain swapped and fold into three α-helices and two β-strands. It remains to be seen whether the secondary structure observed in the crystal structure is a crystallization artifact that might have been induced by the protein truncation or whether the N terminus of Hsp33 indeed serves as a scaffold for the first 57 aa of the C terminus to retain their structure in dimeric Hsp33. That the C terminus of the Hsp33 dimer as seen in the crystal structure might not necessarily reflect the solution structure of the oxidized Hsp33.

**Fig. 7.** Comparison of the NMR signals of Hsp33 and C-terminal constructs. A, 500 MHz HSQC spectrum of reduced, zinc-reconstituted Hsp33 in 10 mM Tris buffer, pH 6.6, 140 mM NaCl, 50 μM ZnSO$_4$, 5 mM DTT (black), overlaid with the HSQC spectrum of reduced and zinc-reconstituted Hsp33-179–287 aa fragment shown in Fig. 6A (red). The arrows highlight some of the cross-peaks that are coincident in the two spectra. B, overlay of the 500 MHz HSQC spectra of reduced, zinc-bound full-length Hsp33 (black) and oxidized full-length Hsp33 (red). C, portion of an overlay of 500 MHz HSQC spectra of reduced, zinc-bound Hsp33 (black), oxidized, disulfide bonded Hsp33 (red), and the reduced, zinc-bound Hsp33-179–287 aa fragment (green).
The dimer is suggested by the observation that the two solved crystal structures of Hsp33 are very similar, although Hsp33 was crystallized either under oxidizing conditions (8) or under reducing, zinc-coordinating conditions (7). In either case, our studies suggest that the N-terminal domain, which harbors the dimerization interface and probably the substrate binding site of Hsp33, is largely unaltered by the redox dependent conformational changes.

Importantly, the unfolding of the C-terminal domain of Hsp33 does not change the solubility of full-length Hsp33 or the isolated C-terminal domain and appears to be fully reversible upon restoration of reducing conditions both in vitro and in vivo. While zinc binding is well known to provide considerable stability to proteins, and zinc-binding domains have often been found to serve as independent folding units (23), such reversible folding and unfolding of complete zinc-binding domains as part of a regulatory mechanism has rarely been observed before. Until recently, high affinity zinc centers have been considered to be redox inert and purely structural (24). Now a number of proteins such as the zinc storage proteins metallothionein (25, 26), protein kinase C (15, 27), and Raf kinase (28) have been found to reversibly bind and release zinc from their high affinity zinc binding sites, depending on the redox conditions of the environment. Especially in proteins where the mechanism of action changes dramatically upon zinc release, it would not be surprising if reversible unfolding of the zinc-binding domain plays an important role as well. Very recently, zinc-dependent folding of an inter-mitochondrial membrane space protein Tim13p has been shown to be highly regulated. Tim13p becomes folded only after translocation across the outer membrane, where it acquires zinc (29). This then prevents reverse translocation back into the cytosol. It remains to be determined whether the unfolded conformation of Tim13p represents a soluble and stable alternative structure, as is the case for unfolded Hsp33, or whether cytosolic chaperones prevent its premature degradation or aggregation in vivo.

Noteworthy, Hsp33 appears not to be the only zinc-dependent redox-regulated protein whose functional regulation has been shown to involve massive structural rearrangements. Under reducing, non-stress conditions, the zinc-binding anti-parallel-β sheet structure of Streptomyces coelicolor RsrA has been shown to be highly regulated. Upon exposure to oxidative stress, disulfide bonds of RsrA form, and zinc is released. This is accompanied by major structural rearrangements that lead to the release of αR, which is then able to activate transcription of its target genes (18, 30). In contrast to Hsp33, where the oxidized conformation unfolds and the reduced zinc-bound conformation is fully structured, disulfide-bonded RsrA appears to contain more α-helical secondary structure than the reduced, zinc-bound form. This then leads to the faster migration of oxidized RsrA on native PAGE (22). This is in stark contrast to Hsp33, where the reduced, zinc-coordinated species was found to exert a significantly faster mobility (2).

Why would Hsp33 and RsrA behave so contrary in regards to their conformational changes? One possibility has to do with the localization of disulfide bonds. In Hsp33, the disulfide bonds are formed between nearby cysteines in the primary sequence. This appears to introduce large conformational restraints, causes the C-terminal domain to lose its structure, and leads to the exposure of otherwise buried hydrophobic residues that are capable of interacting with the unfolding substrate proteins of Hsp33. In RsrA, however, the critical disulfide bonds are formed between cysteines that are far apart in the primary sequence (Cys13 and either Cys13 or Cys43) (22). Formation of disulfide bonds appears to cause RsrA to fold into a more compact folded structure, thereby burying the residues that otherwise interact with αR.

Previously, large differences in the secondary structure of reduced and oxidized Hsp33 have been observed (9). Our results now indicate that these conformational changes are mostly restricted to the C-terminal redox switch domain. The modular nature of Hsp33 allows the N terminus to retain its potential for dimerization and substrate binding even in the reduced, inactive state. All that Hsp33 requires to be activated is the signal from the C-terminal redox switch. This domain is in its “off” position when it is fully folded and blocking the substrate-binding site. The switch is flipped when oxidative stress-induced disulfide bond formation causes zinc release. Then the redox switch domain unfolds, allowing dimerization of two oxidized Hsp33 monomers and supporting the formation of a highly active molecular chaperone.

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Paul C. F. Graf, Maria Martinez-Yamout, Stephen VanHaerents, Hauke Lilie, H. Jane
Dyson and Ursula Jakob

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