

# Chaperone Activity with a Redox Switch

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## Summary

Hsp33, a member of a newly discovered heat shock protein family, was found to be a very potent molecular chaperone. Hsp33 is distinguished from all other known molecular chaperones by its mode of functional regulation. Its activity is redox regulated. Hsp33 is a cytoplasmically localized protein with highly reactive cysteines that respond quickly to changes in the redox environment. Oxidizing conditions like H<sub>2</sub>O<sub>2</sub> cause disulfide bonds to form in Hsp33, a process that leads to the activation of its chaperone function. In vitro and in vivo experiments suggest that Hsp33 protects cells from oxidants, leading us to conclude that we have found a protein family that plays an important role in the bacterial defense system toward oxidative stress.

## Introduction

Every organism responds to a sudden increase of the environmental temperature with the overexpression of a similar set of highly conserved heat shock proteins (Hsps) (Lindquist and Craig, 1988). The majority of these Hsps function either as molecular chaperones to assist other proteins in their folding or as proteases to degrade proteins that are beyond help. Heat shock proteins act both under normal conditions and under stress conditions, where they are overexpressed to cope with the increased concentration of unfolding proteins (Hendrick and Hartl, 1993). Molecular chaperones are able to temporarily stabilize unfolded or partially folded proteins and prevent inappropriate inter- and intramolecular interactions. They reduce the free concentration of aggregation-sensitive folding intermediates and effectively prevent aggregation processes both in vivo and in vitro (Jakob and Buchner, 1994).

Chaperone systems in the cytoplasm of *E. coli* include the GroEL/S complex, the DnaK/J/GrpE system, Hsp90, and small heat shock proteins (Gross, 1996). The most abundant and well-studied member is GroEL and its coworker GroES. Despite their abundance, recent results revealed that GroE's chaperone activity can maximally account for 15% of newly synthesized proteins (Lorimer, 1996; Ewalt et al., 1997). Since the in vivo folding of the majority of proteins is believed to be assisted by chaperones, this leaves an apparent chaperone deficit even under nonstress conditions (Horwich et al., 1993; Lorimer, 1996). We decided to investigate

the possibility that other, yet to be identified, chaperones may play a protective role in protein folding and stress-induced unfolding in the cell.

That the "inventory of heat shock genes and proteins is by no means complete" became evident when Chuang and Blattner identified and mapped 26 new heat shock genes termed heat shock loci (hsl) on the *E. coli* chromosome using a sensitive genomic expression analysis technique (Chuang and Blattner, 1993). Given the tight correlation between heat shock proteins and molecular chaperones, we hypothesized that previously unidentified chaperones might exist amongst this collection of newly identified heat shock proteins.

We describe here the characterization of one of these novel heat shock proteins, Hsp33, a 32.9 kDa protein originally identified as HslO (Chuang and Blattner, 1993). Hsp33 proved to be a highly efficient molecular chaperone with a novel mode of regulation. Hsp33's chaperone function is regulated at the posttranslational level by the redox conditions of the environment. Inactive, reduced Hsp33 has zinc coordinated by its conserved cysteines. Oxidizing conditions like exposure of Hsp33 to H<sub>2</sub>O<sub>2</sub> cause zinc to be released from the cysteines, disulfide bonds to form, and most importantly, the chaperone function to be turned on. Our data suggest that Hsp33 is mostly reduced under normal cellular conditions but turns into an active folding helper protein when subjected to an oxidizing environment. Hsp33 mutants are sensitive to oxidative stress in vivo, and purified Hsp33 prevents the aggregation of proteins exposed to oxidative stress in vitro. Hsp33 appears to be a chaperone with an on-off mode of activity that uses reactive disulfide bonds as a molecular switch.

## Results

### Hsp33, a New Family of Cytoplasmic Heat Shock Proteins

We have initiated study of Hsp33, a novel heat shock protein identified by means of a sensitive genomic expression technique (Chuang and Blattner, 1993) and recently assigned to its open reading frame by the availability of the complete *E. coli* genomic sequence (W. M. et al., unpublished). Hsp33 is encoded on an operon immediately downstream from Hsp15, a newly identified RNA-binding heat shock protein (Korber et al., 1999). Similarity searches using the BLAST program against the nonredundant database and the database of unfinished microbial genomes maintained at the National Center for Biotechnology Information revealed that Hsp33 is a well-conserved protein present in a wide variety of prokaryotic species (Figure 1). Characteristic of this new class of proteins is a motif consisting of four cysteines near the C terminus of the protein. These cysteine residues are arranged in a C-X-C motif separated by 30–31 amino acids from a C-X-Y-C motif (Figure 1).

To verify that Hsp33 is indeed a member of a new heat shock protein family, mRNA hybridization experiments

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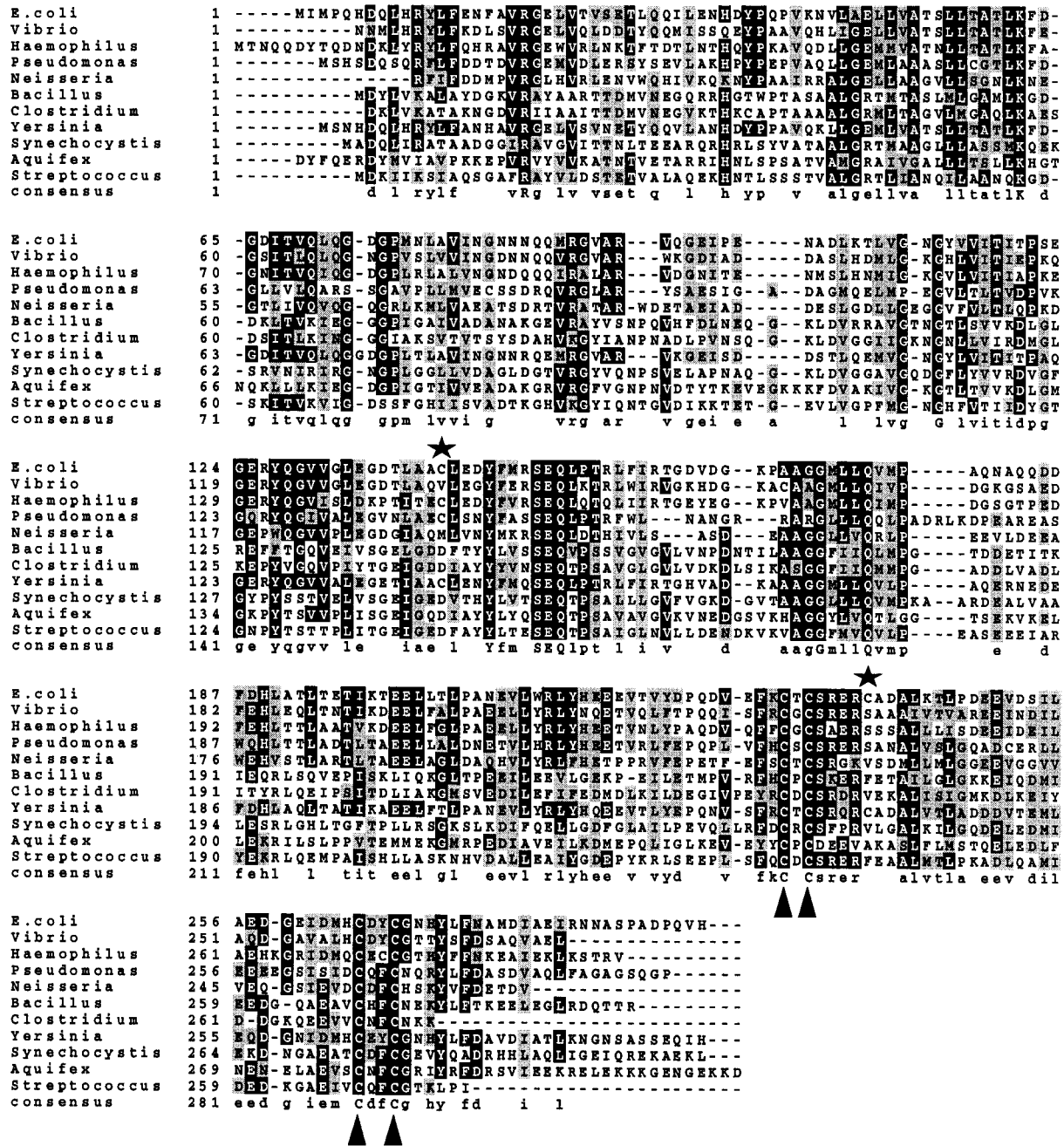


Figure 1. Hsp33 Is a Member of a New, Highly Conserved Prokaryotic Heat Shock Protein Family  
 Sequence homology searches performed on December 10, 1998 revealed 20 members of the Hsp33 family, 11 of which are shown. Sequence alignments of Hsp33 were performed with CLUSTALW. Amino acid residues identical in 60 or more percent of the sequences are highlighted in black; related amino acids are gray shadowed. The highly conserved cysteines are indicated by arrows; the poorly conserved cysteines in *E. coli* Hsp33 are indicated by asterisks.

were performed as described in Chuang and Blattner (1993). mRNA was prepared from log phase *E. coli* cultures grown at 37°C or from the same culture after a 10 min heat shock treatment at 46°C. Hsp33's mRNA was found to be upregulated between 2.6- to 7.8-fold upon a temperature shift from 37°C to 46°C.

Hsp33 is predicted to be localized to the bacterial cytoplasm by the PSORT program. This localization was

confirmed by three different cell fractionation experiments. Quantitative Western blot analysis allowed us to calculate the concentration of Hsp33 in the cell under normal conditions to be approximately  $1.4 \pm 0.3 \mu\text{M}$ . This is similar to the concentration of the GroEL oligomer (Lorimer, 1996).

Based on Hsp33's high conservation, and since no homology to any other family of heat shock proteins or

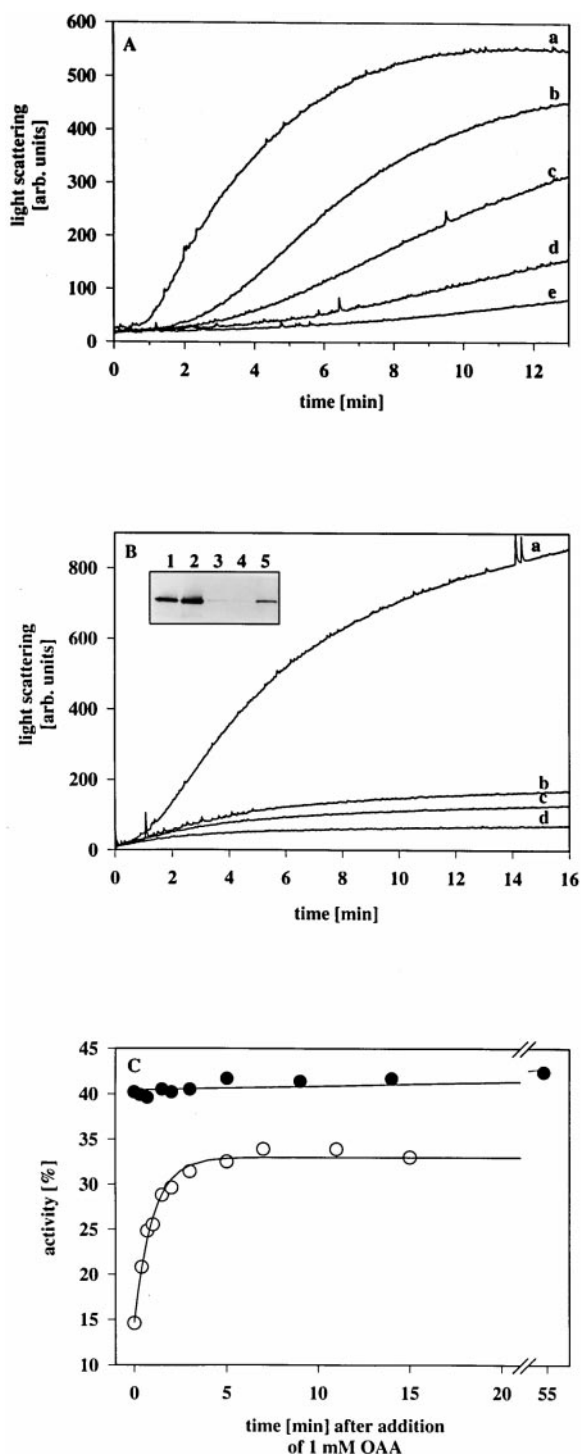


Figure 2. Hsp33 Functions as Molecular Chaperone  
(A) Influence of Hsp33 on the aggregation of citrate synthase (CS) at 43°C. Light scattering measurements of CS (0.11  $\mu$ M) in the (a) absence or the presence of a (b) 0.5:1, (c) 1:1, (d) 2.5:1, or (e) 5:1 molar ratio of Hsp33 to CS.  
(B) Influence of Hsp33 on the thermal aggregation of firefly luciferase at 43°C. Light scattering measurements of luciferase (0.16  $\mu$ M) in the (a) absence or the presence of a (b) 0.5:1, (c) 1:1, or (d) 2:1 molar ratio of Hsp33 to luciferase. (Inset) Hsp33 prevents precipitation of thermally inactivated luciferase. Luciferase (0.16  $\mu$ M) was incubated at 43°C for 15 min in the absence of any protein (lane 1) or in the presence of 12.5  $\mu$ g BSA (lane 2) or 5.6  $\mu$ g Hsp33 (0.16  $\mu$ M) (lane

to any protein of known function could be detected, we believe that we have discovered a novel and well-conserved family of cytoplasmic heat shock proteins.

### Hsp33 Is a Highly Efficient Molecular Chaperone

To analyze the function of this heat shock protein, Hsp33 was overexpressed and purified to apparent homogeneity. The potential role of the purified Hsp33 as molecular chaperone was analyzed using citrate synthase (CS) and luciferase as chaperone substrates. The influence of Hsp33 on the thermally induced aggregation of these enzymes at temperatures that resemble heat shock *in vivo* was used as a standard measure of molecular chaperone activity (Oh et al., 1997; Buchner et al., 1998). In the absence of any additional proteins or in the presence of the nonchaperone control proteins BSA or lysozyme (Buchner et al., 1998), CS unfolds at 43°C, leading to significant aggregation within the first 10 min of incubation (Figure 2A). A 5-fold molar excess of purified Hsp33 is sufficient to almost completely suppress the thermal aggregation of CS (Figure 2A, line e). An equimolar amount of Hsp33 suppresses light scattering by about 50%. This activity appears to be independent of the presence of MgATP in the incubation reaction (see below). The stoichiometry of Hsp33's chaperone action is very similar to that of the known chaperones (Buchner et al., 1998). However, while Hsp33 appears to act as a monomer as analyzed by gel filtration and cross-linking (data not shown), many chaperones, including GroEL, Hsp90, and the small heat shock proteins function as oligomers (Hendrick and Hartl, 1993; Buchner, 1996). Thus, on a mass basis, Hsp33 is at least five times more effective than the well-studied chaperone GroEL (Buchner et al., 1998).

In order to determine if Hsp33's substrate specificity is restricted to CS or if Hsp33 can be considered to be a general chaperone, we tested Hsp33 action with another classical chaperone substrate, luciferase. Like CS, luciferase rapidly inactivates and aggregates upon incubation at 43°C (Oh et al., 1997) (Figure 2B). Hsp33 is also able to prevent this process in a very efficient way. A 2-fold molar excess of Hsp33 completely inhibited aggregation (Figure 2B, line d). Even a 0.5:1 molar ratio of Hsp33 to luciferase is sufficient to almost completely prevent thermal aggregation of the enzyme during the time course of the incubation (Figure 2B, line b). This effect is similar to the influence of a 4-fold molar excess of DnaK on the thermal aggregation of luciferase (Oh et al., 1997).

Chaperone action on thermally unfolding proteins should lead to a decrease in the amount of precipitated protein. Therefore, we visualized the amount of aggregated protein after the 15 min incubation period at 43°C

3) prior to the centrifugation; nonincubated luciferase, lane 4. The insoluble protein pellet is shown. Luciferase standard (lane 5).

(C) Hsp33 forms an apparent stable complex with late CS-unfolding intermediates. Influence of Hsp33 on the oxaloacetic acid-induced reactivation of thermally inactivating CS at 43°C. CS (0.15  $\mu$ M) was incubated at 43°C in the absence (open circles) or the presence (closed circles) of 0.8  $\mu$ M Hsp33. After 3 min (in the absence of Hsp33) or after 6 min (in the presence of Hsp33) of incubation at 43°C, oxaloacetic acid (1 mM) was added to initiate reactivation.

by SDS-PAGE (Figure 2B, inset). In agreement with the results of the light scattering measurements, a significant proportion of luciferase is present as protein aggregates when incubated in the absence of any additional protein or in the presence of BSA. In contrast, incubation of luciferase in the presence of Hsp33 leads to only very small amounts of protein pelleted after a 30 min centrifugation. Similar amounts are detectable when luciferase is not exposed to high temperatures but centrifuged immediately after the dilution into incubation buffer at 4°C. Therefore, Hsp33 seems to be able to completely prevent the thermal aggregation of the model substrate luciferase at elevated temperatures. These results demonstrate that Hsp33 is a novel and highly effective molecular chaperone.

#### Hsp33 Has High Affinity for Unfolding Intermediates

CS is an excellent model substrate, since different chaperones interact with distinct unfolding intermediates of CS depending on the chaperone's functional mechanism (Buchner et al., 1998). To analyze Hsp33's chaperone function, the influence of Hsp33 on the thermal reactivation of CS was examined. During the first 10 min of thermal inactivation, a significant percentage of unfolding intermediates can be reactivated upon the addition of oxaloacetic acid (OAA), a substrate of CS (Jakob et al., 1995). This is due to the binding of OAA to active CS molecules, a process that induces the reactivation of inactive unfolding intermediates that are in equilibrium with the native state. The rate at which these intermediates refold can be taken as a measure of how tightly they are bound to chaperones, since OAA binding indirectly competes with chaperone binding (Buchner et al., 1998). When OAA is added in the absence of Hsp33, these intermediates refold rapidly, as judged by the rapid increase in CS activity (see Figure 2C, open symbols). However, when OAA was added to CS incubated in the presence of Hsp33, the reactivation rate dramatically slowed (Figure 2C, closed symbols). This apparent stable complex formation is most likely due to a kinetic competition between the slow refolding of inactive CS molecules and the fast rebinding to the chaperone and is similar to the complex formation observed with GroEL and unfolding intermediates of CS. The much higher percentage activity at the beginning of the CS reactivation in the presence of Hsp33 (40%) than in the absence of the chaperone (15%) probably reflects Hsp33's interactions with native substrates of CS that are still in a rapid equilibrium with the native state.

#### Reduced Hsp33 Is Inactive

During Hsp33's purification process it became evident that addition of reducing agents to the purification buffers altered the affinity of Hsp33 to hydroxylapatite column material as well as its mobility in SDS-PAGE and native gels. We therefore tested to see if Hsp33 had chaperone activity both in the absence and presence of reducing agents like DTT. We found that the presence of 2 mM DTT dramatically reduced Hsp33's chaperone

activity (Figure 3A). Hsp33's ability to suppress thermal aggregation of both luciferase and CS was almost negligible when reductants were present (Figures 3A and 3B). This was a surprising result because Hsp33 is localized to the cytoplasm of *E. coli*, an environment generally considered to be very reducing (Derman et al., 1993). The DTT-induced inactivation was found to be a fast and quantitative process. Within 45 s of incubation in DTT, the chaperone activity of fully active Hsp33 begins to diminish. After 10 min of incubation, Hsp33 is almost completely inactive (Figure 3A). To exclude the possibility that DTT is affecting the substrates luciferase or CS rather than Hsp33, we confirmed that DTT, in the concentrations used, does not significantly alter the aggregation behavior of luciferase or CS in the absence of Hsp33. The thermal inactivation of CS is also not accompanied by intermolecular disulfide bond formation as analyzed by nonreducing and reducing SDS-PAGE, and we were unable to detect the formation of any stable disulfide-linked complexes between Hsp33 and CS or luciferase-unfolding intermediates (data not shown).

#### Inactivation of Hsp33 Is Reversible

Hsp33 is an effective molecular chaperone in the absence of reductants but is essentially inactive under reducing conditions. This is reminiscent of data obtained for the prokaryotic transcription factors OxyR and SoxR (Ding and Demple, 1997; Zheng et al., 1998). These proteins have the unusual property of being inactive under reducing conditions but are activated when the redox potential of the environment becomes more oxidizing. As a first step to test whether this unique mode of regulation might also apply to a molecular chaperone, reactivation studies using reduced Hsp33 were performed. Reduced Hsp33 was incubated with various concentrations of oxidized glutathione or H<sub>2</sub>O<sub>2</sub>, and the activity of Hsp33 was measured by monitoring the influence of Hsp33 on the thermal aggregation of CS (Figure 3B). Incubation of DTT-reduced Hsp33 in the presence of H<sub>2</sub>O<sub>2</sub> reactivates Hsp33 in less than 30 min of incubation. Reactivation with GSSG reached 50% after 30 min and was completed within 90 min of incubation (data not shown). Reactivation of GSH-reduced Hsp33 is significantly faster. Simple dilution of GSH-reduced, inactive Hsp33 changes the [GSH]<sup>2</sup>/[GSSG] redox value in a more oxidizing direction and is sufficient to reactivate Hsp33 within the first 2 min of incubation (data not shown). These results suggested that Hsp33's chaperone activity is regulated by the redox potential of the cellular environment.

To test whether this regulation takes place in a physiologically relevant redox range, titrations of Hsp33's chaperone activity with mixtures of oxidized and reduced glutathione were performed (Figure 3C). The midpoint of Hsp33's chaperone activity is identical to the midpoint of Hsp33's mobility transition on native PAGE (Figure 3C, inset) and corresponds to its equilibrium constant  $K_{ox}$  with glutathione. The redox potential was calculated from these midpoints and found to be  $-170 \text{ mV} \pm 10 \text{ mV}$  using the Nernst equation and the standard redox potential of  $-240 \text{ mV}$  for the GSH/GSSG redox couple (Gilbert, 1990). This suggests that in the cell,

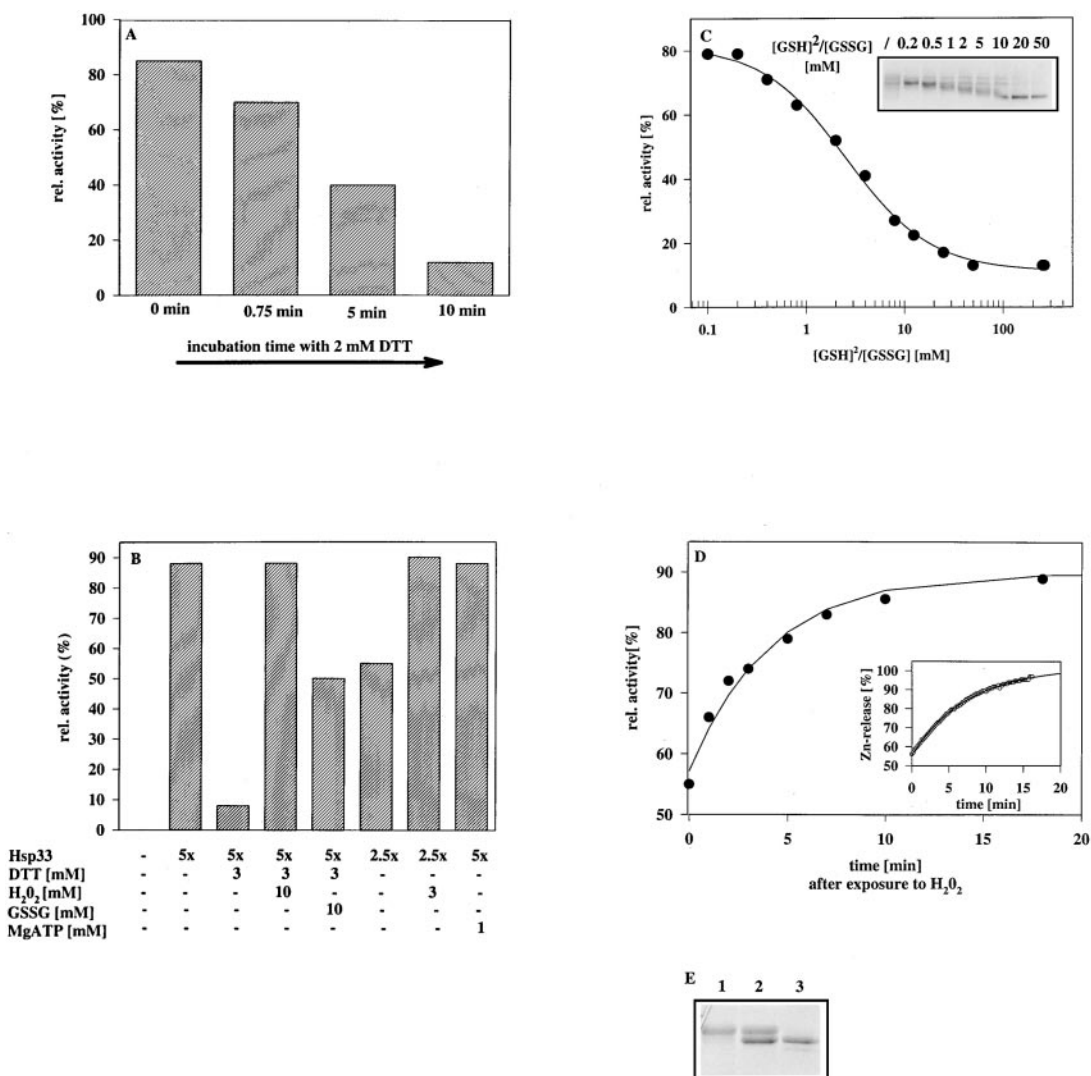


Figure 3. Inactivation and Reactivation of Hsp33 by Reduction and Oxidation

(A) DTT-induced inactivation of Hsp33 is a fast and quantitative process. Thermal aggregation of luciferase (0.16  $\mu$ M) in the presence of a 1:1 molar ratio of Hsp33 to luciferase. Hsp33 was incubated for the time points shown in the presence of 2 mM DTT at 43°C prior to the addition of luciferase. The percent chaperone activity of Hsp33 in suppressing light scattering of luciferase after 10 min incubation at 43°C is shown. One hundred percent activity is defined as corresponding to the "perfect chaperone," that is complete suppression of aggregation. Zero percent activity corresponds to the aggregation level seen for luciferase incubated in the absence of any chaperones.

(B) Inactivation of Hsp33 is reversible. Hsp33 was incubated in the absence or presence of 3 mM DTT for 60 min at 43°C. For reactivation studies, the indicated concentrations of GSSG or H<sub>2</sub>O<sub>2</sub> were added to reduced Hsp33 and incubation was continued for 30 min. To analyze the effects of H<sub>2</sub>O<sub>2</sub> on purified Hsp33, Hsp33 was incubated for 30 min in the presence of 3 mM H<sub>2</sub>O<sub>2</sub>. To investigate the influence of MgATP on the chaperone function of Hsp33, the assay buffer was supplemented with 1 mM MgATP prior to the addition of Hsp33. The relative chaperone activity of Hsp33 in suppressing light scattering of CS (0.112  $\mu$ M) after 13 min incubation at 43°C is shown.

(C) Redox titration of Hsp33 with oxidized and reduced glutathione. The percent chaperone activity of an 8-fold molar excess of Hsp33 to CS after incubation in various concentrations of GSH and GSSG is shown. A representative titration is shown. (Inset) Redox titration of Hsp33 on native PAGE. Redox transition of Hsp33 C141D/C239S mutant.

(D) Activation of Hsp33 by H<sub>2</sub>O<sub>2</sub>. Purified Hsp33 contains about 55% oxidized, active species (zero time point). The remaining 45% reduced, inactive Hsp33 species can be reactivated with H<sub>2</sub>O<sub>2</sub> in a time-dependent manner. Purified Hsp33 (0.3  $\mu$ M) was incubated in 3 mM H<sub>2</sub>O<sub>2</sub> at 43°C for the time points indicated. The percent chaperone activity of Hsp33 in suppressing light scattering of CS (0.112  $\mu$ M) at 43°C is shown. (Inset) Activation is accompanied by zinc release. Purified Hsp33 contains approximately 55% noncysteine-associated zinc that reacts immediately with the zinc complexing reagent PAR upon addition of the protein to the PAR-containing buffer (0 min time point). This probably corresponds to the amount of oxidized, active species in the preparation. Addition of 3 mM H<sub>2</sub>O<sub>2</sub> leads to the release of cysteine-associated zinc. The same complete zinc release is observed within 1 min after addition of the thiol-specific reagent PMPS (1 mM), indicating that the zinc is coordinated via cysteines.

(E) Activation and inactivation of Hsp33 by formation and breakage of disulfide bonds. Combined iodoacetamide/AMS trapping was performed on H<sub>2</sub>O<sub>2</sub>-oxidized (lane 1), nontreated (lane 2), and DTT-reduced (lane 3) Hsp33. The trapped samples were loaded onto a nonreducing SDS-PAGE.

Hsp33 is mostly inactive under normal cellular redox conditions ( $-250$  mV to  $-280$  mV) but becomes readily activated under oxidizing conditions ( $>-150$  mV), as is the case during oxidative stress (Gilbert, 1990).

Interestingly, the activity of  $H_2O_2$ -reactivated Hsp33 appeared to be even higher than the activity of non-treated Hsp33 (Figure 3B). This suggested that a mixture of reduced and oxidized Hsp33 species is present in our preparation. Purified Hsp33 was therefore incubated in 3 mM  $H_2O_2$  and the activity monitored (Figure 3D). Within 10 min of incubation at  $43^\circ\text{C}$ , the activity of Hsp33 increased 2-fold, suggesting that, as purified, about half of Hsp33 is oxidized.

Our experiments demonstrate that inactivation of Hsp33 is a reversible process and reactivation is possible by just making the redox potential of the incubation buffer more oxidizing.

### Hydrogen Peroxide Causes Activation of Hsp33 by Disulfide Bond Formation

The redox-dependent chaperone activity of Hsp33 suggested that disulfide bond formation is involved in the activation process of Hsp33. Thiol titrations of reduced, inactive, and oxidized active Hsp33 preparations implied that the activation of Hsp33 is accompanied by formation of two disulfide bonds. Reduced and inactive Hsp33 has  $4.8 \pm 0.2$  accessible cysteines, while incubation of Hsp33 in 3 mM  $H_2O_2$  yielded less than 0.5 titratable cysteines. Similar results were obtained with GdnHCl-denatured Hsp33. The total number of cysteines present in Hsp33 is six, four of which are highly conserved in a C-X-C and C-X-Y-C motif. Thiol titrations of a Hsp33 mutant protein whose nonconserved cysteines were replaced by Asp and Ser, respectively, (M. E. and U. J., unpublished) were consistent with the proposed formation of two disulfide bonds in the active, oxidized state of Hsp33.  $H_2O_2$ -oxidized Hsp33 C141D/C239S mutant possesses less than 0.2 free thiol groups, while  $3.7 \pm 0.2$  free thiol groups were titrated in the reduced, inactive protein.

To visualize the disulfide bond formation upon treatment with  $H_2O_2$  and disulfide bond reduction upon incubation with DTT, we employed a combined iodoacetamide/AMS trapping method. Iodoacetamide was first used to alkylate all free cysteines present in the preparation. Next, the existing disulfide bonds were reduced with DTT and the newly exposed sulfhydryl groups were trapped with AMS. AMS adds a molecular mass of 500 Da per thiol group and causes a mobility change in SDS-PAGE. As shown in Figure 3E, a significant mass difference exists between  $H_2O_2$ -oxidized and DTT-reduced Hsp33, indicating the formation of disulfide bonds in the active, oxidized species. The thiol-trapping experiments also confirmed our earlier observation that purified Hsp33 exists in an equilibrium between reduced and oxidized species.

EDTA influences the reactivation of Hsp33, suggesting that metal binding might be involved in the stabilization and reactivation of reduced Hsp33. Inductively coupled plasma atomic emission spectroscopy (ICP) metal analysis of different Hsp33 preparations revealed the presence of equimolar concentrations of zinc in our

preparations. To analyze the role of zinc in the regulation of Hsp33, the association state of zinc during the activation process of Hsp33 was monitored. The  $H_2O_2$ -induced activation of Hsp33's chaperone function almost exactly parallels the release of zinc from Hsp33 (Figure 3D, inset). This release can be also challenged by the addition of the small thiol specific reagent p-hydroxymercuric phenylsulfonate (PMPS), a reagent that has been successfully employed to identify and analyze cysteine-ligated zinc coordination in proteins (Hunt et al., 1984). The formation of a mercaptide bond between PMPS molecules and Hsp33's cysteines causes the immediate dissociation of zinc. This clearly indicates that cysteines play a part in the zinc coordination of reduced Hsp33. Zinc binding analysis of the Hsp33 C141D/C239S mutant protein that eliminates the two nonconserved cysteines but maintains redox regulation (Figure 3C, inset) and chaperone activity (data not shown) implies that the conserved cysteines are involved in the proper zinc coordination under reducing conditions. The oxidation of Hsp33 does not seem to cause the complete dissociation of zinc from Hsp33 but rather a displacement from the reactive cysteines. A significant amount of noncysteine coordinated zinc is still present in the oxidized form of Hsp33 (Figure 3D, inset).

Our results explain the fast attenuation of Hsp33's chaperone function in response to the redox conditions of the environment. Under reducing conditions, the reactive cysteines are coordinated by a zinc molecule and Hsp33 is inactive. Upon exposure of Hsp33 to more oxidizing conditions such as  $H_2O_2$  and concomitant with zinc release from the cysteines, the disulfide bonds form and transform the protein into a very potent molecular chaperone.

### Hsp33 Confers Resistance to $H_2O_2$ - and Heat-Induced Killing

Cellular heat shock is accompanied by oxidative stress (Benov and Fridovich, 1995; Davidson et al., 1996), and oxidative stress induces the expression of heat shock proteins (VanBogelen et al., 1987; McDuffee et al., 1997). Since Hsp33's chaperone function is quickly turned on with exposure to more oxidizing conditions *in vitro*, we investigated whether Hsp33 plays a role during heat stress and oxidative stress *in vivo*. In order to determine the role of Hsp33 in the growth of *E. coli* at high temperatures, the maximal growth temperatures of strains with and without the Hsp33 null mutation were determined. This was done by using a large thermal gradient LB agar plate whose temperature range encompassed the maximal growth temperatures (Bardwell and Craig, 1988). The maximal growth temperature of the wild-type *E. coli* K12 strain MG1655 was found to be reduced  $0.5^\circ\text{C}$  by the deletion of the gene encoding Hsp33 (*hsfO*). This phenotype is similar to the temperature sensitivity shown by deletion of the gene for other heat shock proteins in *E. coli*, such as Hsp90 (Bardwell and Craig, 1988). Next, we examined the phenotype of the *hsfO* mutant in a strain that contains a mutation in the thioredoxin reductase gene *trxB* (Russel and Model, 1986). This oxidizing strain background is known to contain potentially destructive disulfide bonds in cytoplasmic

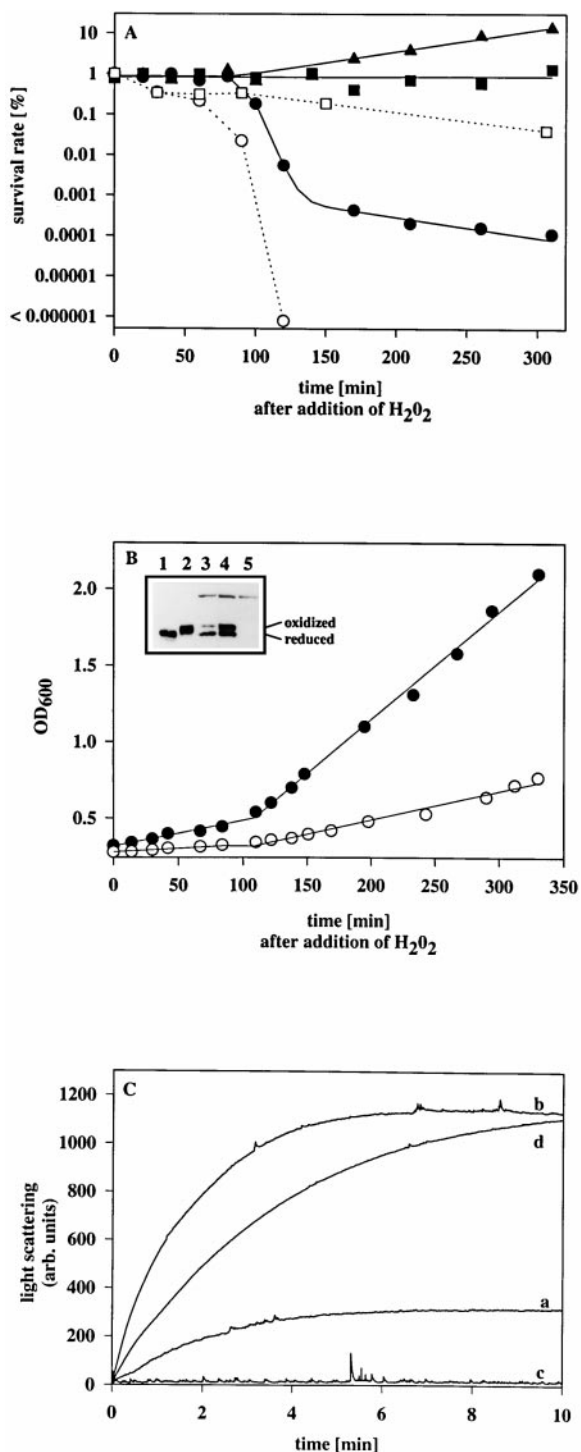


Figure 4. Hsp33 Plays a Role under Oxidative Stress Both In Vivo and In Vitro

(A) Hsp33 confers resistance to H<sub>2</sub>O<sub>2</sub>-induced cell killing at 37°C and 43°C. Survival rate of Hsp33-deficient strains after H<sub>2</sub>O<sub>2</sub> treatment. WM93 (*trxB*<sup>-</sup>) (closed squares), WM97 (*trxB*<sup>-</sup> *hslO*<sup>-</sup>) (closed circles), and WM97 (triangles) containing the *hslO*-encoding plasmid pUJ30 after addition of 4 mM H<sub>2</sub>O<sub>2</sub> at 37°C. WM93 (open squares) and WM97 (open circles) after addition of 4 mM H<sub>2</sub>O<sub>2</sub> and shift to 43°C. The expression level of Hsp33 in the pUJ30-containing cells in the absence of T7 polymerase reached a very similar level to that produced by the chromosomal copy of Hsp33 in wild-type cells as determined by Western blot analysis of whole cell extracts.

proteins (Derman et al., 1993) and is expected to cause the accumulation of Hsp33 in the active, oxidized state. Deletion of the Hsp33 gene in the *trxB*<sup>-</sup> strain caused a 1.4°C ± 0.4°C reduction in the maximal growth temperature, and this temperature sensitivity cotransduced with the *hslO* null mutation. These in vivo results are consistent with the ability of Hsp33 to protect oxidatively damaged proteins in vitro (see below).

Oxidative stress is known to be one major reason for heat shock-induced cell death (Benov and Fridovich, 1995; Davidson et al., 1996). We thus investigated whether Hsp33 plays a role in oxidative stress protection per se. Deletion of the Hsp33 gene *hslO* leads to a slightly higher sensitivity of cells toward H<sub>2</sub>O<sub>2</sub> exposure at elevated temperatures compared to wild-type cells (data not shown). A highly sensitive oxidative stress phenotype was observed when the previously employed strains *trxB*<sup>-</sup> (WM93) and *trxB*<sup>-</sup> *hslO*<sup>-</sup> (WM97) were exposed to oxidative stress. In this strain background, the *hslO* deletion confers a 10,000-fold increase in H<sub>2</sub>O<sub>2</sub> sensitivity (Figure 4A). Complementation studies revealed that the increased sensitivity toward oxidants is due to the absence of the Hsp33 gene. The presence of an *hslO*-encoding plasmid in *trxB*<sup>-</sup> *hslO*<sup>-</sup> strains fully restores and may even enhance the bacteria's resistance toward H<sub>2</sub>O<sub>2</sub> (Figure 4A). Even more severe effects of H<sub>2</sub>O<sub>2</sub> treatment on the survival rate of *trxB*<sup>-</sup> *hslO*<sup>-</sup> strains could be observed when oxidative stress was combined with heat stress (Figure 4A). H<sub>2</sub>O<sub>2</sub> treatment at 43°C led to a very rapid decline in the survival rate of *trxB*<sup>-</sup> *hslO*<sup>-</sup> cells compared to the isogenic wild-type strains. Within 120 min of H<sub>2</sub>O<sub>2</sub> treatment at 43°C, no surviving colonies of *trxB*<sup>-</sup> *hslO*<sup>-</sup> cells could be detected. This corresponds to a greater than 10<sup>6</sup>-fold higher sensitivity of the *hslO* deletion toward H<sub>2</sub>O<sub>2</sub> treatment compared to the isogenic *trxB*<sup>-</sup> cells.

Cells mutant in genes for both thioredoxin (*trxA*) and glutathione reductase (*gor*) also provide a highly oxidizing cytoplasmic environment by negatively affecting the two major redox-balancing systems in the cytoplasm of *E. coli* (Prinz et al., 1997). This leads to an accumulation of disulfide bonds in the cytoplasm and possibly to the induction of the oxidative stress response. In agreement with the previous results, we observed that the H<sub>2</sub>O<sub>2</sub> resistance of *trxA*<sup>-</sup> *gor*<sup>-</sup> cells diminishes significantly in the absence of functional Hsp33. *trxA*<sup>-</sup> *gor*<sup>-</sup> *hslO*<sup>-</sup> cells grow almost 4-fold slower after H<sub>2</sub>O<sub>2</sub> exposure than do the isogenic *trxA*<sup>-</sup> *gor*<sup>-</sup> cells (Figure 4B).

These results show that Hsp33 plays a significant protective role under oxidative stress and heat stress in *E.*

(B) *hslO* deletion results in growth disadvantage after H<sub>2</sub>O<sub>2</sub> treatment. Growth of WP843 (*trxA*<sup>-</sup> *gor*<sup>-</sup>) (closed circles) and WM104 (*trxA*<sup>-</sup> *gor*<sup>-</sup> *hslO*<sup>-</sup>) (open circles) after addition of 15 mM H<sub>2</sub>O<sub>2</sub> at 37°C. (Inset) WP843 (*trxA*<sup>-</sup> *gor*<sup>-</sup>) provides a highly oxidizing environment. AMS thiol trapping from reduced Hsp33 (lane 1) and oxidized Hsp33 (lane 2). In vivo trapping from Hsp33 in DHB4 wild-type cells (lane 3), *trxA*<sup>-</sup> *gor*<sup>-</sup> (lane 4), and *trxA*<sup>-</sup> *gor*<sup>-</sup> *hslO*<sup>-</sup> (lane 5) cells. A Western blot with polyclonal antibodies against Hsp33 is shown. (C) Hsp33 protects FtsJ from oxidative damage in vitro. FtsJ (final concentration 0.27 μM) was incubated in the (a) absence or (b) presence of 8 mM H<sub>2</sub>O<sub>2</sub>. The incubation reactions supplemented with additional protein contained a (c) 6-fold molar excess (106 μg) of Hsp33 or (d) 106 μg BSA in the presence of 8 mM H<sub>2</sub>O<sub>2</sub>.

*coli*, stress conditions known to be intimately linked in vivo via their damaging effects on cellular proteins.

#### Oxidized Form of Hsp33 Accumulates In Vivo under Oxidative Stress

We compared the in vivo ratio of oxidized and reduced Hsp33 in wild-type and *trxA<sup>-</sup> gor<sup>-</sup>* mutant cells using AMS as a thiol-trapping reagent (inset in Figure 4B). While in the isogenic wild-type strain, only about 20% of Hsp33 could be trapped in the oxidized form, the *trxA<sup>-</sup> gor<sup>-</sup>* mutants provided an environment where ~60% of Hsp33 is in the oxidized form. This result suggests that under normal conditions, Hsp33 is mainly in the reduced, inactive state. Changing the redox environment in a more oxidizing direction causes a shift in the equilibrium resulting in the accumulation of oxidized, active Hsp33. These results are in good agreement with the in vitro studies of the redox regulation of Hsp33's chaperone activity.

#### Hsp33 Protects Proteins from Oxidative Stress In Vitro

Oxidative stress-induced aggregation of certain cellular proteins leads to the induction of the heat shock response both in pro- and eukaryotes (VanBogelen et al., 1987; McDuffee et al., 1997). To elucidate whether oxidatively damaged proteins serve as substrates for Hsp33, we first analyzed a number of purified proteins in respect to their sensitivity toward reconstituted oxidative stress conditions in vitro. We discovered that the *E. coli* protein FtsJ rapidly forms large aggregates when exposed to oxidants like H<sub>2</sub>O<sub>2</sub> (Figure 4C, line b). Hsp33 is remarkably efficient at preventing H<sub>2</sub>O<sub>2</sub>-induced aggregation of FtsJ. A 6-fold molar excess of Hsp33 to FtsJ completely inhibits the formation of any detectable aggregates (Figure 4C, line c). BSA, used to exclude unspecific protein effects, fails to significantly alter the extent of FtsJ's aggregation (Figure 4C, line d). These results imply that Hsp33 is a highly efficient chaperone for oxidatively damaged proteins. Hsp33 is activated under reconstituted oxidative stress conditions in vitro and can prevent oxidatively damaged proteins from aggregating. We therefore propose that Hsp33 is a chaperone that is very effective in dealing with the consequences of oxidative stress.

#### Discussion

Most heat shock proteins function as molecular chaperones and most chaperones are heat inducible. Given this tight correlation, we have decided to exploit the rich and largely untapped source of potential new chaperones that may exist among the 26 recently identified heat shock proteins in *E. coli* (Chuang and Blattner, 1993). We describe here Hsp33, a member of a novel heat shock family, which acts as a very efficient molecular chaperone, protecting both thermally unfolding and oxidatively damaged proteins from irreversible aggregation. All twenty members of this highly conserved protein family sequenced to date share a four-cysteine motif

that is involved in the functional regulation of the chaperone. These cysteines are sensitive to the redox conditions of the environment and function as a redox switch that turns the chaperone on and off. Hsp33's cysteines quickly respond to an oxidizing environment such as H<sub>2</sub>O<sub>2</sub> exposure by the formation of disulfide bonds. This leads to the activation of the chaperone function of Hsp33. In vivo thiol-trapping experiments showed that the majority of Hsp33 is reduced in the absence of stress and becomes oxidized when cells are subject to oxidative stress. Since activated Hsp33 appears to bind with high affinity to certain folding intermediates, this regulation might be necessary to prevent stable complex formation between Hsp33 and folding intermediates during normal cellular conditions. The high affinity of Hsp33 to folding intermediates as well as the apparent ATP independence of Hsp33's chaperone action leads us to speculate that Hsp33 works as a "holdase" rather than a "foldase" (Bohen et al., 1995). "Holdases" like the ATP-independent small Hsps bind stably to folding intermediates and suppress otherwise lethal aggregation. Their subsequent interactions with "foldases" like Hsp70 allow the efficient release of the intermediates and their successful refolding to the native state when conditions return to normal. Chaperones that stably bind proteins may also serve as holdases for the subsequent action of proteases (Sherman and Goldberg, 1996). Since proteases specialized for the degradation of oxidatively damaged proteins have been identified in *E. coli* (Davies and Lin, 1988), it will be interesting to determine the in vivo fate of substrate proteins stably associated with Hsp33.

Hsp33's ability to be functionally regulated in an on-off mode depending on the redox conditions of the environment is an unusual form of regulation and, to our knowledge, a unique form of regulation amongst molecular chaperones. A central role is played by the conserved cysteines in Hsp33, which are extremely reactive and quickly adjust Hsp33's activation state to changes in the environment by forming or reducing disulfide bonds. The midpoint of the redox transition is -170 mV, assuring that Hsp33 is reduced and inactive under normal redox conditions. In vitro, activation of reduced Hsp33 can be achieved very rapidly using physiological oxidants like H<sub>2</sub>O<sub>2</sub>, and this activation seems to occur at a rate limited only by the rate of the thiol/disulfide exchange. This allows preexisting Hsp33 to respond rapidly to changes in the cell's redox status, much more rapidly than would be allowed by regulation of Hsp33 exclusively at the transcriptional or translational level. This fast reaction might be due in part to metal coordination in Hsp33. Reduced Hsp33 has zinc coordinated via its conserved cysteines. This coordination could enhance the reactivity of Hsp33 toward peroxides by stabilizing the thiolate form of the cysteine. Upon oxidation with H<sub>2</sub>O<sub>2</sub>, the zinc is released from the cysteines and disulfide bonds form. Therefore, the zinc coordination, the oxidation state, and most importantly, the activation state of Hsp33 is directly linked to the redox state of the environment. Interestingly, a somewhat similar form of redox regulation has recently been established for the zinc storage protein metallothioneine (Maret and Vallee, 1998). Oxidizing agents cause the zinc transfer from



metallothionine to zinc-depleted enzymes with concomitant disulfide bond formation in the donor protein. Unlike Hsp33, where this oxidation leads to a functional activation, for metallothionine it simply leads to a zinc release. We believe that Hsp33 is the first protein known to utilize this fast redox mechanism for its activation.

Reactive oxygen species have been implicated in a number of pathological processes, including aging and cancer (Berlett and Stadtman, 1997). This is in part due to the deleterious effects of oxidative damage on proteins that affect their enzymatic activity and increase their susceptibility to proteolysis and aggregation. We believe that the fast response of Hsp33 may represent a first line of defense against oxidative protein damage in prokaryotes. One source of exogenously induced oxidative stress for pathogenic bacteria is their attack by host phagocytic cells (Miller and Britigan, 1997). To kill pathogenic bacteria, phagocytes release high concentrations of superoxide radicals that subsequently dismutate to H<sub>2</sub>O<sub>2</sub>. On protein level, these highly reactive oxidants lead to side chain oxidation, nonspecific disulfide bond formation, unfolding, thermal instability, and finally protein aggregation in the cell (Gilbert, 1990; McDuffee et al., 1997). We show Hsp33 to be a potent protector of both thermally unfolded and oxidatively damaged proteins in vitro. The increased sensitivity of Hsp33 deletion mutants toward H<sub>2</sub>O<sub>2</sub> suggests that this is also the case under oxidative stress conditions in vivo. Hsp33 is effective in preventing H<sub>2</sub>O<sub>2</sub>-induced cell killing. This function becomes especially important under conditions when cells are exposed to more oxidizing conditions such as occur when the host cell is lacking the thioredoxin and glutathione genes (Derman et al., 1993; Prinz et al., 1997). Cells carrying these mutations have a significantly higher resistance toward oxidative stress than wild-type cells (Takemoto et al., 1998). This resistance appears to be at least in part due to Hsp33 and its accumulation in the oxidized and active state, since deletion of the Hsp33 gene causes a loss of this resistance and expression of Hsp33 from a plasmid leads to higher resistance of the cells toward the H<sub>2</sub>O<sub>2</sub> treatment.

Hsp33's regulation takes place on both transcriptional and posttranslational levels. Heat shock induces the expression of Hsp33, while oxidative stress induces the activation of its chaperone function. However, since heat and oxidative stress responses are so intimately linked in the cell, it is not surprising that we found increased expression of Hsp33 under oxidative stress conditions (data not shown) and a temperature-sensitive phenotype under heat shock conditions. These overlapping effects of heat shock and oxidative stress on Hsp33 may be due to their common cellular consequences: protein damage and accumulation of unfolding, aggregation-sensitive protein folding intermediates. These intermediates are potential substrates for the highly effective chaperone Hsp33. One of the possible reasons for heat shock-induced oxidative stress is thought to be the thermal denaturation of cellular antioxidant proteins that results in the accumulation of endogenous oxidants (Davidson et al., 1996). This could explain why a number of antioxidant proteins are induced by heat stress (VanBogelen et al., 1987). Even so, oxidative damage was

found to be one major cause of heat shock-induced cell death in both pro- and eukaryotes (Benov and Fridovich, 1995; Davidson et al., 1996). Oxidative stress, on the other hand, induces the expression of a number of heat shock proteins in both eu- and prokaryotes (VanBogelen et al., 1987; McDuffee et al., 1997). This includes Hsp33, whose steady-state protein concentration increases more than 2-fold within the first 20 min after exposure to H<sub>2</sub>O<sub>2</sub> (data not shown). This is very similar to the observed 2-fold increase in steady-state level of classical Hsps following temperature shift. This implies that oxidative stress is accompanied by the accumulation of unfolded proteins, a signal known to trigger the heat shock response (Parsell and Sauer, 1989). It is interesting to note that DnaK, a chaperone important in mediating the heat shock response in *E. coli* (Liberek et al., 1992), is one of the major targets of protein oxidation in *E. coli* (Tamarit et al., 1998). Given the relative abundance of Hsp33 in the cell, which is in the same range as the concentration of active GroEL oligomers (Lorimer, 1996), it is tempting to speculate that Hsp33 is able to take over the function of certain chaperones such as DnaK that may be damaged following oxidative stress conditions in vivo.

In summary, both our in vitro and in vivo studies show that Hsp33 is a novel molecular chaperone that is directly activated by oxidation and which appears to play an important role in the bacterial defense system toward oxidative stress.

#### Experimental Procedures

##### Cloning and Purification of Hsp33

The forward and reverse primers used for amplifying the Hsp33 gene *hslO* from pTHZ13 (W. M. et al., unpublished) by PCR were 5' ctgcaagacatgatgattgccc 3' and 5' ctgccggatccactcattaatgaa 3', respectively. The cloned *hslO* was introduced into pET11a to generate the plasmid pUJ30 that was transformed into *E. coli* BL21. Cells were grown at 37°C in the presence of 1 mM ZnCl<sub>2</sub> and harvested 6 hr after induction with 1 mM IPTG. Cells were resuspended in buffer A (40 mM HEPES-KOH, 0.2 M KCl [pH 7.5], 1 tablet Boehringer complete protease inhibitor mix, 2 mM PMSF) and lysed (French Press, two cycles, 14,000 psi). The cleared supernatant was applied onto a 40 ml Q-Sepharose column (Pharmacia). Hsp33 eluted between 450 and 600 mM KCl in buffer A. Hsp33-containing fractions were applied onto a 40 ml hydroxylapatite column. Hsp33 elutes between 10 and 50 mM potassium phosphate (pH 6.8). Hsp33-containing fractions were loaded onto a Superdex 75 (Pharmacia) equilibrated in buffer A. Highly purified Hsp33 fractions from the Superdex column were dialyzed against storage buffer (40 mM HEPES-KOH, 20 mM KCl [pH 7.5]). For concentration determination, the extinction coefficient of 0.54 for a 1 mg/ml solution at OD<sub>280</sub> was used (Gill and von Hippel, 1989). The OD<sub>280/260</sub> ratio of the purified Hsp33 was 1.8, revealing the presence of less than 1% nucleotide impurity in our preparation.

##### Cell Fractionation and Cellular Concentration

###### Determination of Hsp33

Three different cell fractionations to determine the subcellular localization of Hsp33 were performed (Thorstenson et al., 1997) and gave identical results. The concentration determination of Hsp33 in MC4100 cells was performed according to Korber et al. (1999).

##### Chaperone Activity of Hsp33

Citrate synthase (Boehringer Mannheim) was diluted into preincubated 40 mM HEPES-KOH (pH 7.5) at 43°C. Light scattering was monitored in a Hitachi Fluorimeter F4500 equipped with thermostated cell holder and stirrer and the measurements were performed

Table 1. Strains Used in This Work

Strains	Relevant Genotype	Source of Reference
MC4100	<i>araD139 Δ(argF-lac)U169 rpsL150 relA flbB5301 deoC1 ptsF25 rbsR</i>	lab collection
WM28	<i>F<sup>-</sup>, recD1903::Tn10d Tet, hslO::Km zhh-198::Tn10dSpcRCP2 λhslPRO*</i>	this work
WM51	MC4100 <i>hslO::Km</i>	MC4100 × P1 (WM28); this work
A313	HfrC (1) <i>trxA::Km metE::Tn10</i>	Russel and Model (1986)
WM93	A313 <i>zhh-198::Tn10dSpcRCP2</i>	A313 × P1 (WM28); this work
WM97	WM93 <i>hslO::Km zhh-198::Tn10dSpcRCP2</i>	A313 × P1 (WM28); this work
DHB4	<i>F lac-pro lacP (ara-leu)7697 araD139 lacX74 galE galK rpsL phoR (phoA) PvuII malF3 thi</i>	Prinz et al. (1997)
WP843	DHB4 <i>trxA gor522::mini-Tn10Tc<sup>R</sup></i>	Prinz et al. (1997)
WM104	WP843 <i>hslO::Km</i>	WP843 × P1 (WM51); this work

\**λhslPRO*, 7 kb Sall fragment from Kohara  $\lambda$  621 cloned into Sall in  $\lambda$ gtWES.

as described (Buchner et al., 1998). Inactivation and reactivation studies with oxaloacetic acid were performed according to Jakob et al. (1995). For the luciferase assay, firefly luciferase (Boehringer Mannheim) was diluted under constant stirring into preequilibrated 40 mM HEPES-KOH (pH 7.5) at 43°C in the absence or presence of the respective additives.  $\lambda_{\text{ex/em}}$  were set to 350 nm, the excitation slit width was set to 2.5, and the emission slit width was set to 5.0 nm. After 15 min of thermal inactivation, 2 ml samples were centrifuged for 30 min at 4°C (15,000 × g), the protein pellets were resuspended in 100  $\mu$ l SDS-PAGE buffer, and 30  $\mu$ l was applied to a 14% NOVEX-SDS-PAGE. Purified FtsJ (H. Buegl and U. J., unpublished) was incubated at 30°C in 40 mM HEPES-KOH (pH 7.5) in the absence or presence of 8 mM H<sub>2</sub>O<sub>2</sub>.  $\lambda_{\text{ex/em}}$  were set to 350 nm and the slit widths set to 2.5 nm (ex) and 5.0 nm (em), respectively.

#### Redox Titration and Equilibrium Constant $K_{\text{ox}}$

Light scattering measurements of CS at 43°C were performed as described. A concentration of CS was chosen in which a linear relationship exists between the light scattering signal of thermally inactivating CS and the concentration of CS used (correlation coefficient: 0.9996). Oxidized Hsp33 (22.8  $\mu$ M) was incubated in various concentrations of GSH and GSSG for 2 hr at 37°C prior to diluting it 1:26 into the assay buffer. To prevent alteration of the oxidation status of Hsp33 during the time course of the assay, the assay buffer was supplemented with concentrations of GSH/GSSG, yielding redox potentials identical to the ones in the incubation reaction. Control experiments in the absence of Hsp33 revealed no influence of the GSH and GSSG concentrations used on the light scattering signal of CS. The equilibrium constant  $K_{\text{ox}}$  was determined from the redox titration and used to calculate the redox potential of Hsp33 (Grauschopf et al., 1995). For the analysis of the redox state on native PAGE, 0.15 mg/ml Hsp33 C141D/C239S mutant was incubated in mixtures of GSSG and GSH for 2 hr at 37°C. The samples were supplemented with 1/10 of their volume native sample buffer and loaded immediately onto 14% native NOVEX PAGE.

#### Determination and Trapping of Free Thiols In Vitro and In Vivo

Titration of free thiols in native and in 6 M GdnHCl denatured Hsp33 and Hsp33 C141D/C239S mutant were performed according to Creighton (1989). For in vitro trapping experiments, 30  $\mu$ g purified Hsp33 was incubated in 100  $\mu$ l 40 mM HEPES (pH 7.5) in the absence or presence of either 10 mM DTT or 5 mM H<sub>2</sub>O<sub>2</sub> for 1 hr at 37°C to generate a homogenous preparation of a reduced and oxidized Hsp33 species. Iodoacetamide (in 100 mM Tris, 10 mM EDTA [pH 9.5]) was added to a final concentration of 100 mM, and the reaction was incubated at 37°C for 2 min. The reaction was stopped by the addition of 122  $\mu$ l 20% v/v ice-cold TCA and incubation on ice (30 min).

Fifty milliliters of LB medium was inoculated with fresh overnight cultures of DHB4, WP843, and WM104, and the cells were grown at 37°C until OD<sub>600</sub> = 0.4 was reached. Cultures (1.4 ml) were taken

and mixed with 0.4 ml of preequilibrated iodoacetamide (final concentration, 100 mM). The samples were incubated at 37°C for 2 min and the reaction was stopped by addition of 0.2 ml 100% ice-cold TCA and incubation on ice (30 min). The in vitro and in vivo alkylated samples were centrifuged at 4°C for 30 min (14,000 × g), washed twice with TCA, resuspended in 50  $\mu$ l 10 mM DTT, 100 mM Tris, 10 mM EDTA, 0.5% SDS (pH 9.5), and incubated at 44°C for 1 hr. The cysteines exposed by these reducing conditions were trapped with the thiol-trapping agent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) (Molecular Probes). Twenty microliters of 50 mM AMS in 100 mM Tris, 10 mM EDTA (pH 9.5) was added to 20  $\mu$ l of the reduction reaction and incubation continued for 90 min at room temperature. The reaction was stopped by the addition of 10  $\mu$ l 5× nonreducing Laemmli buffer and boiling. The samples were applied onto a 14% SDS-Tris-Glycine PAGE (Novex) and analyzed by Western blot using polyclonal antibodies against Hsp33.

#### Metal Analysis and Zinc Determination

The ICP metal analysis was performed by Garrat-Callahan Company (Millbrae, CA). Hsp33 was purified from exogenous metals by gel filtration against 18 M $\Omega$  deionized water. The protein concentration was between 18 and 26  $\mu$ M Hsp33, depending on the preparation. Three different Hsp33 preparations, purified from overexpressing cells grown in the absence and presence of zinc, were submitted for analysis. Buffer samples without protein as well as Hsp33 samples treated with EDTA prior to the gel filtration were used as controls and did not reveal detectable amounts of zinc in the preparation. ICP analysis tested for 23 different metals. Hsp33 prepared from overexpressing cells grown in the absence of zinc contained 0.3 M iron and 0.7 M zinc per mol Hsp33. Addition of 1 mM zinc to the growth medium resulted in an equimolar zinc to protein content. No other metals were detected in our preparations.

The H<sub>2</sub>O<sub>2</sub>-induced release of zinc was monitored according to Hunt et al. (1984). This assay is based on the complex formation of released zinc with the zinc complexing dye 4-(2-pyridylazo) resorcinol (PAR),  $\epsilon_{500} = 66000 \text{ M}^{-1} \text{ cm}^{-1}$ . Since PAR usually does not interact with cysteine-liganded zinc, this assay allowed us to distinguish between thiol- and nonthiol-associated zinc. Hsp33 was purified by gel filtration and added to 40 mM HEPES-KOH, supplemented with 0.1 mM PAR (final concentration of Hsp33, 1.0  $\mu$ M). The increase of OD<sub>500</sub> upon addition of the protein can be taken as the amount of nonthiol-associated zinc present in the preparation. H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 3 mM, and the zinc release was monitored at 43°C in a Beckman spectrophotometer. H<sub>2</sub>O<sub>2</sub> or PMPS treatment after 30 min of the reaction did not lead to any additional zinc release and therefore also excluded the presence of zinc impurities in the H<sub>2</sub>O<sub>2</sub> solution.

#### Chromosomal Deletion of the Hsp33 Gene and H<sub>2</sub>O<sub>2</sub> Survival

The chromosomal deletion of the Hsp33-encoding *hslO* gene was generated by the homologous recombination of a gene disruption. This disruption consists of a 737 bp internal deletion tagged with a

km<sup>R</sup> resistance cassette. Recombinants were isolated in a *recD::Tn10*, *hslO*<sup>+</sup> merodiploid strain after electroporation. Subsequently, the disrupted *hslO::km* locus was transduced to MC4100 by following km<sup>R</sup> resistance. The *hslO::km* mutant allele together with a nearby spectinomycin resistance cartridge was moved via P1vir into the *trxB*<sup>-</sup> strain A313 (Russel and Model, 1986). Transductants were screened by Western blot analysis for the presence and absence of Hsp33, and isogenic strain pairs (WM93, WM97) were isolated. The *hslO::km* mutant allele was moved into *trxA*<sup>-</sup> *gor*<sup>-</sup> strain WP843 (Prinz et al., 1997) via P1vir following the km<sup>R</sup> resistance and generating the strain WM 104 (Table 1).

LB medium was inoculated 1:100 with fresh overnight cultures, and the cells were grown at 37°C to an OD<sub>600</sub> of 0.3–0.4. The cultures were supplemented with 4 mM H<sub>2</sub>O<sub>2</sub> and 15 mM H<sub>2</sub>O<sub>2</sub>, respectively. Samples were taken at various time points during the incubation and either survival rate or optical density at OD<sub>600</sub> was determined.

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