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Redox Switch of Hsp33 Has a Novel Zinc-binding Motif*

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Ursula Jakob‡§, Markus Eser‡¶, and James C. A. Bardwell‡‖

From the ‡Department of Biology, University of Michigan, Ann Arbor, Michigan 48109-1048

The chaperone activity of the heat shock protein Hsp33 is regulated by reversible disulfide bond formation. Oxidized Hsp33 is active, and reduced Hsp33 is inactive. We show that zinc binding is essential for the function of this redox switch. Our results reveal that Hps33 contains a new, high affinity ($K_a > 10^{17}$ M^{-1}), zinc-binding motif in the form Cys-*X*-Cys-$X_{27–32}$-Cys-*X*-*X*-Cys. All four conserved cysteines within this motif act to coordinate a single zinc atom. Experiments where reduced wild type Hsp33 is reconstituted with cobalt or cadmium demonstrate that the metal-coordinating cysteines are present as highly reactive thiolate anions. This ionization may allow for the fast and successful activation of the chaperone function of Hsp33 upon incubation in oxidizing agents.

Hsp33 is a novel heat shock protein present in a wide variety of prokaryotic species. To date over 30 different Hsp33 homologues have been identified, all of which share a conserved cysteine motif located near the C terminus of the protein. These cysteines, which are arranged in a C*X*C$X_{27–32}$C*XX*C motif, play an important role in the functional regulation of Hsp33 (1–3). Under oxidizing conditions, Hsp33 functions as a potent molecular chaperone, capable of recognizing and binding aggregation-sensitive folding intermediates (1). This leads to the efficient suppression of protein aggregation, a common and irreversible side reaction of protein folding and thermal unfolding events (4). Under reducing conditions, however, Hsp33 is inactive. The molecular switch of this activation/inactivation process is mediated by the cysteine-containing motif of Hsp33 (1). Under reducing conditions, all 4 conserved cysteines are present as thiol groups. They are titratable with Ellman's thiol reagent and accessible to thiol modifications such as alkylation by iodoacetamide (5). Under oxidizing and activating conditions, these cysteines form two intramolecular disulfide bonds connecting the next two neighbor cysteines Cys^{232}–Cys^{234} and Cys^{265}–Cys^{268} (5). The activation process of Hsp33 appears to be accompanied by major conformational changes in the protein. Active and inactive Hsp33 preparations show substantially different mobility on native gels (1). Cys^{239}, a non-reactive, non-conserved cysteine localized in the vicinity of the zinc-binding motif of Hsp33 has been shown to become completely buried upon oxidation and activation. This is additional evidence of a major conformational change accompanying the disulfide bond formation in Hsp33 (5). Other molecular chaperones like DnaK and GroEL show significant conformational changes upon cofactor or co-chaperone binding that appear to constitute a major regulative part of their chaperone function (6, 7).

Reduced Hsp33 coordinates zinc via its cysteines (1). Upon activation of Hsp33, the disulfide bonds form, and zinc is released. Zinc-coordinating cysteine centers, such as present in Hsp33 or zinc finger proteins, are prime targets for reversible oxidative modification processes and constitute a novel regulatory element of proteins. Zinc finger transcription factors such as members of the Sp1 family have been demonstrated to be redox-regulated both *in vitro* and *in vivo* (8). The zinc finger of replication protein A as well as the zinc ring finger protein SAG have been shown to be highly sensitive toward oxidation and reduction processes (9, 10). Metallothionein, an important cytosolic zinc storage protein, has been shown to utilize disulfide bond formation for its directed zinc donation (11, 12). This allows the efficient transfer of zinc from the high affinity zinc centers of metallothionein ($K_a = 3.2 \times 10^{13}$ M^{-1}, pH 7.4) to proteins with significantly lower zinc affinity (11, 12). Reversible disulfide bond formation is a very fast and elegant way to translate changes in environmental conditions into changes in protein activity. Given that a large number of signal-transducing proteins contain zinc finger domains, it is of interest to analyze what properties determine the redox sensitivity of zinc centers.

Although it has been shown that reduced Hsp33 binds zinc, the precise role of the zinc binding, particularly the question whether zinc plays a primary structural role or is involved in the activation process, is not yet known. Neither the stoichiometry of zinc binding nor the identity of the cysteines involved in the zinc coordination in Hsp33 has been investigated so far. Here we show that Hsp33 has a high affinity zinc-binding motif consisting of the 4 highly conserved cysteines. Reconstitution experiments with cobalt and cadmium were performed to evaluate the composition and geometry of the zinc binding. Reactivation of Hsp33 in the presence and absence of zinc revealed that zinc coordination plays an important role in the efficient reactivation of Hsp33.

MATERIALS AND METHODS

Cysteine Mutants of Hsp33—The cysteine mutants in *hsp33* were constructed using the site-specific mutagenesis system GeneEditor™ (Promega). Wild type *hsp33* cloned into the expression vector pET11a (pUJ30) was used as template for all single or double cysteine mutants (1). The primers were used individually or in combinations to introduce the respective single or double mutation. We designated our mutants as follows: D indicates the cysteine to aspartic acid substitution and S indicates the cysteine to serine substitutions. Thus C141D has cysteine substituted for aspartic acid at position 141. The following primers were used: for C141D, 5′ GAT ACC CTG GCG GCC GAT CTA GAA GAT TAC TTT ATG CGT TC 3′; for C239S, 5′ ACC TGC TCG CGT GAA CGT TCC GCG GAT GCG CTG AAA ACG CTG 3′; for C232S, 5′ CGC AGG ATG TGG AGT TCA AGT CGA CCT GCT CGC GTG AAC G3′; for C234S, 5′

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§ To whom correspondence should be addressed: Dept. of Biology, University of Michigan, Ann Arbor, MI 48109-1048. Tel.: 1-734-764-8028; Fax: 1-734-647-0884; E-mail: ujakob@biology.lsa.umich.edu.

¶ Present address: School of Biosciences, Cardiff University, Cardiff, CF 103US, UK.

‖ PEW scholar.

GTG GAG TTC AAA TGC ACT AGT TCG CGT GAA CGT TGC GCC G 3′; for C265S, 5′ GGC GAA ATT GAC ATG CAT TCT GAT TAC TGC GGT AAC CAC 3′; and for C268S, 5′ GAC ATG CAT TGT GAT TAC TCC GGA AAC CAC TAT CTG TTC AAT GCG 3′.

All introduced mutations were confirmed by sequencing. BL21 strains were transformed with the respective expression plasmids. The Hsp33 overexpressing strains were grown in LB medium, supplemented with 1 mM $ZnCl_2$, and Hsp33 expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Purification of the Hsp33 mutant proteins was performed in the absence of reducing agents, according to the purification protocol of wild type Hsp33 (1).

Preparations of Zinc-reconstituted and Metal-free Hsp33—1.5 ml of purified Hsp33 (100 μM, in 40 mM HEPES-KOH, pH 7.5) was incubated in 2 mM DTT[1] and 20–100 μM $ZnCl_2$ for 30 min at 37 °C. To prepare metal-free Hsp33, 1.5 ml of purified Hsp33 (50 μM, in 40 mM HEPES-KOH, pH 7.5) was incubated in 2 mM DTT and 2 mM *N,N,N′,N′*-tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN) for 4 h at 43 °C. Zinc-reconstituted and metal-free samples were supplemented with 1 ml of 40 mM HEPES-KOH, pH 7.5, and applied onto a PD10 column (Amersham Pharmacia Biotech). The PD10 columns were either equilibrated in metal-free 40 mM HEPES-KOH buffer (5 g of Chelex and 100 ml of 40 mM HEPES-KOH, pH 7.5, incubated for 1 h at 37 °C) for zinc measurements or in untreated 40 mM HEPES-KOH for activity measurements. The protein was eluted with 2.5 ml of metal-free or untreated 40 mM HEPES-KOH buffer, respectively, and the protein concentration was determined using an extinction coefficient of 0.545 mg^{-1} cm^{-1} (1). When metal determinations were performed, only new plastic ware was used.

Determination of Accessible Thiol Groups in Wild Type Hsp33 and the Mutants—Accessible thiol groups in wild type Hsp33 and the mutant proteins were determined in 6 M Gdn·HCl according to Creighton (13).

PAR-PMPS Assay—Zinc determination and analysis of the number of zinc-coordinating cysteines were performed according to Hunt *et al.* (14). The assay is based on the complex formation of free zinc with the zinc-complexing dye 4-(2-pyridylazo) resorcinol (PAR), which produces an intense red dye (ϵ_{500} = 66 000 M^{-1} cm^{-1}). To analyze the amount of free or surface-bound zinc in wild type Hsp33, untreated or zinc-reconstituted Hsp33 was incubated with 100 μM PAR in 40 mM metal-free HEPES-KOH, pH 7.5, and the A_{500} was monitored using a Beckman DU600 spectrophotometer. To determine the number of cysteines involved in the zinc coordination, defined volumes of 0.2–0.5 mM *p*-hydroxymercuriphenylsulfonic acid (PMPS) titration solution (in 100 μM PAR, metal-free 40 mM HEPES-KOH, pH 7.5) were added, and the changes in A_{500} were recorded. PMPS forms mercaptide bonds with thiols, leading to the release of zinc into the solution where it is immediately complexed by PAR, thereby turning the solution red (14). The PMPS titration solution was added until all zinc was released from the proteins, as indicated by a constant A_{500} signal. To initiate the reassociation of zinc to apoHsp33, 40 μM DTT was added to the incubation reaction to displace PMPS from the cysteines. The change in A_{500} over time was monitored. To exclude the possibility that DTT re-extracts zinc from PAR rather then reducing the thiol-mercaptide bonds in Hsp33, a control experiment was performed in which 40 μM DTT were added to a 20 μM zinc solution in 100 μM PAR, 40 mM HEPES-KOH, pH 7.5. No significant decrease in A_{500} could be monitored after the addition of DTT, indicating that DTT, at this low concentration used, was unable to re-extract zinc from the $Zn(PAR)_2$ complex.

To determine the number of thiols in Hsp33, thiol titrations with PMPS in the absence of PAR were performed. Mercaptide thiol bonds show a strong absorption at A_{250}. To titrate the thiol groups in wild type Hsp33 and the mutants, defined volumes of 0.2 mM PMPS (in 40 mM HEPES-KOH, pH 7.5) titration solution were added to Hsp33 (3 μM in the same buffer), and the changes in A_{250} were monitored until further addition of PMPS failed to change the absorption. This indicated that all of the thiols have been titrated. Changes in protein and PMPS concentration due to the volume changes during the titrations were calculated accordingly.

Determination of Zinc Binding Constant—Hsp33 wild type and mutant proteins were reconstituted with zinc as described, and DTT and unbound zinc were removed using PD10 gel filtration columns that had been equilibrated in metal-free 40 mM HEPES-KOH, pH 7.5, buffer. To determine the equilibrium between Hsp33 and zinc, competition experiments between Hsp33 and the metal-complexing agent TPEN (K_a = 10^{16} M^{-1}) were performed. Zinc coordinating wild type Hsp33 (40 μM) was incubated in metal-free 40 mM HEPES, pH 7.5, 0.2 mM DTT and increasing concentrations of TPEN (20 μM to 5 mM) for 16 h at 23 °C. The effective TPEN concentration present at pH 7.5 was calculated according to the pK_a of 7.19 of TPEN (15). After equilibrium was reached, 100-μl aliquots were taken, supplemented with metal-free 40 mM HEPES-KOH applied to a PD10 column, and equilibrated in the same buffer. The protein was eluted with 2 ml of metal-free 40 mM HEPES-KOH, and the protein concentration was determined. 540 μl of the eluate was tested with the PAR/PMPS assay for the remaining amount of zinc bound to the cysteine center of Hsp33. To exclude the presence of residual TPEN in the sample, which would immediately bind to the zinc released from the cysteine centers of Hsp33 upon PMPS addition, a defined amount of zinc was added after the PMPS addition. The absorption corresponded well with the amount of zinc added and excluded the presence of even submicromolar concentrations of TPEN present in the gel-filtered sample. Based on the binding constant of TPEN (K_a = 10^{16} M^{-1}) (Equation 1), the binding constant of Hsp33 for zinc (Equation 2) was calculated according to Equation 3.

$$K_a(\text{TPEN}) = \frac{[\text{TPEN} \cdot \text{Zn}]}{[\text{TPEN}_{\text{free}}] \cdot [\text{Zn}_{\text{free}}]} \quad \text{(Eq. 1)}$$

$$K_a(\text{Hsp33}) = \frac{[\text{Hsp33}\cdot\text{Zn}]}{[\text{Hsp33}_{\text{free}}] \cdot [\text{Zn}_{\text{free}}]} \quad \text{(Eq. 2)}$$

$$K_a(\text{Hsp33}) = \frac{K_a(\text{TPEN}) \cdot [\text{Hsp33} \cdot \text{Zn}] \cdot [\text{TPEN}_{\text{free}}]}{[\text{TPEN} \cdot \text{Zn}] \cdot [\text{Hsp33}_{\text{free}}]} \quad \text{(Eq. 3)}$$

where [Hsp33·Zn] corresponds to the amount of zinc released from Hsp33 by the addition of PMPS; $[\text{Hsp33}_{\text{free}}]$ equals $[\text{Hsp33}_{\text{total}}] - [\text{Hsp33}\cdot\text{Zn}]$; $[\text{TPEN}_{\text{free}}]$ equals $[\text{TPEN}_{\text{total}}] - [\text{TPEN}\cdot\text{Zn}]$; and [TPEN·Zn] corresponds to the amount of non-complexed Hsp33 $[\text{Hsp33}_{\text{free}}]$.

Similar experiments were performed with the Hsp33 mutant proteins; however, PAR was used in the competition experiments instead of TPEN (16). PAR has a lower affinity for zinc than TPEN does, allowing the measurement of Hsp33 mutants that have a decreased affinity for zinc. The K_a for $Zn(PAR)_2$ complexes had been calculated to be 2×10^{12} M^{-1} at pH 7.0, when the PAR concentration is 100 μM and the zinc concentration is below 12 μM (14, 16). Various concentrations of Hsp33 mutant proteins (2, 5, and 10 μM) were incubated in metal-free 400 mM KCl, 40 mM HEPES-KOH, pH 7.0, and 100 μM PAR at room temperature. Equilibrium was reached within the mixing time. The absorption at A_{500} was determined. The calculated amount of $Zn(PAR)_2$ corresponded directly to the fraction of zinc-free Hsp33 $[\text{Hsp33}_{\text{free}}]$. The remaining zinc bound to Hsp33 was released by the addition of 32 μM PMPS. This corresponded to the fraction of zinc-complexed Hsp33 [Hsp33·Zn]. The apparent zinc association constant of the Hsp33 mutant proteins was calculated according to Equation 4.

$$K_a(\text{Hsp33}) = \frac{K_a(\text{PAR}) \cdot [\text{Hsp33} \cdot \text{Zn}] \cdot [\text{PAR}_{\text{free}}]}{[\text{Zn(PAR)}_2] \cdot [\text{Hsp33}_{\text{free}}]} \quad \text{(Eq. 4)}$$

To extract zinc from Hsp33 wild type and the mutants by PAR, the various reduced and zinc-reconstituted Hsp33 preparations (3 μM each) were incubated in 40 mM metal-free HEPES-KOH, 400 mM KCl, pH 7.0, and increasing concentrations of PAR. One minute after addition of PAR, the A_{500} was determined to allow calculation of the amount of $Zn(PAR)_2$ complexes formed. Then, 20 μM PMPS was added to release all residual zinc from the cysteine centers corresponding to the fraction of zinc-complexed Hsp33.

Absorption Spectra of Metal-free, Co(II)- and Cd(II)-reconstituted Hsp33—Metal-free Hsp33 (15 μM) in 40 mM HEPES-KOH, pH 7.5, was supplemented with increasing amounts of fresh $CoCl_2$ or $CdCl_2$, and the absorption spectra were immediately recorded using a Beckman spectrophotometer DU600. At the end of the titration, 30 μM $ZnCl_2$ solution was added.

To analyze the number of thiolate anions present in wild type Hsp33 and the mutants, the respective proteins (15 μM) were incubated in a 2-fold molar excess of fresh cadmium chloride, and the absorption of the cadmium-thiolate charge transfer band was determined at A_{250}. This allowed the determination of the number of cysteines that coordinate the metal in the form of highly reactive thiolate anions. The extinction coefficient at 250 nm per CysS–Cd bond is 5300 M^{-1} cm^{-1} (17).

Proteolytic Digests of Hsp33—Zinc-reconstituted and metal-free Hsp33 were prepared as described. To determine the proteolytic sensi-

[1] The abbreviations used are: DTT, dithiothreitol; TPEN, *N,N,N′,N′*-tetrakis(2-pyridyl-methyl)ethylenediamine; PAR, 4-(2-pyridylazo) resorcinol; PMPS, p-hydroxymercuri-phenylsulfonic acid; Gdn·HCl, guanidinium hydrochloride.

tivity of both protein preparations, proteolytic digests with trypsin and proteinase K were performed. 0.3 mg/ml Hsp33 was incubated in a final volume of 170 μl in the presence of 2 μg of trypsin or 0.2 μg of proteinase K for 0, 2, 5, 7, and 10 min at 37 °C. After the respective incubation times, 30-μl aliquots were taken, and the tryptic digest was terminated by the addition of 5 mM PMSF (in chloroform) and incubation on ice (2 min). The samples were supplemented with non-reducing 5× Laemmli buffer and boiled. The proteinase K digests were terminated by immediate boiling of the samples for 5 min in the presence of Laemmli buffer. The samples were loaded onto a 14% SDS-PAGE (NOVEX), and the protein bands were visualized using a fast, highly sensitive Coomassie staining technique (18).

Activity Measurements of Hsp33—To analyze the chaperone activity of Hsp33, the influence of Hsp33 on the aggregation of refolding luciferase was tested (5). Firefly luciferase (Roche Molecular Biochemicals) was denatured in a final concentration of 8 μM in 4.3 M Gdn·HCl for 2 h at room temperature. To initiate the refolding of luciferase, the unfolded enzyme was diluted 1:80 (final concentration 0.1 μM) into 1600 μl of 40 mM HEPES, pH 7.5, at 32 °C in the absence or presence of Hsp33. Light scattering was monitored using a Hitachi F4500 fluorimeter equipped with thermostated cell holder and stirrer. Excitation and emission wavelengths were set to 350 nm, and the excitation and emission slit widths were set to 2.5 nm.

To initiate the activation of Hsp33, reduced zinc-reconstituted or metal-free Hsp33 (35 μM) was incubated in the presence of 2 mM H_2O_2 at 43 °C in the absence or presence of freshly prepared $CuCl_2$. Defined time points after addition of H_2O_2, aliquots were taken and diluted 1:44 (0.8 μM final concentration) into the refolding buffer, present in the cuvette. Then denatured luciferase was added, and light scattering measurements were performed. To exclude reactivation by incubation at elevated temperature alone, activity of Hsp33 was determined after incubation of Hsp33 ± $CuCl_2$ in the absence of H_2O_2 for 5 min at 43 °C.

RESULTS

Hsp33 Coordinates Zinc via Its Four Universally Conserved Cysteines—Hsp33 is a recently identified chaperone whose activity is redox-regulated (1). Under reducing conditions, Hsp33 is inactive. All six cysteines of Hsp33 are in the thiol state, and zinc has been shown to be cysteine-coordinated (1). Upon activation of the chaperone function of Hsp33 with oxidizing agents like H_2O_2, two intramolecular disulfide bonds form, connecting the invariant cysteines Cys^{232} with Cys^{234} and Cys^{265} with Cys^{268} (5). This activation process of Hsp33 is paralleled by the release of zinc (1). To analyze the zinc-binding properties of reduced Hsp33 in detail, an assay was applied that allows the sequential determination of free and cysteine-coordinated zinc within the same sample. This assay exploits the unique properties of two reagents, PAR and PMPS (14). The metal chelator PAR interacts with free zinc to form a $Zn(PAR)_2$ complex ($K_a = 2 \times 10^{12}$ M^{-1}), which strongly absorbs light at 500 nm. To detect zinc molecules that are coordinated by high affinity cysteines, the mercurial agent PMPS is added. PMPS forms mercaptide bonds with thiols, a process that can be monitored by an increase in absorption at 250 nm. Within the mixing time of the experiment, this thiol modification induces the release of zinc into solution. Zinc is then rapidly chelated by PAR, producing an intense red color. A PMPS titration experiment in the presence of PAR, therefore, allows one to evaluate the number of cysteine residues involved in the zinc coordination and to determine the total amount of zinc bound to the protein.

To obtain a homogeneous preparation of reduced and metal-coordinated protein, purified wild type Hsp33 (zinc content ~80%) was incubated in DTT in the presence of additional zinc. Excess DTT and metal was removed by PD10 gel filtration of the sample in metal-free HEPES-KOH buffer. Incubation of 3 μM reduced Hsp33 in the presence of 100 μM PAR resulted in a slight increase of A_{500}, indicating that reduced Hsp33 had small amounts of zinc associated via the surface (data not shown). The remaining zinc could only be released by addition of PMPS indicating that it is cysteine-associated (Fig. 1*A*). Stepwise addition of PMPS led, after a short lag phase, to an increase in A_{500} that was directly proportional to the amount of PMPS added. In the linear range of the titration, approximately four thiol groups had to be titrated with PMPS to induce complete zinc release (Fig. 1*A*).

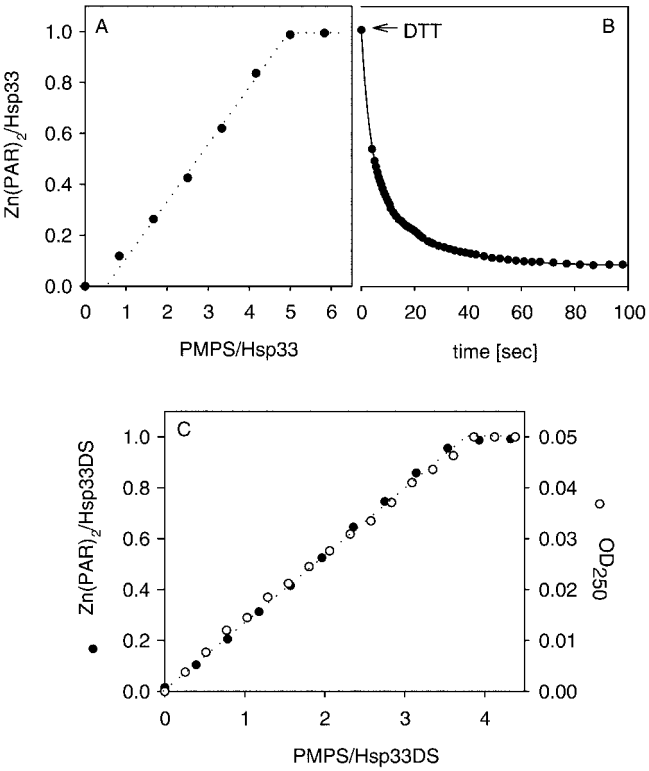

FIG. 1. **Hsp33 coordinates zinc via its 4 conserved cysteines.** *A,* PMPS titration of reduced, zinc-reconstituted wild type Hsp33. Hsp33 (3 μM) was incubated in 40 mM metal-free HEPES-KOH, pH 7.5, in the presence of 100 μM PAR. PMPS was titrated in 2.5 μM aliquots, and the absorption change at A_{500} was monitored. The ratio of $Zn(PAR)_2$ complexes formed during the titration to molecules Hsp33 was calculated. The ratio of molecules PMPS to molecules Hsp33 corresponds to the number of thiol groups that have to be titrated by PMPS to induce zinc release. *B,* DTT treatment re-extracts zinc from the $Zn(PAR)_2$ complexes back into the Hsp33 apoprotein. After PMPS titration was complete (final concentration 20 μM PMPS) (*A*), 40 μM DTT was added, and the decrease in A_{500} was monitored. This decrease reflects the DTT-induced reduction of all mecaptide–thiol bonds formed during the PMPS titration, and the ability of reduced Hsp33 to reassociate zinc. *C,* thiol titration and concomitant zinc release of Hsp33-C141D239S (Hsp33DS) mutant protein. PMPS titration (1.5 μM per titration) of zinc-reconstituted, reduced Hsp33-C141D/C239S (2.7 μM in 40 mM metal free HEPES-KOH, pH 7.5) was performed in the presence of 100 μM PAR to monitor zinc release (A_{500}) or in the absence of PAR to monitor the formation of mercaptide–thiol bonds (A_{250}).

We next tested if the PMPS-induced zinc release was reversible by adding a 2-fold molar excess of DTT to the Hsp33/PMPS/PAR mixture present at the titration end point. DTT should reduce all mercaptide–thiol bonds formed during the PMPS titration in Hsp33 and should allow the thiol groups in Hsp33 to re-extract zinc from the $Zn(PAR)_2$ complex. This should lead to a decrease in the amount of $Zn(PAR)_2$ complex and, therefore, to a decrease in A_{500}. As shown in Fig. 1*B*, after addition of DTT, zinc that has been released from Hsp33 upon formation of mercaptide-thiol adducts was indeed immediately re-coordinated by the reduced thiols in apoHsp33. The total amount of zinc released by PMPS and re-coordinated by apoHsp33 upon DTT treatment accounted to one zinc atom per molecule Hsp33, establishing a 1:1 stoichiometry of zinc binding to Hsp33 (Fig. 1, *A* and *B*).

Six cysteines exist in Hsp33. Four of these cysteines (Cys^{232}, Cys^{234}, Cys^{265}, and Cys^{268}) are invariant residues, present in

TABLE I
Zinc content and thiol status of purified wild type Hsp33 and Hsp33 mutants

The number of cysteine residues present in the respective proteins is shown in parentheses.

Protein as purified	Zinc coordinated via cysteines	Accessible cysteines in Gdn · HCl
	%	
Wild type	>75	3.6 ± 4.2 (6)
C141D	>75	3.6 ± 0.2 (5)
C239S	>75	3.2 ± 0.2 (5)
C232S	<5	<1 (5)
C234S	<5	<1 (5)
C265S	<5	<1 (5)
C268S	<5	<0.5 (5)
Wild type (reduced, zinc-reconstituted)	100	5.1 ± 0.2 (6)
Wild type (reduced, TPEN-treated)	<2	5.1 ± 0.2 (6)
Wild type (H_2O_2 oxidized)	<2	<1 (6)

all 31 organisms from which Hsp33 has been sequenced. One cysteine, Cys^{141}, is moderately conserved and present in 11 of the 31 organisms. The final cysteine, Cys^{239}, is very poorly conserved and only present in four Hsp33 homologues. All 6 cysteines in reduced Hsp33 were able to form mercaptide–thiol bonds, as monitored by PMPS titrations of Hsp33 in the absence of PAR (data not shown). PMPS/PAR titrations of wild type Hsp33 revealed an initial lag phase, which is most likely due to the PMPS titration of accessible cysteines that are not involved in zinc coordination (Fig. 1*A*). A mutant protein of Hsp33, lacking the two less-conserved cysteines C141D and C239S, showed no detectable lag phase during the PMPS titration, and zinc was completely released by the addition of exactly 4 mol eq of PMPS (Fig. 1*C*). This implied that one of the less conserved cysteines, which is highly accessible for PMPS modification and not involved in zinc coordination, is responsible for the observed lag phase. The zinc titration of the C141D/C239S mutant corresponded with the titration of thiol groups in this protein (Fig. 1*C*). These results strongly suggested that all 4 invariant cysteines are involved in the high affinity zinc coordination of Hsp33.

To confirm that the Cys-*X*-Cys-$X_{27–32}$-Cys-*X*-X-Cys motif of Hsp33 is required for zinc binding, cysteine mutants were constructed in which each of the 6 cysteines present in Hsp33 were individually altered. In 5 of the 6 mutants constructed, the generally conservative, but chemically inactive, serine substitution was chosen. For Cys^{141}, we chose to make an aspartic acid substitution, because this residue was present in this position in nearly 50% of all Hsp33 homologues. The relatively conserved nature of this position in the protein family suggested that this residue might play a role in Hsp33 folding or stability. The mutant proteins were overexpressed in the presence of 1 mM $ZnCl_2$, purified to homogeneity, and tested for their zinc-binding properties. As shown in Table I, all cysteine mutants that had one of the 4 conserved cysteines substituted for a serine residue completely lacked detectable zinc in the purified protein preparation. This strongly suggested that all 4 conserved cysteines are important for zinc binding. In contrast, wild type Hsp33, the individual mutants in the less conserved cysteines C141D and C239S as well as the double mutant C141D/C239S showed between 75 and 90% zinc present in the protein after purification. This showed that neither of the less conserved cysteines is essential for zinc coordination.

Although all proteins were purified in the absence of reducing agents, wild type Hsp33, the C141D, C239S, and the C141D/C239S mutants were predominantly in the reduced, zinc associated state. All other mutant proteins showed very few accessible cysteines (Table I). Despite the fact that these mutants lacked one of the conserved cysteines and thus could not form both native disulfide bonds, less then one cysteine was accessible in these mutants even when they were tested under denaturating conditions (Table I). This suggested either the formation of mixed disulfides with small molecules such as glutathione or the presence of extra, non-native, disulfide bond(s) that involve the non-conserved cysteines Cys^{141} or Cys^{239}. Preliminary analysis of the chaperone activities of these mutant proteins revealed significantly different chaperone activities, suggesting distinct roles for the individual cysteine/disulfide bonds in chaperone function and redox regulation. A detailed analysis of the respective cysteine mutants is underway which will allow us to dissect further the functional roles of each of the individual conserved cysteines in Hsp33.

TABLE II
Zinc content and coordination of reduced, zinc-reconstituted wild type Hsp33 and Hsp33 mutants

Protein after zinc reconstitution	Zinc bound	Mol equivalent PMPS for zinc release
	%	
Wild type	100	5.2
C141D	100	4.8
C239S	100	4.2
C232S	70–100	3.6
C234S	60–100	3.7
C265S	70–100	3.1
C268S	70–100	3.6

After reduction and zinc treatment, all mutant proteins were capable of approximately equimolar zinc binding (Table II). The C239S mutant protein showed a nearly identical PMPS titration as the wild type protein does, despite the absence of one cysteine residue in the protein. This suggested that the Cys^{239} residue is buried and titrated with PMPS only after the zinc release, allowing this cysteine to go unnoticed in the titration of the wild type protein. The C141D protein, on the other hand, had no lag phase and contained 4 titratable cysteines despite the presence of 5 cysteines in the protein. We interpret this as meaning that in wild type Hsp33, Cys^{141} is the highly accessible, reduced thiol group which reacts with PMPS prior to the titration of the zinc-coordinating cysteines and that Cys^{141} is responsible for the observed lag phase. All the mutant proteins that lacked one of the conserved cysteines had the initial lag phase in the PMPS titration, consistent with the interpretation that this lag is due to the presence of Cys^{141} and needed approximately 3 additional mol eq of PMPS for complete zinc release. This agreed well with the assumption that all four conserved cysteines of the C*X*C$X_{27–32}$C*XX*C motif are involved in the zinc coordination of wild type Hsp33.

Binding Affinity of Zinc to Wild Type Hsp33 and the Mutants—Hsp33 mutants that lack any one of the 4 conserved cysteines are unable to retain zinc throughout the purification process but are able to bind to zinc that has been added after reduction of the purified protein. To analyze and compare the zinc affinity of wild type Hsp33 and the mutant proteins, their zinc binding constants were determined. To extract zinc from the wild type protein, the very high affinity zinc chelator TPEN ($K_a = 10^{16}\ M^{-1}$) needed to be used. Not even millimolar concentrations of EDTA ($K_a = 10^{14}\ M^{-1}$) were sufficient to outcompete micromolar amounts of wild type Hsp33 for zinc binding. This suggested that the zinc binding constant of Hsp33 is higher than $10^{14}\ M^{-1}$. Zinc-reconstituted Hsp33 was incubated in increasing concentrations of TPEN and allowed to equilibrate for 16 h at 23 °C. Zinc-free and zinc-complexed TPEN were separated from Hsp33 by PD10 gel filtration, and the ratio of zinc-bound to zinc-free Hsp33 was determined using the PAR/PMPS assay (Fig. 2*A*). On the basis of Equation 3, the association constant for zinc binding of Hsp33 at 23 °C and pH

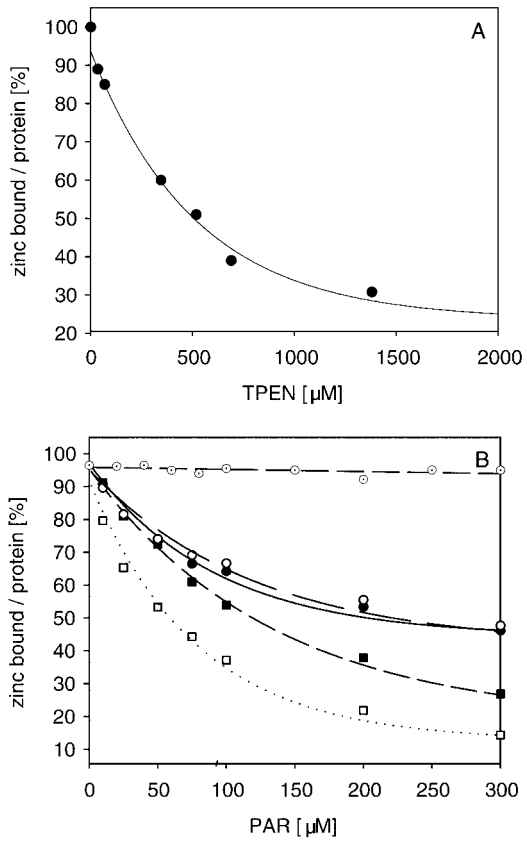

FIG. 2. **Zinc binding constant of wild type Hsp33 and Hsp33 single cysteine mutants.** *A,* zinc binding constant of wild type Hsp33. Wild type Hsp33 (40 μM) was incubated in 40 mM metal-free HEPES-KOH in the absence or presence of increasing concentrations of TPEN. After 16 h of incubation at 23 °C, aliquots were taken and purified with PD10 gel filtration. The combined PMPS/PAR assay was applied to determine the amount of residual zinc bound to wild type Hsp33. The K_a was calculated to be 2.5×10^{17} M^{-1}. *B,* zinc extraction from Hsp33 wild type and mutants by PAR. Wild type Hsp33 and the mutant Hsp33 proteins (3 μM each) were incubated in 40 mM metal-free HEPES-KOH, 400 mM KCl, pH 7.0, and increasing concentrations of PAR. 1 min after addition of PAR, A_{500} was determined to calculate the amount of $Zn(PAR)_2$ complexes formed. Then 20 μM PMPS was added to release all residual zinc from the cysteine centers to determine the total amount of zinc bound. PAR was unable to release significant amounts of zinc from (⊙) wild type Hsp33 but induced the zinc release from (○) Hsp33 C232S, (●) Hsp33 C234S, (■) Hsp33 C265S, and from (□) Hsp33 C268S.

7.5 was calculated to be 2.5×10^{17} M^{-1}. This is similar to the zinc binding constant determined for methionine synthase MetE ($K_a > 10^{17}$ at pH 7.0, 25 °C) (16) and significantly higher than the zinc association constants reported for the neural zinc finger factor NZF-1 ($K_a = 1.4 \times 10^{10}$ M^{-1} at pH 6.9, 25 °C) (19) or carbonic anhydrase ($K_a = 0.8 \times 10^{12}$ M^{-1} at pH 7.0, 30 °C) (20). Noteworthy, incubation of 40 μM wild type Hsp33 in 2 mM TPEN showed that the apparent half-time of zinc release was only 160 ± 10 min at 23 °C. This fast dissociation rate of zinc indicated that TPEN functions by "catalyzing" zinc release from Hsp33 rather than by trapping zinc that has dissociated from Hsp33. Similar observations have been made when the zinc release of carbonic anhydrase was studied using dipicolinic acid (21) and explains the fast equilibrium established between Hsp33 and TPEN in competition experiments.

To determine the zinc binding constant of the mutant proteins, a different approach needed to be used since zinc dissociation experiments with TPEN indicated that the zinc binding constant of the mutant proteins is several magnitudes lower than the binding constant of the wild type protein (data not shown). Therefore, the metal chelator PAR ($K_a = 2 \times 10^{12}$ M^{-1}, at pH 7.0) was employed as a competitor for zinc binding (16). Whereas PAR was unable to extract zinc from the wild type protein, significant amounts of zinc were released from the mutant proteins under the same assay conditions, indicating that the mutants bound zinc less tightly (Fig. 2*B*). Equilibrium was reached within a few minutes of incubation in PAR. This was similar to zinc binding measurements of the MetE protein with PAR, where the equilibrium between PAR and the protein was achieved within the mixing time of the experiment (16). To determine the association constants for the mutant proteins, various concentrations of the proteins (2–10 μM) were incubated with 100 μM PAR at pH 7.0. The concentrations of [$Zn(PAR)_2$], [Hsp33·Zn], and [$Hsp33_{free}$] were determined using the PAR/PMPS assay (16). The binding constants for zinc were calculated and found to be between 0.9 and 2.5×10^{14} M^{-1} at 23 °C, pH 7.0, for all four mutants (Table III). These experiments showed that although substitution of one of the four cysteines did not abolish the ability of Hsp33 to coordinate zinc via the remaining cysteines, it did result in a greater than 1000-fold decrease in the zinc binding constant. This shows that all 4 cysteines in the C*X*C*X*$_{27–32}$C*XX*C motif are important and about equally so in the zinc binding activity of Hsp33.

TABLE III
Zinc association constants of wild type Hsp33 and Hsp33 mutants

Protein after zinc reconstitution	K_a
	M^{-1}
Wild type[a]	2.5×10^{17}
C232S[b]	$0.94 \pm 0.2 \times 10^{14}$
C234S[b]	$2.2 \pm 0.3 \times 10^{14}$
C265S[b]	$0.96 \pm 0.1 \times 10^{14}$
C268S[b]	$1.06 \pm 0.03 \times 10^{14}$

[a] pH 7.5, 23 °C.
[b] pH 7.0, 23 °C.

Absorption Spectrum of Metal-reconstituted Hsp33—Substitution of Co^{2+} into the zinc-binding site of proteins provides a useful method to evaluate the characteristics of the metal polyhedron using spectroscopic techniques (22, 23). Metal-free Hsp33 was obtained by incubating wild type Hsp33 in the presence of 2 mM TPEN and 2 mM DTT for 4 h at 43 °C. The metal binding affinity of Hsp33 was retained upon removal of zinc from the protein, since addition of zinc to metal-free Hsp33 resulted in an immediate reassociation of zinc into the apoprotein as monitored with the PAR assay. Thiol titration with PMPS revealed that all 4 cysteines were able to coordinate zinc in the reconstituted wild type protein.

Metal-free Hsp33 was incubated with increasing amounts of cobalt, and the absorption spectra were monitored immediately after the cobalt addition. As shown in Fig. 3, *A* and *B*, two distinct new absorption bands appeared that exhibited absorption maxima at 340 and 685 nm with absorption shoulders at 620 and 710 nm. These absorption bands increased in intensity until equimolar ratios of cobalt to Hsp33 were titrated (Fig. 3, *A* and *B*). Higher concentrations of cobalt did not change the absorption spectra of Hsp33. Subsequent addition of zinc resulted in the immediate disappearance of these absorption bands suggesting that zinc was capable of quickly displacing cobalt from the cysteine center. This suggested an extremely fast exchange reaction between the metals. The resulting absorption spectra closely resembled the spectra of zinc-treated Hsp33 in the absence of cobalt. The position of the 340 nm band in the absorption spectra of cobalt-reconstituted Hsp33 is consistent with a ligand-metal charge transfer band which indicates a charge transfer between Co(II) and thiolate ligands (22, 24). The intensity of the absorption band at 340 nm was 3 600 M^{-1} cm^{-1}. Given the calculated molar extinction coefficients of

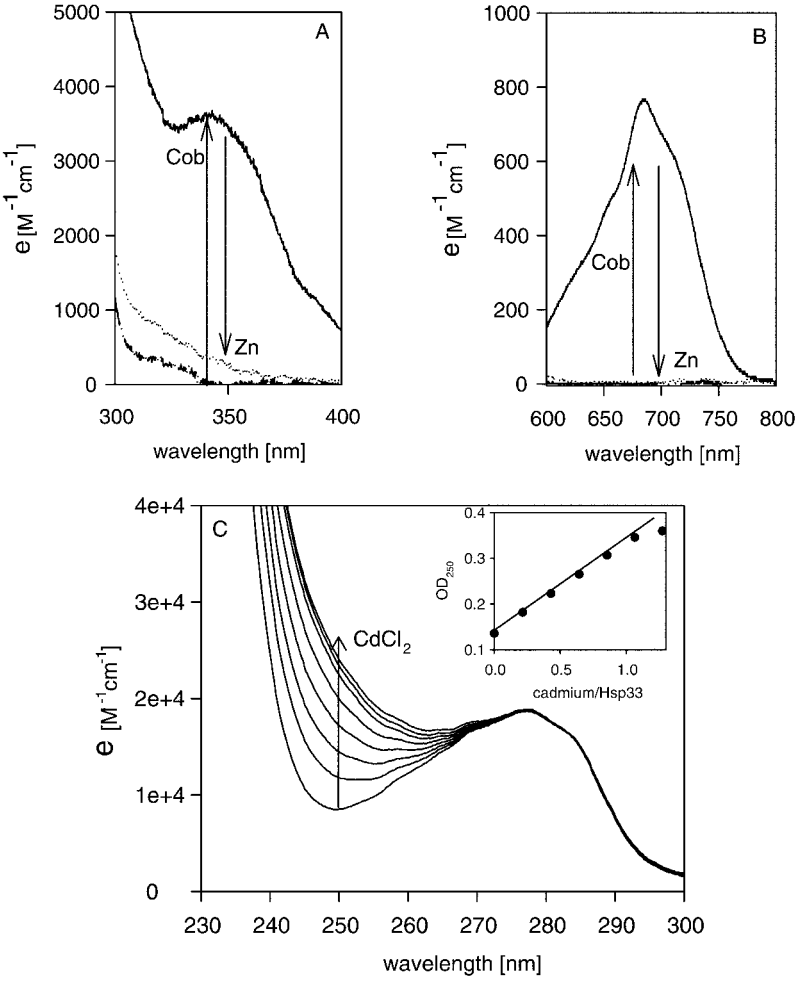

FIG. 3. **Metal coordination induces thiolate anion formation of the conserved cysteines of Hsp33.** *A* and *B*, cobalt reconstitution of Hsp33. Absorption spectra (300–800 nm) of metal-free wild type Hsp33 (15 μM) in 40 mM HEPES-KOH, pH 7.5 (- - -) before and (—) immediately after addition of 20 μM freshly prepared $CoCl_2$. This was followed by the (···) addition of 30 μM $ZnCl_2$, which immediately displaced cobalt from the cysteine center as monitored by the decrease in absorbance. The ligand-metal charge transfer band region is shown in *A*, and the visible range of the spectrum is shown in *B*. *C,* cadmium reconstitution of Hsp33. Absorption spectrum of metal-free wild type Hsp33 (15 μM) was recorded. Then, freshly prepared $CdCl_2$ (3 μM each) was titrated, and the formation of the cadmium-thiolate (Cys-Cd) band at 250 nm was monitored (*inset*).

the ligand-metal charge transfer band with 900–1300 M^{-1} cm^{-1} per Co(II)–CysS bond (25), it is likely that at least 3 and possibly all 4 zinc-coordinating cysteines in Hsp33 are in the highly reactive thiolate anion state. The intensities of the d-d ligand field transition bands in the visible range of the spectrum (*e.g.* ϵ_{685} = 780 M^{-1} cm^{-1}) indicated a tetrahedral high spin cobalt system (22).

Similar results were obtained when cadmium was used to reconstitute Hsp33. Hsp33 has an even higher affinity toward cadmium than toward zinc, like model thiolate complexes and many other metalloproteins that possess cysteine ligands (17). Hsp33 binds these metals with the following order of affinity Co(II) < Zn(II) < Cd(II) (data not shown). When cadmium was titrated, an immediate increase in the cadmium thiolate charge transfer band at 250 nm was noticeable. Again, equimolar amounts of cadmium to Hsp33 were necessary for the complete titration (Fig. 3*C, inset*). The extinction coefficient of cadmium-substituted Hsp33 at 250 nm was 21,500 M^{-1} cm^{-1} and revealed a value of 5300 M^{-1} cm^{-1} per Cd–CysS bond. This was very similar to the molar extinction coefficient of Cd–CysS bonds reported for a number of other cadmium-substituted metalloproteins (ϵ_{250} = 5 300–5 500 M^{-1} cm^{-1}) (17, 26) and confirmed that all 4 cysteines are in the thiolate anion state.

Similar extinction coefficients were obtained when zinc-reconstituted Hsp33 was used, and cadmium was added subsequently. Within the mixing time of the experiment, cadmium successfully competed with and replaced zinc from this kinetically labile cysteine-containing metal site. The molar extinction coefficient of the cadmium-reconstituted conserved cysteine mutants was found to be lower by ~5500 M^{-1} cm^{-1}. This was very consistent with the presence of three thiolate anions remaining in the mutant proteins, which are able to coordinate the reconstituted metal.

Zinc Coordination Confers Major Protection of Hsp33 Against Proteolytic Digestion—Hsp33 coordinates zinc with high affinity. Zinc-free and reduced Hsp33 migrates on native gels with a very different mobility than zinc-containing and reduced Hsp33. This mobility difference may be either due to the presence of additional charges in the zinc-coordinated protein or due to major changes in the conformation or to a combination thereof. To compare the conformation of zinc-coordinated and metal-free Hsp33, proteolytic digests with trypsin and proteinase K were performed. As shown in Fig. 4*A*, zinc coordination in Hsp33 confers a high resistance toward trypsin. Metal-free and reduced Hsp33 was degraded within the first 2 min of incubation in the presence of trypsin. On the other hand, treatment of zinc-coordinated protein with identical concentrations of trypsin did not cause significant proteolytic digestion even after 10 min of incubation with trypsin at 37 °C. No stable tryptic fragments larger than 5–8 kDa were observed when metal-free Hsp33 was digested. This suggests that zinc binding strongly stabilizes Hsp33. Since the entire Hps33 molecule is stabilized by zinc binding, the conformational changes triggered by zinc dissociation appear to propagate outside of the zinc-coordinating center, which is localized within the last 50 amino acids of the C terminus of Hsp33. Zinc-containing Hsp33 also showed a high resistance toward proteinase K digestion, whereas metal-free Hsp33 was immediately degraded into numerous fragments (Fig. 4*B*). The proteolytic pattern obtained with zinc-free Hsp33 was significantly different from the frag-

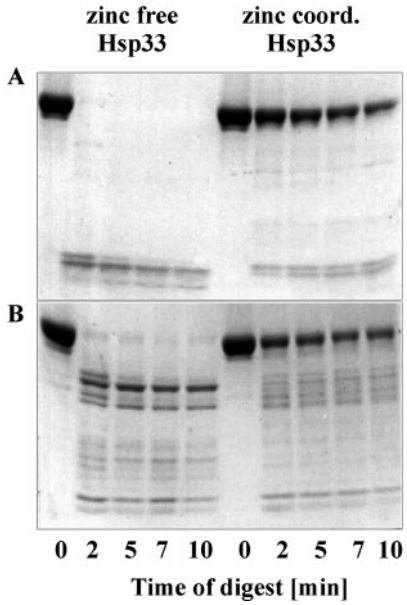

FIG. 4. **Zinc coordination confers resistance toward proteolytic degradation.** *A,* tryptic digest of metal-free (*1st to 5th lanes*) and zinc-coordinated (*6th to 10th lanes*) wild type Hsp33. For defined time points after addition of trypsin, aliquots were taken, and the digest was terminated by the addition of 5 mM PMSF. *B,* proteinase K digest of metal-free (*1st to 5th lanes*) and zinc-coordinated (*6th to 10th lanes*) wild type Hsp33. For defined time points after addition of proteinase K, aliquots were taken, and the digest was terminated by immediate boiling of the samples in Laemmli buffer.

ment pattern obtained when metal-coordinated Hsp33 was digested for an extended time. Our results show that zinc coordination efficiently protects Hsp33 from proteolytic digestion.

Zinc Coordination Is Important for Activation Process of Hsp33 by H_2O_2—Hsp33 coordinates the redox-inactive metal zinc with high affinity in the reduced state, and all 4 cysteines appear to be in the highly reactive thiolate state. To analyze the potential role of zinc in the activation process of Hsp33, activity measurements were performed. Active and oxidized Hsp33 suppresses the aggregation of chemically denatured luciferase in a concentration-dependent manner (5). Reduced and zinc-coordinating Hsp33 and reduced and metal-free Hsp33 were both inactive. Even a 10-fold molar excess of either reduced protein preparation was unable to influence the aggregation behavior of refolding luciferase. Activation of reduced Hsp33 was induced by the addition of H_2O_2 and incubation at elevated temperatures. Within 60 min of incubation in H_2O_2, significant reactivation occurred when reduced Hsp33 was zinc-coordinated (Fig. 5*B*). Absence of zinc in Hsp33, on the other hand, led to a very slow and incomplete reactivation, indicating that the zinc coordination not only plays a major role in stabilizing Hsp33 but is also directly involved in the activation process of Hsp33 (Fig. 5*A*).

The reactivation process of Hsp33 was slower in these experiments compared with earlier measurements where the ability of Hsp33 to suppress the thermal aggregation of luciferase was analyzed (1). These reactivation reactions were in part carried out in quartz cuvettes, UV light, and at elevated temperatures, environmental conditions where hydroxyl radicals are known to be produced. To analyze whether hydroxyl radicals may indeed accelerate the activation process of Hsp33, Fenton reagents (27) were added to the reactivation reaction. Unfortunately, the "classical" hydroxyl radical-producing reagents Fe(II) and Cu(I) but could not be used due to the fast oxidation of Fe(II) to Fe(III) in HEPES buffer and due to interference of the Cu(I) solvent with the chaperone assay. Therefore, the

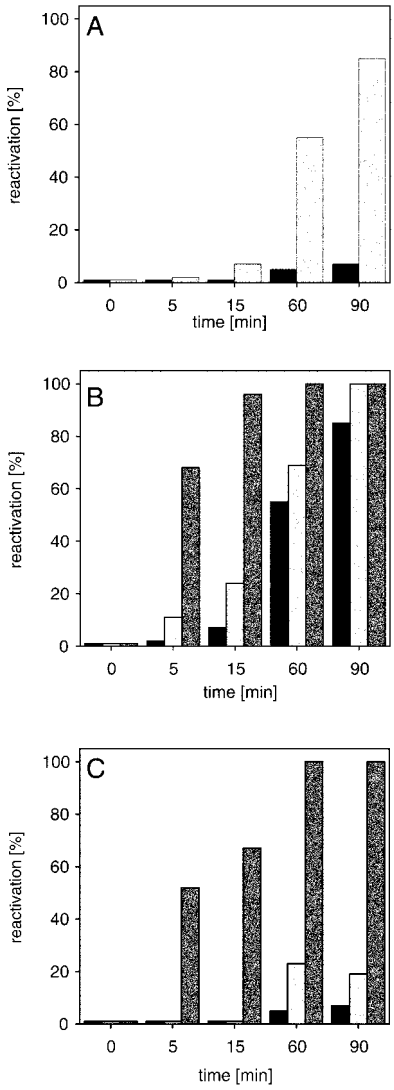

FIG. 5. **Zinc coordination is essential for fast and successful reactivation of Hsp33 upon H_2O_2 treatment.** *A,* reactivation of reduced metal-free (*black bars*) or zinc-reconstituted (*light gray bars*) wild type Hsp33 (35 μM) was initiated by the addition of 2 mM H_2O_2 and incubation at 43 °C. To determine the reactivation yields and rates, Hsp33 aliquots were taken after the time points indicated and added to the activity assay (final concentration 0.8 μM). Chemically denatured luciferase was added (0.1 μM), and the influence of Hsp33 on the light scattering of renaturating luciferase was monitored. The light scattering signal reached after 6 min of incubation in the absence of molecular chaperones was set to 0% activity. The light scattering signal reached after 6 min in the presence of fully active Hsp33 was set to 100%. *B,* reactivation of Hsp33 is accelerated by the presence of $CuCl_2$. H_2O_2-induced reactivation of zinc reconstituted wild type Hsp33 (35 μM) was performed as described in the absence (*black bars*) or in the presence of 3.5 μM $CuCl_2$ (*light gray bars*) or 35 μM $CuCl_2$ (*dark gray bars*). Activity assays were performed as described in *A*. *C,* reactivation of metal-free Hsp33 in the presence of stoichiometric amounts of $CuCl_2$. H_2O_2-induced reactivation of metal-free wild type Hsp33 (35 μM) was performed as described in the (*black bars*) absence or in the presence of (*light gray bars*) 3.5 μM $CuCl_2$ or (*dark gray bars*) 35 μM $CuCl_2$.

water-soluble Cu(II) was used since it is known to become easily reduced to the hydroxyl radical-producing Cu(I) in thiol-disulfide reactions (28, 29). As shown in Fig. 5*B*, substoichiometric amounts of Cu(II) present in the incubation reaction with H_2O_2 were sufficient to significantly increase the reactivation rate of Hsp33. They did so without changing the final activity of Hsp33. Within 5 min of incubation in equimolar concentrations of Cu(II) and H_2O_2, the reactivation reaction of Hsp33 was 75% complete. Cu(II) alone was unable to reactivate

TABLE IV
Zinc-binding motifs
For simplicity, only those residues that have been directly implicated as zinc ligands are shown, in all cases additional residues are conserved.

Name	Sequence motif	Prosite ID
Hsp33	$CXCX_{27-32}XCX_2C$	
Zinc finger, C2H2 type	$CX_{2-4}CX_{12}HX_{3-5}H$	PDOC00028
Zinc finger, C3HC4 type	$CXHX_2CX_2C$	PDOC00449
GATA-type zinc finger	$CX_2CX_{17-19}CX_2C$	PDOC00300
Poly(ADP-ribose) polymerase zinc finger	$CX_2CX_{28-30}HX_2C$	PDOC00360
Prokaryotic dksA/traR C4-type zinc finger	$CX_2CX_{17}CX_2C$	PDOC00846
TFIIS zinc ribbon	$CX_2CX_{2-4}CX_{2-5}C$	PDOC00383
Cytochrome *c* oxidase subunit Vb, zinc-binding region	CX_nCX_2C	PDOC00663
Prokaryotic zinc-dependent phospholipase C signature	HX_3DX_5H	PDOC00357
Zinc carboxypeptidases, zinc-binding region	HX_2EX_nH	PDOC00123
Neutral zinc metallopeptidases	HX_3H	PDOC00129
Cytidine and deoxycytidylate deaminases zinc-binding region	$[CH]X_{24-40}CX_{2-8}C$	PDOC00702

Hsp33 within the time frame of the experiment, excluding the possibility of a direct oxidation process of cysteines by copper. Moreover, metal competition experiments revealed that copper is unable to replace zinc from the zinc-binding site (data not shown). However, given the numerous negatively charged amino acids that are present in Hsp33, nonspecific copper association in the vicinity of the cysteines, cannot be excluded. The significant acceleration of reactivation of Hsp33 in the presence of copper suggested that hydroxyl radicals are indeed involved in the activation process of this chaperone.

Metal-free Hsp33 was also reactivated when equimolar amounts of copper and H_2O_2 were present in the incubation reaction, revealing that metal-free Hsp33 is still capable of reactivation. The reactivation reaction was, however, reproducibly slower than the reactivation reaction of zinc-associated Hsp33 and required the presence of equimolar amounts of copper (Fig. 5*C*). In this case, we hypothesize that Cu(II) binds directly to the cysteine center and, in cooperation with hydrogen peroxide, induces the oxidation process and formation of the correct disulfide bonds.

DISCUSSION

Hsp33 is the first molecular chaperone whose activity is known to be directly regulated by the redox conditions of the environment. Deletion mutants of Hsp33 are highly sensitive toward oxidative stress treatment, suggesting that the chaperone action of Hsp33 plays a major role in protecting cellular proteins against the deleterious effects of reactive oxygen species (Ref. 1 and reviewed in Refs. 2 and 3). Under normal conditions, the majority of cytosolic Hsp33 is reduced and presumably inactive in the cell. Under oxidizing conditions, as is the case during oxidative stress or heat treatment, two intramolecular disulfide bonds are formed, and Hsp33 accumulates in the active, oxidized state (1, 5). In the past few years, a growing number of pro- and eukaryotic proteins have been identified that seem to be regulated by similar means in response to changes in the redox potential of the cytosol. Under oxidizing conditions, OxyR, a prokaryotic transcription factor responsible for the transcription of antioxidant genes, forms an intramolecular disulfide bond (30). This activation process is accompanied by conformational changes that lead to the initiation of gene transcription. On the other hand, RsrA, an anti-sigma factor in *Streptomyces coelicolor*, is inactivated by disulfide bond formation (31). In its oxidized state, RsrA is no longer capable of interacting with the sigma factor R, which, in its non-complexed form, induces the transcription of the thioredoxin system, an important redox balancing system of the cytosol (31).

One factor that distinguishes Hsp33 from these other two redox-regulated proteins is its high zinc binding affinity in the reduced, inactive state. We showed that Hsp33 coordinates one zinc atom via its highly conserved $CXCX_{27-32}CXXC$ motif in presumably tetrahedral geometry. This motif is clearly distinct from all other zinc-binding motifs present in the Prosite data base (Release 16, updated June 1, 2000), and we consider Hsp33 to contain a previously undescribed zinc-binding motif (Table IV). Somewhat similar structural zinc-binding motifs in which zinc is coordinated by two pairs of cysteines in a tetrahedral geometry have been found in a number of other metalloproteins including DksA/TraR C4-type zinc finger, TFIIS zinc ribbon, and GATA-type zinc finger.

The zinc binding affinity of reduced Hsp33 is very high (2.5×10^{17} M^{-1}), and zinc binding confers considerable stability to Hsp33 against proteolytic digests as well as denaturating conditions. Not even incubation in 6 M Gdn·HCl permits the zinc-chelating agent PAR ($K_a = 2 \times 10^{12}$ M^{-1}) to extract zinc from the wild type protein (data not shown) indicating that at least the zinc-binding domain in Hsp33 is an extremely stable folding unit. Oxidation of the conserved cysteines to disulfide bonds, however, leads to the immediate loss of zinc. This is accompanied by major conformational changes in Hsp33 and activation of the chaperone function of Hsp33.

Zinc is a redox inert metal, and is often considered to play a purely structural role when coordinated by 4 ligands. However, zinc-coordinating cysteine centers can serve as targets for reactive oxygen species such as H_2O_2, hydroxyl radicals, and nitric oxide (NO) both *in vitro* and *in vivo*. For instance, zinc levels increase dramatically in nucleus and cytoplasm of eukaryotes upon NO stress, indicating that a number of zinc-binding proteins lose their metal presumably due to the oxidation of these cysteines (32). High affinity zinc-binding proteins such as metallothionein (11) as well as certain zinc finger transcription factors (33) have been shown to release their bound metal upon oxidation. Oxidation of metal clusters in zinc finger proteins causes the inactivation of the protein even when the zinc finger is not directly involved in DNA recognition and binding (9). It appears, however, that inactivation is reversible and does not lead to irreversible unfolding or aggregation of the proteins. This suggests that structural, zinc-coordinating cysteine centers are able to serve an additional role by quickly sensing and responding to reactive oxygen species, thereby leading to changes in protein conformation and activity. To define whether an intact metal center is indeed needed for the oxidation process of Hsp33, metal-free Hsp33 was generated, and reactivation experiments with H_2O_2 were performed. It was shown that reactivation of metal-free Hsp33 was very slow and incomplete. Several mechanisms could explain the role zinc plays in enhancing the activation process of Hsp33. First, zinc coordination may keep the reactive cysteines in a conformation that allows the correct disulfide bonds to be formed. The extreme protease sensitivity of the metal-free, reduced Hsp33

is consistent with this mechanism. Alternatively, zinc may play a direct or indirect role in the catalytic mechanism of the activation process of Hsp33. Zinc is capable of coordinating additional substrates such as H_2O_2 leading to structurally flexible systems with five coordination sites (34). This may allow zinc to bring this reactive oxidant into close proximity to the reactive cysteines of Hsp33. Zinc may also alter the reactivity of the conserved cysteines by reducing their pK_a thereby keeping them in the activatable thiolate anion state.

The question now arises, what distinguishes the highly reactive metal center of Hsp33 from that of zinc finger proteins? The thiolate anions of Hsp33 coordinate zinc with high affinity in presumably tetrahedral coordination. This is very similar to zinc finger proteins. The stability of zinc finger domains are equally high, and the spacing of the cysteines are comparably close. Could the observed regulation of Hsp33 by reactive oxygen species be not only similar to the subset of redox-regulated zinc finger proteins studied so far but represent a general mechanism of modulating zinc finger proteins? With the development of *in vivo* thiol-trapping techniques (2), it will soon be possible to monitor the *in vivo* thiol status of cysteine-containing proteins under oxidative stress conditions like aging and cancer (35, 36). This might contribute to our understanding toward the severe changes observed in signal transduction pathways during these pathological processes.

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