The heat shock protein Hsp33 is a very potent molecular chaperone with a distinctive mode of functional regulation; its activity is redox-regulated. In its reduced form all six cysteinyl residues of Hsp33 are present as thiols, and Hsp33 displays no folding helper activity. Exposure of Hsp33 to oxidizing conditions like \( \text{H}_2\text{O}_2 \), however, rapidly converts Hsp33 into an efficient molecular chaperone. Activated Hsp33 binds tightly to refolding intermediates of chemically denatured luciferase and suppresses efficiently their aggregation in vitro. Matrix-assisted laser desorption/ionization-mass spectrometry peptide mapping in combination with in vitro and on target protein chemical modification showed that this activation process of Hsp33 is accompanied by the formation of two intramolecular disulfide bonds within Hsp33: Cys\(^{2352}\)-S—S—Cys\(^{2354}\) and Cys\(^{265}\)-S—S—Cys\(^{268}\). Cys\(^{141}\), although not involved in disulfide bond formation, was found highly reactive toward chemical modifications. In contrast, Cys\(^{239}\) is readily accessible under reducing conditions but becomes poorly accessible though still reduced when Hsp33 is in its active state. This indicates a significant conformational change during the activation process of Hsp33. Mass spectrometry, thus, unraveled a novel molecular mechanism by which alteration of the disulfide bond structure, as a result of changes in the cellular redox potential, results in the activation of a molecular chaperone.

Hsp33 is a newly discovered heat shock protein that functions as a highly efficient molecular chaperone (1). Hsp33 protects bacterial cells from deleterious effects caused by oxidants like \( \text{H}_2\text{O}_2 \) showing that it plays a role in the bacterial defense system against oxidative stress. Hsp33 is distinguished from all other known chaperone proteins by the finding that Hsp33 is functionally regulated at posttranslational level, by the redox conditions of the environment (1) (reviewed in Refs. 2 and 3). Elevated \( \text{H}_2\text{O}_2 \) concentrations induce the chaperone functions of Hsp33 (1), but the precise molecular mechanism that translates the changes of the cellular redox environment into differences in chaperone activity is not yet understood.

The Hsp33 amino acid sequence contains a novel conserved motif consisting of four cysteinyl residues near the C terminus of the protein. These cysteinyl residues form a C-X-C-C motif (Fig. 1), separated by 27–30 amino acid residues in \textit{Escherichia coli} Hsp33 and its homologues. These four cysteinyl residues are present in all 27 known Hsp33 homologues suggesting that they play an important role in the function of Hsp33. In addition, two further cysteinyl residues, Cys\(^{141}\) and Cys\(^{239}\), are present in Hsp33. Cys\(^{239}\) is very poorly conserved occurring in only 4 of the 27 known Hsp33 homologues. Cys\(^{141}\) is moderately conserved and is present in 10 of the 27 Hsp33 homologues known so far. We have postulated that disulfide bond formation is involved in the activation process of Hsp33. Thus, analysis of the disulfide bond status and connectivities in inactive and active Hsp33 seemed very important to further understand the regulation of this efficient molecular chaperone.

Two complementary mass spectrometry-based strategies are suitable for determining the presence and locations of disulfide bonds in proteins. Which one is superior is dependent on the proximity of the involved cysteinyl residues in the amino acid sequence. Both approaches involve cleavage of the protein by enzymatic or chemical means under conditions that minimize disulfide bond scrambling (4–6). The first approach can be used when the proteolytically derived peptides contain zero or one cysteinyl residue. The peptide mixtures are directly analyzed by mass spectrometric peptide mapping. Molecular mass analyses identify the peptides and disulfide-bonded dipeptides by assigning the observed ion signals to the corresponding calculated masses from the amino acid sequence. To confirm whether or not an observed peak in the recorded mass spectrum represents a disulfide-linked dipeptide, a chemical reduction reaction is performed afterward with the sample either in vitro (4, 5, 7) or on target (8–11). Subsequent re-analyses of these samples by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS)\(^1\) should show the disappearance of the ion signals from dipeptides that are disulfide-linked and the appearance of signals corresponding to the resulting pairs of reduced peptides. The second approach has been developed for the situation where proteolytic cleavage results in individual peptides that contain more than one cysteinyl residue. The absence or presence of disulfide bonds in these peptides is detected by first alkylating free thiols in the intact protein followed by proteolytic cleavage and subsequent detection of the peptide masses (4, 12–14). Thiol-containing peptides are found alkylated and are clearly distinguished by the corresponding mass shifts from unmodified and, thus, disulfide bond-carrying peptides. Thiol selective modification reagents such as iodoacetic acid and iodoacetamide are widely used for cysteinyl-selective modification of reduced proteins (4, 15). They rapidly and irreversibly modify free thiol groups but do not...
Assignment of Disulfide Bonds in Active Hsp33 Chaperone

Chemical Modification of Hsp33

Reduction of Hsp33—Hsp33 was reduced by adding 6 μl of a 44 mM DTT solution (in 50 mM NH₄HCO₃, pH 8) to 50 mM of a solution containing 100 μg of Hsp33 (3 nmol, in 40 mM HEPES-KOH, 20 mM KCl, 5% glycerol, pH 7.5). The mixture was immediately diluted with 50 mM NH₄HCO₃, pH 8, to yield a final protein concentration of 1 μg/μl. The molar ratio of DTT to cysteinyl residues was 14:1. Reduction was performed for 1 h at 37 °C and was complete as judged by Ellman’s assay.

H₂O₂ Oxidation of Hsp33—Hsp33 was oxidized using 4 mM H₂O₂ at room temperature as follows: 1.6 μl of 800 mM H₂O₂ (in 50 mM NH₄HCO₃, pH 8) was added to 320 μl of a solution containing 1 μg/μl of Hsp33 solution (50 mM NH₄HCO₃, pH 8). The mixture was incubated at room temperature for 2 h. Aliquots (100 μl) were desalted using an Amicon microconcentration device (cutoff: 10,000 Da). Retentates were washed three times with 200 μl of 50 mM NH₄HCO₃, pH 8. The final protein concentration was 2 μg/μl.

Air Oxidation of Hsp33—Air oxidation of Hsp33 was performed in ammonium bicarbonate buffer at 4 °C. The Hsp33 solution (2 μg/μl in 40 mM HEPES-KOH, 20 mM KCl, 5% glycerol, pH 7.5) was diluted 1:2 with 50 mM NH₄HCO₃, pH 8. Aliquots containing 100 μg of Hsp33 were pipetted into Eppendorf tubes and covered with cellulose membrane (molecular mass cutoff 3500 Da). Gentle stirring at 8 °C allowed oxygen exchange between the reservoir (3 liters of air-saturated 50 mM NH₄HCO₃, pH 8) and the sample solution. Samples were removed after 1 h and 24 h, respectively, and stored frozen at −20 °C.

Carboxamidomethylation of Hsp33 after Reduction, H₂O₂ Oxidation, and Air Oxidation—Hsp33 (100 μg = 3 nmol) was incubated in 100 mM iodoacetamide, 50 mM NH₄HCO₃, pH 8, in the dark for 30 min to carboxamidomethylate all accessible thiogroups. This iodoacetamide concentration represents a 1000-fold molar excess over the cysteinyl residues in Hsp33. The reaction was terminated by adding glacial acetic acid, which shifted the pH to ~4. Buffer exchange was performed using microconcentrator devices as described above. Aliquots (20 μl) were lyophilized after the addition of 20 μl of 0.1% 2,2,2-trifluoroacetic acid.

Proteolytic Degradation of Hsp33 and Carboxamidomethylated Derivatives

Proteolytic degradations of Hsp33 and its carboxamidomethylated derivatives (40 μg each) were carried out in 50 mM NH₄HCO₃, pH 8, 2.5 mM EDTA, 10% (v/v) CH₃CN. Protease stock solutions of Lys-C (EC 3.4.99.30, Roche Molecular Biochemicals) and trypsin (1 μg/μl, EC 3.4.21.4, Sigma) were prepared with a concentration of 1 μg/μl in 50 mM NH₄HCO₃. Enzyme to substrate ratios were 1:80 for Lys-C and 1:50 for trypsin. Digestions were performed for 2 h at 37 °C. The

Chaperone Activity Assays of Hsp33

The chaperone activity of oxidized and reduced Hsp33 was tested with luciferase as substrate protein. Firefly luciferase (12.6 μl) (Roche Molecular Biochemicals) was denatured with 5 μl guanidine-HCl in 40 mM HEPES-KOH, pH 7.5, for 90 min at room temperature. Refolding was initiated by diluting denatured luciferase 1:133 into 40 mM HEPES-KOH, pH 7.5, at 25 °C in the absence or presence of Hsp33. To monitor aggregation, light scattering measurements were performed using a Hitachi F4500 fluorescence spectrophotometer equipped with a thermostated cell holder under constant stirring. Excitation and emission wavelengths were set to 350 nm, and the slit widths were 2.5 nm. To test for the effects of reduction on the chaperone activity of Hsp33, oxidized Hsp33 was reduced with 2 mM DTT for 25 min at 37 °C prior to the addition to the reaction mixture. Control experiments showed that the residual concentration of DTT present in the reaction mixture (0.2 mM) did not influence the aggregation behavior of luciferase in the absence of any additional protein.

FIG. 1. Hsp33 amino acid sequence. Cys residues are depicted in bold and italic letters. Disulfide bonds as in oxidized Hsp33 are shown (black lines). Tryptic cleavage sites, T, (filled arrows), and cleavages by Lys-C, L, (open arrows), are indicated. Tryptic peptide cleavages given in parentheses refer to peptides to the left.

![Hsp33 Amino Acid Sequence](http://www.jbc.org)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Peptide</th>
<th>Mass [Da]</th>
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<tr>
<td>1-10</td>
<td>NMTAEGKLKDQTLKCAHWDQKKDNSTENAVTG</td>
<td>1237</td>
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<tr>
<td>11-20</td>
<td>LQDADDYKDLLGKLKDKYDQKKDNSTENAVTG</td>
<td>1237</td>
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<tr>
<td>21-30</td>
<td>KDDQDDYKDLLGKLKDKYDQKKDNSTENAVTG</td>
<td>1237</td>
</tr>
<tr>
<td>31-40</td>
<td>SQTDYKDLLGKLKDKYDQKKDNSTENAVTG</td>
<td>1237</td>
</tr>
<tr>
<td>41-50</td>
<td>QNKSDYKDLLGKLKDKYDQKKDNSTENAVTG</td>
<td>1237</td>
</tr>
<tr>
<td>51-60</td>
<td>DQKDDYKDLLGKLKDKYDQKKDNSTENAVTG</td>
<td>1237</td>
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</tbody>
</table>

Assignment of Disulfide Bonds in Active Hsp33 Chaperone
reactions were terminated by adding glacial acetic acid, shifting the pH to \( \sim 4 \).

**On Target Reduction of Disulfide Bonds**

On target reduction in the presence of 4-hydroxy-\( \alpha \)-cyano-cinnamic acid (HCCA) matrix was carried out with the Lys-C-derived Hsp33 peptides after MALDI-MS peptide mapping (see below) according to published procedures (8, 10, 11).

**Mass Spectrometry**

For nanoelectrospray ionization (nano-ESI)-MS analyses, samples containing freshly isolated Hsp33, oxidized and reduced Hsp33, as well as the carboxamidomethylated derivatives were redissolved in 200 \( \mu L \) of acetic acid trifluoroethanol (3 volumes of 10% aqueous acetic acid mixed with 7 volumes of neat trifluoroethanol), pH 2 (final protein concentration was \( \approx 0.2 \mu g/\mu L \)). Nano-ESI-MS was performed with a Vestec-501A quadrupole mass spectrometer as described previously (10). Mass calibration was performed with the 8+ to 12+ charged ions of hen egg white lysozyme (\( M_r \), 14305.1), and raw data were analyzed using a Vector-2 data system (Teikinen).

For MALDI-MS peptide mapping analyses 1-\( \mu L \) aliquots from the proteolytic digests were used either without further purification or after elution from C-18-reversed phase material deposited in gel loader pipette tips (8). Matrix solution (1 \( \mu L \) = 10 \( \mu g/\mu L \); HCCA) dissolved in CH\(_3\)CN/0.1% trifluoroacetic acid (2:1, v/v), pH 2, was added directly on the target. Spectra were recorded after evaporation of the solvent with a Bruker Biflex linear time-of-flight mass spectrometer equipped with a UV-nitrogen laser (357 nm) and a dual microchannel plate detector. The acceleration voltage was set to 20 kV. Spectra were calibrated with insulin and processed using the X-MASS data system.

**RESULTS**

**Molecular Mass Determinations of Completely Reduced and of \( H_2O_2 \)-oxidized Hsp33**

To characterize the thiol status of Hsp33, homogenous preparations of reduced and of oxidized protein were prepared. Hsp33 was incubated with 2 \( \text{mM} \) DTT in the presence of equimolar amounts of zinc chloride to saturate the zinc binding capabilities of Hsp33. Titration experiments revealed the presence of 5.7 \( \pm 0.3 \) freely accessible cysteinyl sulphydryl groups (data not shown). The molecular mass of this Hsp33 derivative was determined by MALDI-MS to be 32,782 \( \pm 5 \) Da, which is in good agreement with the 32,779 Da mass calculated from the translated amino acid sequence for Hsp33 in a fully reduced form. Carboxamidomethylation of this Hsp33 preparation resulted in a Hsp33 derivative with a molecular mass of 33,119 \( \pm 8 \) Da (\( M_r \), 33,121), consistent with the complete modification of all cysteinyl residues.

Fully oxidized Hsp33 was prepared from the reduced protein sample by incubation in excess amounts of \( H_2O_2 \). Gel filtration was performed to remove DTT, Zn\(^{2+} \), and \( H_2O_2 \). An Ellman’s assay revealed that less than 0.5 freely accessible thiol groups were present in this sample (data not shown). The molecular mass for \( H_2O_2 \)-oxidized Hsp33 of 33,032 \( \pm 4 \) Da was calculated from the nano-ESI mass spectrum (Fig. 2). This molecular mass is consistent with the addition of 16 oxygen atoms (\( M_r \), 33,029), presumably because of oxidation of methionyl, tryptophanyl, and cysteinyl residues in monomeric Hsp33.

**Chaperone Activity of Completely Reduced and of \( H_2O_2 \)-oxidized Hsp33**

To analyze the chaperone activity of DTT-reduced and of \( H_2O_2 \)-oxidized Hsp33, these two species were tested for their ability to interact with refolding intermediates of chemically denatured luciferase as substrate. Denatured luciferase immediately aggregates upon dilution into renaturation buffer (17). Molecular chaperones are able to recognize and bind these refolding intermediates of luciferase and efficiently prevent the otherwise irreversible aggregation processes. Oxidized Hsp33 is able to nearly completely suppress the aggregation of refolding intermediates of luciferase as monitored with light scattering (Fig. 3a). This implies that Hsp33 is capable of recognizing and binding to very early, unstructured folding intermediates, thereby keeping their free concentration low and preventing their aggregation. Reduction of Hsp33 by incubation of the active, oxidized Hsp33 in 2 \( \text{mM} \) DTT for 25 min at 37 °C was sufficient to completely abolish the chaperone activity of Hsp33 in vitro (Fig. 3b). Reduced Hsp33 is no longer able to recognize these folding intermediates. The light scattering kinetics of luciferase in the presence of reduced Hsp33 is nearly indistinguishable from the kinetics monitored in the absence of any additional protein (Fig. 3c).

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**Fig. 2.** Nano-ESI-MS molecular mass analysis of Hsp33 after \( H_2O_2 \)-oxidation. The spectrum shows a series of multiply charged molecular ions centering around the [M+27H]\(^{27+} \) molecular ion. The observed molecular mass indicates the presence of 16 additional oxygen atoms. Only traces of dimeric Hsp33 are observed. Analysis was performed in a solution containing 10% acetic acid/2,2,2-trifluoroethanol (7:3; v/v), pH 2.
MALDI-MS Peptide Mapping of DTT-reduced and of 
\( \text{H}_2\text{O}_2 \)-oxidized Hsp33

To obtain the peptide maps, reduced and \( \text{H}_2\text{O}_2 \)-oxidized Hsp33 were carboxamidomethylated and subsequently cleaved proteolytically with either Lys-C or trypsin, respectively. The resulting peptide mixtures were analyzed by MALDI-MS peptide mapping. Lys-C cleavage of reduced Hsp33 produced 8 peptides (L1–L8; cf. Fig. 1), trypsin cleavage yielded a pattern of 20 different peptides (T1–T20; cf. Fig. 1). All six cysteinyl residues of reduced Hsp33 were found completely alkylated (Table I). An ion signal at \( m/z \) 683, for example, represented the doubly carboxamidomethylated peptide T16 (amino acids 232–236) that carries residues Cys\(^{232}\) and Cys\(^{234}\) (Fig. 4A and Table I). Similarly, the ion signal at \( m/z \) 5706 found unmodified. In addition, an ion signal at \( m/z \) 5706 was observed at \( m/z \) 5706 (Fig. 4B). Both less conserved cysteines were found carboxamidomethylated in the peptides T9 (Cys\(^{141}\)) and T17 (Cys\(^{238}\)).

MALDI-MS peptide mapping after tryptic digestion of the fully oxidized Hsp33 showed the presence of all individual peptides indicating that intermolecular disulfide bond formation is not involved in the activation process of Hsp33. This result also ruled out the formation of disulfides between cysteines located on different tryptic peptides. Two ion signals, though, were significantly different from the ion signals of the corresponding reduced peptides. Peptide T16, carrying Cys\(^{232}\) and Cys\(^{234}\), was found unalkylated at \( m/z \) 568 (Table I) compared with \( m/z \) 568 of the corresponding peptide in reduced and carboxamidomethylated Hsp33. This indicated that these two cysteines are linked with a disulfide bridge and, thus, were found unmodified. In addition, an ion signal at \( m/z \) 584 was observed that was consistent with a derivative of peptide T16 with an additional oxygen atom. This ion signal may represent a subpopulation of Hsp33 in which T16 bears a thiosulfinate group (-SO\(_3\)S\(_2\)) instead of a disulfide bridge (Table I). Peptide T19, harboring Cys\(^{265}\) and Cys\(^{268}\), was found at \( m/z \) 490 instead of \( m/z \) 454, indicating that a second disulfide bond had occurred upon \( \text{H}_2\text{O}_2 \) incubation in Hsp33 linking Cys\(^{265}\) and Cys\(^{268}\) (Table I). Peptide T19 was found 32 Da higher in mass than calculated for unmodified T19 (\( M_\text{r} \), 4458). This indicates additional oxidation of the two methionine residues, Met\(^{263}\) and Met\(^{277}\), in this peptide. None of the cysteine residue-carrying peptides, including the peptides harboring the less conserved cysteines, were found alkylated in the \( \text{H}_2\text{O}_2 \)-oxidized form of the protein. Peptide T9, which contains Cys\(^{141}\) was observed at \( m/z \) 2516, consistent with the addition of four oxygen atoms. Cys residues are forming one intramolecular disulfide bond. Calculated mass as in footnote \( c \) plus the addition of one oxygen atom.

# Table I

<table>
<thead>
<tr>
<th>Cys-residue/no. of alkyl groups</th>
<th>Peptide/(amino acid range)</th>
<th>[M + H](^\text{+}) ((\text{calculated}))</th>
<th>( m/z ) ((\text{observed}))</th>
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</thead>
<tbody>
<tr>
<td>Cys(^{141})/1</td>
<td>T9/(127–148)(^f)</td>
<td>2505.8</td>
<td>2509 (-)</td>
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<tr>
<td>Cys(^{141})/0</td>
<td>T9/(127–148)(^g)</td>
<td>2515.8</td>
<td>(-) 2516 (-)</td>
</tr>
<tr>
<td>Cys(^{232})/2</td>
<td>T16/232–236(^b)</td>
<td>683.7</td>
<td>683 (-)</td>
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<tr>
<td>Cys(^{232})/0</td>
<td>T16/232–236(^c)</td>
<td>567.7</td>
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</tr>
<tr>
<td>Cys(^{234})/2</td>
<td>T16/232–236(^g)</td>
<td>585.7</td>
<td>(-) 584 (-)</td>
</tr>
<tr>
<td>Cys(^{239})/1</td>
<td>T18/239–244(^d)</td>
<td>676.7</td>
<td>676 (-)</td>
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<tr>
<td>Cys(^{239})/0</td>
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<tr>
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<td>Cys(^{265})/0</td>
<td>T19/245–283(^k)</td>
<td>4489.9</td>
<td>4490 (-)</td>
</tr>
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</table>

\( ^a \) Average masses.
\( ^b \) Calculated mass for carboxamidomethylated Cys residue(s).
\( ^c \) not detected.
\( ^d \) Calculated mass for addition of four oxygen atoms.
\( ^e \) Cys residues are forming one intramolecular disulfide bond.
\( ^f \) Calculated mass as in footnote \( c \) plus the addition of one oxygen atom.
\( ^g \) Cys residue as thiol.
\( ^h \) Calculated mass as in footnote \( d \) plus the addition of two oxygen atoms.
We conclude that activation of Hsp33 by H$_2$O$_2$ results in the formation of two intramolecular disulfide bonds linking Cys$^{232}$–S–S–Cys$^{234}$ and Cys$^{265}$–S–S–Cys$^{268}$. These results are consistent with those obtained by ESI-MS molecular mass determinations that showed most of the active Hsp33 as monomeric but heavily oxidized species in this sample (see above).

Assignments of Disulfide Bonds in Hsp33 after Air Oxidation

As H$_2$O$_2$ oxidation not only caused formation of disulfide bonds in Hsp33 but also lead to the uptake of additional oxygen atoms, we attempted to prepare solely disulfide bond-carrying Hsp33 species by air oxidation.

Short Air Oxidation—When reduced Hsp33 was incubated in air-saturated buffer for 1 h and was subsequently alkylated with iodoacetamide, mixtures of differently carboxamidomethylated Hsp33 derivatives were observed after proteolytic digestions. This was very similar to the results obtained with freshly harvested Hsp33. Ellman’s titration of freshly isolated and untreated Hsp33 revealed the presence of 2.4–3.0 accessible cysteinyl residues (1). As the sequence of Hsp33 codes for six cysteinyl residues (cf. Fig. 1), this result may be explained by partial formation of disulfide bonds. Alternatively, this result is consistent with the presence of a protein mixture consisting of (partially) disulfide bond-carrying and completely reduced species.

Proteolytic digestion of Hsp33 after 1 h of air oxidation using Lys-C protease and mass spectrometric peptide mapping showed peptide ion signals that covered the entire amino acid sequence of Hsp33 (Fig. 5). Interestingly, some of the cysteine-containing peptides resulted in multiple ion signals differing by the mass increment of 57 Da, showing the presence of cysteinyl-carboxamidomethyl groups. Peptide L7 that carries residues Cys$^{232}$, Cys$^{234}$, and Cys$^{239}$ was found triply and singly carboxamidomethylated, giving rise to ion signals at $m/z$ 1626 and $m/z$ 1511, respectively. Similarly, peptide L8 that harbors Cys$^{265}$ and Cys$^{268}$ showed ion signals representing doubly carboxamidomethylated and unmodified peptide at $m/z$ 5706 and $m/z$ 5590, respectively. In contrast, peptide L4, which contains Cys$^{141}$, was only found alkylated giving rise to an ion signal at $m/z$ 6511. This result clearly showed that this Hsp33 sample presented a mixture of disulfide bond-containing (oxidized) and completely reduced protein species. It further is consistent with the finding that on native gel electrophoresis Hsp33 migrates as multiple bands. These bands can be converted into single but distictively migrating bands when either strictly reducing or oxidizing conditions are used (1).

Long Air Oxidation—Incubation for 24 h in air-saturated buffer yielded in fully oxidized Hsp33; however, a mixed population of monomeric and dimeric proteins was formed. The latter Hsp33 derivative harbored both intra- and intermolecular disulfide bonds. Small ion signals corresponding to dimeric Hsp33 were observed in the ESI spectra of this sample together with dominant ion signals of monomeric Hsp33. The molecular mass of 65,523 ± 30 Da for dimeric Hsp33 was determined from the spectra ($M$, 65,546).

Proteolytic digestion of this Hsp33 mixture after carboxamidomethylation unambiguously determined the connectivities of the disulfide bridges. An ion signal for T16 (amino acids 232–236) at $m/z$ 568 was observed (Table II), in agreement with an intramolecular disulfide bond that connected Cys$^{232}$ with Cys$^{234}$. Further, an ion signal at $m/z$ 5590 represented peptide L8 in which a disulfide bridge was formed between residues Cys$^{265}$ and Cys$^{268}$ (Table II).

The subpopulation of air-incubated Hsp33 that contained both intra- and intermolecular disulfide bonds was distinguished by the appearance of an additional ion signal at $m/z$ 2906. This ion signal was assigned as a disulfide-bonded dipeptide, L7–S–S–L7 (Table II and Fig. 6A). It contained 6 cysteinyl residues; two times Cys$^{232}$, Cys$^{234}$, and Cys$^{239}$ from each of the two covalently linked L7 peptides. The determined molecular mass ($m/z$ 2906) indicated that all cysteinyl residues in this dipeptide were involved in disulfide bridges as no addition of alkyl groups were detected. Hence, the small ion signal at $m/z$ 1455 in this spectrum may be because of MALDI-induced cleavage of disulfide bonds (19–21). After recording of the mass spectrometric data, the sample was reduced with β-mercaptoethanol on the MALDI target in the presence of the HCCA matrix. Subsequent acquisition of a further MALDI spectrum showed that the ion signal at $m/z$ 2906 had disappeared completely, and four new strong ion signals at $m/z$ 1454 (L7), $m/z$ 1532 (L7 + 1β-mercaptoethanol), $m/z$ 1610 (L7 + 2β-mercaptoethanol), and $m/z$ 1688 (L7 + 3β-mercaptoethanol) had appeared (Fig. 6B). These results show that the dipeptide L7–S–S–L7 had in fact contained three disulfide bonds. The number of β-mercaptoethanol adducts reveals the number of disulfide bridges in the peptide (22). Further, after tryptic digestion of this Hsp33 sample, an ion signal for a disulfide-bridged T17/18–S–S–T17/18 peptide (amino acids 237–244) was observed at $m/z$ 1810 (Table II) and proved the existence of the intermolecular disulfide bridge connecting Cys$^{239}$–S–S–Cys$^{239}$. Interestingly, this is the same cysteine that, after H$_2$O$_2$ oxidation, was found inaccessible to any thiol modifications.
Hsp33 is the first member described of a new family of molecular chaperones (1). The regulation of Hsp33 appears to be under both transcriptional and post-translational control. Transcription of Hsp33, like most _E. coli_ heat shock proteins, is directly regulated by the concentration and activity of the heat shock transcription factor σ32. Hsp33 is also regulated on the posttranslational level directly by the redox conditions of the cell (reviewed in Refs. 3 and 23). This latter mode of functional regulation distinguishes Hsp33 from all other molecular chaperones characterized so far. Hsp33 is present in the reduced form under in _vivo_ normal conditions and, in this form, does not show significant chaperone activity. It is unable to interact with thermal unfolding intermediates of citrate synthase or luciferase (1), and it is also not capable of recognizing refolding intermediates of chemically denatured luciferase (this study). H_2O_2 oxidation, however, quickly activates the chaperone activity of Hsp33. Oxidized Hsp33 binds with high affinity to highly structured, late folding intermediates as well as to unstructured, early folding intermediates of various substrate proteins. It decreases their free concentration and significantly suppresses otherwise irreversible aggregation processes _in vitro_. Hsp33 probably plays a similar role under oxidative stress conditions _in vivo_. Deletion of the _hsp33_ gene leads to a higher sensitivity of cells toward H_2O_2 treatment as well as toward incubation of the cells at elevated temperatures (1).

Thiol analysis and thiol-trapping experiments of active and inactive Hsp33 suggested that reversible disulfide bond formation might represent the molecular switch that turns Hsp33 on (1). Because Hsp33 contains 6 cysteines, of which 5 are arranged within close proximity in a 35-amino acid region of the protein, thiol trapping with iodoacetamide and subsequent mass spectrometric analysis of the peptides was used to uncover the precise thiol status of the active Hsp33 species. The individual two neighbor cysteines Cys^232_ and Cys^234_ as well as Cys^265_ and Cys^268_ were found to rapidly form two intramolecular disulfide bonds upon H_2O_2 oxidation and activation of Hsp33. The Cys^262_Y-Z-Cys^266_motif in Hsp33 is reminiscent of the reactive cysteine motifs found in oxidoreductases like thioredoxin or disulfide bond isomerase A. Hsp33 does not appear to function as an oxidoreductase but uses the same motif to quickly translate changes in the redox potential into changes in the thiol status. The second disulfide bond forms between two cysteines, separated by only one amino acid (Cys^232-X-Cys^234_). The formation of a disulfide bond between the corresponding thiol groups is considered to be sterically hindered and likely to be accompanied by conformational changes in the protein (24). Interestingly, redox titration of the chaperone activity of Hsp33 was consistent with the formation and breakage of a single disulfide bond (1). It is, therefore, possible that only one of the two disulfide bonds formed upon oxidation is responsible for the chaperone activity of Hsp33.

The mass spectrometric analysis of the H_2O_2-oxidized wild type Hsp33 showed the presence of additional oxygen atoms in the oxidized species. One oxygen atom was observed within the peptide bearing the Cys^232-S-S-Cys^234_disulfide bond. This could reflect the presence of a thiosulfinate group between the two cysteinyI groups (25). We speculate this was an intermediate state during the H_2O_2 oxidation. H_2O_2 is known to oxidize thiol groups to the highly reactive sulfenic acid (26). The most common reaction of sulfenic acids is the formation of thiosulfinate, if the two sulfenic acids are in close proximity within the protein (26). Sulfenic acid formation was thought to be vital for the thiol status. The second disulfide bond forms between two cysteines, separated by only one amino acid (Cys^232-X-Cys^234_). The formation of a disulfide bond between the corresponding thiol groups is considered to be sterically hindered and likely to be accompanied by conformational changes in the protein (24). Interestingly, redox titration of the chaperone activity of Hsp33 was consistent with the formation and breakage of a single disulfide bond (1). It is, therefore, possible that only one of the two disulfide bonds formed upon oxidation is responsible for the chaperone activity of Hsp33.

**DISCUSSION**

Hsp33 is the first member described of a new family of molecular chaperones (1). The regulation of Hsp33 appears to be under both transcriptional and post-translational control. Transcription of Hsp33, like most _E. coli_ heat shock proteins, is directly regulated by the concentration and activity of the heat shock transcription factor σ32. Hsp33 is also regulated on the posttranslational level directly by the redox conditions of the cell (reviewed in Refs. 3 and 23). This latter mode of functional regulation distinguishes Hsp33 from all other molecular chaperones characterized so far. Hsp33 is present in the reduced form under in _vivo_ normal conditions and, in this form, does not show significant chaperone activity. It is unable to interact with thermal unfolding intermediates of citrate synthase or luciferase (1), and it is also not capable of recognizing refolding intermediates of chemically denatured luciferase (this study). H_2O_2 oxidation, however, quickly activates the chaperone activity of Hsp33. Oxidized Hsp33 binds with high affinity to highly structured, late folding intermediates as well as to unstructured, early folding intermediates of various substrate proteins. It decreases their free concentration and significantly suppresses otherwise irreversible aggregation processes _in vitro_. Hsp33 probably plays a similar role under oxidative stress conditions _in vivo_. Deletion of the _hsp33_ gene leads to a higher sensitivity of cells toward H_2O_2 treatment as well as toward incubation of the cells at elevated temperatures (1).
Finding that reducing agents seem to be unable to cause the dissociation of Hsp33-substrate complexes is probably because of nearly lacking accessibility of the disulfide bonds in oxidized Hsp33. These results tempt us to speculate that this region of the protein was involved in substrate binding. Burying Cys\(^{239}\) might be accompanied by exposing hydrophobic surfaces, the main forces in chaperone-substrate interactions. Interestingly, a subpopulation of Hsp33 that has been air-exposed for an extended time forms an intramolecular disulfide bond between two Cys\(^{239}\) residues. This dimer formation might be caused by the partial unfolding of one Hsp33 molecule and the binding of an active Hsp33 molecule to prevent aggregation. This is reminiscent of Hsp70, a well studied molecular chaperone, which is known to form dimers and to bind to other Hsp70 molecules “as if it were an unfolded protein” (28).

The position of the intramolecular disulfide bond could serve as a further indication that the C terminus of Hsp33 corresponds to the substrate binding site of Hsp33.

Thiol-disulfide exchange reactions appear to play a mechanistic role in an increasing number of cytoplasmic proteins in bacteria (23). The transcription factors OxyR and Flp as well as the very recently identified anti-\(\sigma\) factor RsrA are all regulated by reversible disulfide bond formation. Interestingly, whereas Hsp33, OxyR, and Flp are activated upon disulfide bond formation (18, 29), RsrA is inactivated as soon as the thiol becomes engaged in disulfide bridges (30). This seems plausible in the light of the individual functions of these proteins in the cell. Because Hsp33 is also a Zn\(^{2+}\)-binding protein, we assume that metal coordination might play a role in keeping Hsp33 in the reduced, inactive form both in \(\text{in vivo}\) and \(\text{in vitro}\). \(\text{H}_2\text{O}_2\) oxidation, or prolonged dialysis of Hsp33, could result in the dissociation of zinc and the subsequent oxidation of the disulfide bonds. It will be interesting to determine the binding constant of zinc and the role of zinc for oxidation and/or reduction processes in Hsp33.

Taken together, Hsp33 is a member of a new group of proteins that share a high sensitivity toward the redox potential of the environment and that use reactive disulfide bonds as molecular switches. Further study of proteins with closely spaced cysteines or cysteine-coordinating zinc centers may reveal that thiol-disulfide exchanges play a much more widespread role in protein regulation at the posttranslational level than had previously been anticipated (23).

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\(^2\) U. Jakob, unpublished results.
Assignment of Disulfide Bonds in Active Hsp33 Chaperone

Mass Spectrometry Unravels Disulfide Bond Formation as the Mechanism That Activates a Molecular Chaperone
Stefanie Barbirz, Ursula Jakob and Michael O. Glocker

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