Title: The redox-switch domain of Hsp33 functions as dual stress sensor

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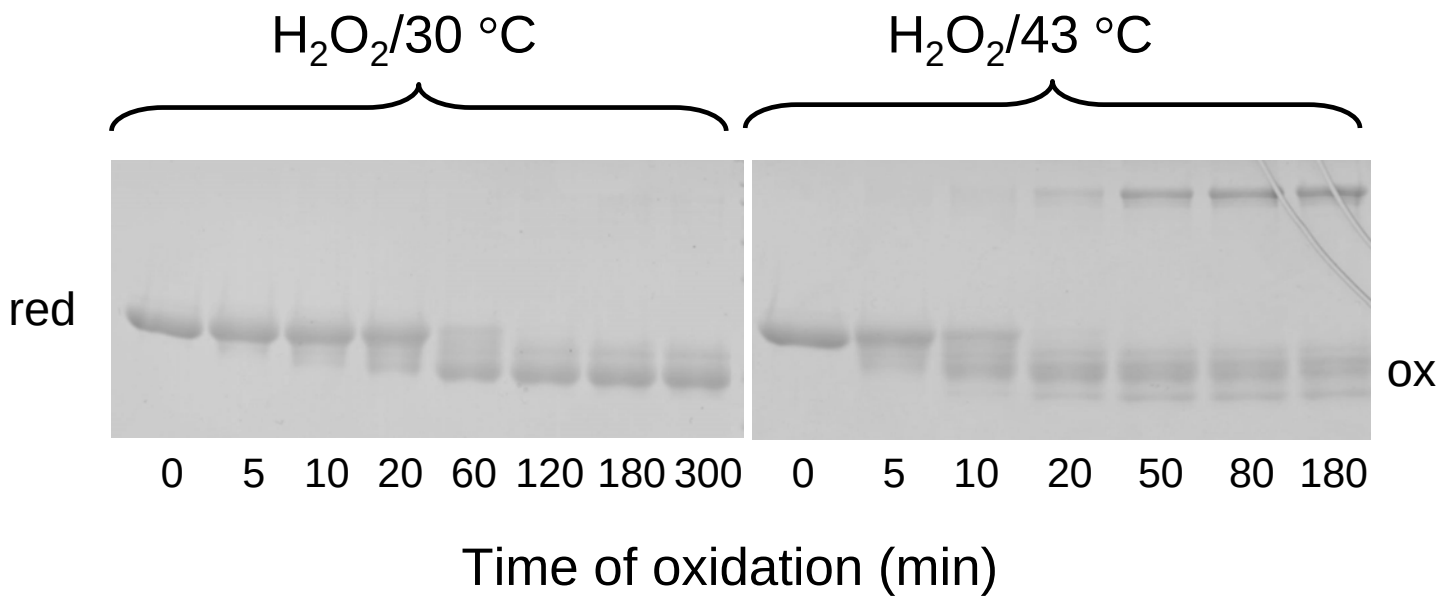
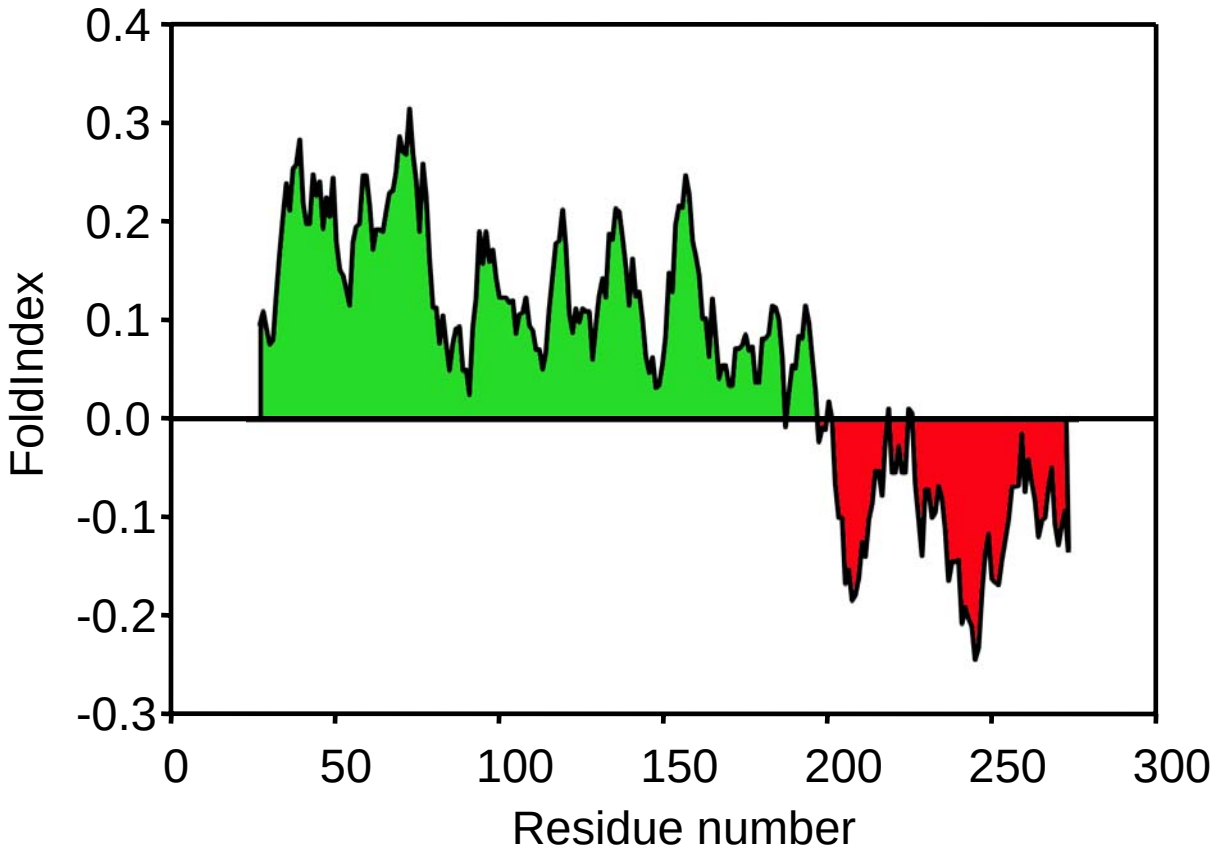


Supplementary Fig. 1. AMS-trapping reveals differences in thiol modification pattern of Hsp33 incubated in H_2O_2 at 30 °C or 43 °C. 50 µM $Hsp33_{red}$ was incubated in 2 mM H_2O_2 at either 30 °C or 43 °C. At the time points indicated, aliquots were taken and TCA-precipitated to stop thiol-disulfide exchange reactions. The free thiol groups were then covalently modified with the 490 Da thiol-specific molecule AMS and differences in the mobility of the proteins were detected using non-reducing 14 % SDS-PAGE. $Hsp33_{red}$ modified with AMS migrates significantly more slowly than oxidized non-AMS modified Hsp33.



Supplementary Fig. 2. Hsp33's C-terminus is predicted to be natively unfolded in the absence of cofactors. To predict the folded *versus* unfolded regions of Hsp33, the amino acid sequence of full length Hsp33 was used in the FoldIndex prediction program [1] . The folded regions are represented in green whereas the unfolded regions are represented in red. This method is based on the average of hydrophobic amino acids present and on the absolute value of the net charge. They do not account for cofactor binding. A window size of 51 residues was used to avoid false positives (for more details see [2]).

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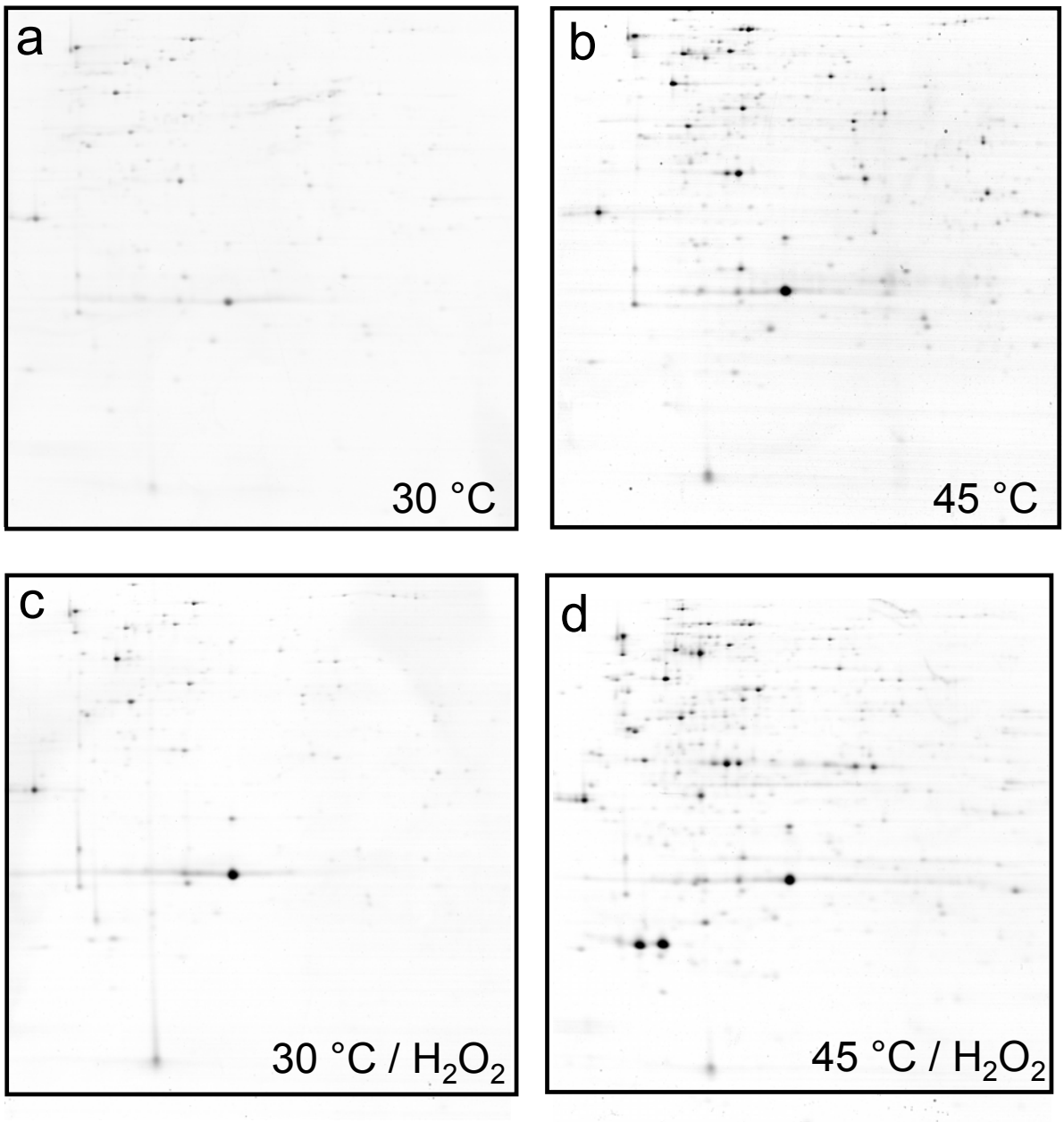

Supplementary Fig. 3. Peroxide stress does not induce protein aggregation *in vivo*. Δ*rpoH* cells were incubated at 30ºC (**a**, **c**) or 45ºC (**b, d**) in either the absence (**a, b**) or presence of 4 mM H_2O_2 (**c, d**) for 40 min. Aggregated proteins were isolated, separated on 2D gels and visualized with colloidal Coommassie.

	Calculated α-helix content according to X-ray [%]		Predicted α-helix content according to CD [%][a]
Zinc bound folded protein	34.7	$Hsp33_{red}$	37
Unfolding of zinc center (α8 - 10)	24.5	$Hsp33_{ox30°C}$	30
Unfolding of complete C-terminal domain (α5 - 10)	18.7	$Hsp33_{ox43°C}$	19

Supplementary Table 1. α-helix content in reduced and oxidized Hsp33. The crystal structure of Hsp33 was used to calculate the percentage of alpha helices in reduced Hsp33 [1]. Unfolding of the zinc-center would affect α-helices 8 to 10, while unfolding of the complete C-terminal domain would affect α-helices 5 to 10. [a]The CD spectra presented in Fig. 3 were used to calculate the percent α-helices in $Hsp33_{red}$, $Hsp33_{ox30°C}$ and $Hsp33_{ox43°C}$ using the prediction program K2D [2].

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2. Andrade, M.A., Chacon, P., Merelo, J.J. & Moran, F. Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. *Protein Eng* **6**, 383-90 (1993).

SUPPLEMENTARY DATA

Oxidation at 30°C is insufficient to form correct oxidative thiol modifications and dimers

AMS-thiol trapping experiments were performed to monitor the oxidation state of Hsp33's thiol groups upon incubation in 2 mM H_2O_2 at 30°C or 43°C. AMS is a thiol reactive agent, which reacts with accessible thiols and leads to the addition of 490 Da per reduced thiol group. We found that incubation of Hsp33 in H_2O_2 leads to the disappearance of the reduced Hsp33 species and the simultaneous appearance of several faster migrating bands, which correspond to the oxidized species (**Supplementary Fig. 1**). We furthermore observed that the kinetics of these oxidation processes are very similar to the kinetics of zinc release **(Fig. 2b**). Interestingly, however, the oxidized species that develops during the H_2O_2 incubation at 30°C reveals a migration pattern on SDS PAGE, which is distinct from the pattern observed at 43°C. These results indicate that incubation at 30°C leads to oxidation processes on Hsp33's cysteines but suggest that either the number of modified cysteines or their specific oxidative modifications might differ from those occurring upon incubation at 43°C.

We have shown previously that oxidation of Hsp33 at 43°C leads to the non-covalent association of two oxidized Hsp33 monomers and the formation of highly chaperone-active Hsp33 dimers [1]. A small subpopulation of these active Hsp33 dimers appear to undergo additional intermolecular disulfide bond formation upon prolonged incubation at 43°C, which cannot be resolved on non-reducing SDS-PAGE (**Supplementary Fig. 1)**. This has been observed before and involves the non-conserved Cys239 [2]. Analysis of the $Hsp33^{C141D/C239S}$ mutant protein, which lacks both non-conserved cysteines Cys^{141} and

Cys^{239} revealed that this mutant protein behaves indistinguishable from wild type Hsp33 but does not accumulate any disulfide-linked dimers (data not shown). These experiments excluded that disulfide-linked Hsp33 dimers play a significant role in the activation process of Hsp33, a finding that agreed with *in vivo* studies where no disulfide-linked Hsp33 dimers were detected under oxidative heat stress conditions.

Oxidation of Hsp33 at 30°C in H_2O_2, on the other hand, does not lead to the formation of any disulfide-linked Hsp33 dimers (**Supplementary Fig. 1)**, suggesting that $Hsp33_{ox30°C}$ might be unable to dimerize. Ultracentrifugation studies confirmed this finding and showed that $Hsp33_{ox30°C}$ sediments as monomer while $Hsp33_{ox43°C}$ sediments as dimer (data not shown).

Peroxide stress at non-stress temperatures does not cause protein aggregation in vivo

To investigate to what extent cells exposed to peroxide stress accumulate protein folding intermediates that require a potent chaperone such as Hsp33, we investigated protein aggregation in oxidatively stressed *E. coli* cells at 30°C and 45°C. In order to observe the highest possible extent of protein aggregation, we conducted these experiments in sigma 32 deletion strains (Δ*rpoH*), which are unable to respond to stress conditions with the expression of heat shock proteins and have significantly lower concentrations of most *E. coli* chaperones [3]. We treated Δ*rpoH* cells with 4 mM H_2O_2 at either 30°C or 43°C and analyzed the insoluble protein aggregates 40 min after start of the treatment by 2D gel analysis (**Supplementary Fig. 3**). At 45°C, oxidative stress aggravated protein aggregation induced by elevated temperatures alone and led to the accumulation of a considerable amount of protein aggregates. At non-stress

temperatures, however, H_2O_2-induced oxidative stress apparently lacks the ability to unfold proteins as no significant increase in protein aggregation was observed in H_2O_2 treated cells as compared to cells grown at 30°C in the absence of oxidants (**Supplementary Fig. 3**). These results suggest that strains that are exposed to high concentrations of H_2O_2 at non-stress temperatures do not suffer greatly from protein unfolding and aggregation and apparently do not require the presence of a potent chaperone like Hsp33 for protection. The combination of oxidative stress and unfolding conditions, i. e. elevated temperatures, however, leads to the accumulation of unfolding proteins and requires the presence of Hsp33 to protect the proteins against non-specific aggregation [4].

References:

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SUPPLEMENTARY METHODS

Plasmid Construction- To generate the Hsp33$^{F187W/W212F}$ and Hsp33$^{W212F/Y267W}$ mutant variants, the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene) was performed using a pET11a vector containing wild type Hsp33 (pUJ30) as template [1]. The plasmids pHJ4 (pET11a/Hsp33$^{F187W/W212F}$) and pHJ5 (pET11a/ Hsp33$^{W212F/Y267W}$) were transformed into JH13, a BL21 strain containing an insertion in the Hsp33 encoding gene *hslO* [1], thereby generating the expression strains HJ10 and HJ11, respectively. The C-terminal truncation mutant Hsp33^{N1-235} was obtained using standard PCR methods. The coding sequence was inserted into a pET11a overexpression plasmid, generating the plasmid pPG9. For protein expression, pPG9 was transformed into JH13, generating strain PG25. All inserts were confirmed by sequencing.

Analysis of the thiol status of Hsp33-The extent of thiol modifications in Hsp33 was determined under denaturating conditions by using the alkylating agent 4-acetamido-4'-maleimidylstilbene-2'-disulfonate (AMS) (Molecular Probes). At defined time points during the H_2O_2 oxidation of 50 µM Hsp33 at either 30°C or 43°C, aliquots were removed and thiol-disulfide exchange reactions were stopped by the addition of an equal volume of ice-cold 20% trichloroacetic acid (TCA). After 20 min incubation on ice, the protein was pelleted by centrifugation at 16 000× g at 4°C and TCA was removed. The protein pellet was resuspended in 25 µl of 15 mM AMS in DAB buffer (6 M urea, 200 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% SDS) and incubated for 1 h at 25°C in the dark under vigorous shaking. The samples were then supplemented with non-reducing Laemmli buffer and analyzed on 14% SDS-PAGE (Novex).

Preparation of samples for 2D gels

An overnight culture of MC4100 *ΔrpoH* (JW49) was grown in Luria Broth (LB) was diluted 1:100 into fresh LB medium and cultivated at 30^{o}C until an OD_{600} of 0.5 was reached. Cultures were then diluted 1:20 into fresh LB medium, which was pre-warmed at either 30°C or 45°C in the absence or presence of 4 mM H_2O_2. After 40 min of incubation, cells from 30 ml cultures were harvested and aggregated proteins were isolated and analyzed by 2D gel electrophoresis as previously described [2].

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