The anti-sigma factor RsrA responds to oxidative stress by reburying its hydrophobic core

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Redox-regulated effector systems that counteract oxidative stress are essential for all forms of life. Here we uncover a new paradigm for sensing oxidative stress centred on the hydrophobic core of a sensor protein. RsrA is an archetypal zinc-binding anti-sigma factor that responds to disulfide stress in the cytoplasm of Actinobacteria. We show that RsrA utilizes its hydrophobic core to bind the sigma factor \( \sigma^R \) preventing its association with RNA polymerase, and that zinc plays a central role in maintaining this high-affinity complex. Oxidation of RsrA is limited by the rate of zinc release, which weakens the RsrA-\( \sigma^R \) complex by accelerating its dissociation. The subsequent trigger disulfide, formed between specific combinations of RsrA’s three zinc-binding cysteines, precipitates structural collapse to a compact state where all \( \sigma^R \)-binding residues are sequestered back into its hydrophobic core, releasing \( \sigma^R \) to activate transcription of anti-oxidant genes.
All organisms must contend with the toxic effects of reactive oxygen species (ROS), which include superoxide anion ($\text{O}_2^-\text{H}^+$), hydrogen peroxide ($\text{H}_2\text{O}_2$) and the hydroxyl radical ($\text{OH}^-$), that covalently damage proteins, lipids and DNA. ROS are by-products of aerobic metabolism, which in mammals are implicated in the ageing process and diseases such as type 2 diabetes. To minimize the build-up of disulfide bonds, one of the toxic consequences of ROS, organisms maintain a reducing cytoplasm through the production of millimolar concentrations of small-molecule reducing agents such as glutathione. A second line of defence comprises detoxification enzymes that decompose ROS and the glutaredoxin/thioredoxin system of redox proteins that reduce cytoplasmic disulfide bonds. Oxidative stress sensor proteins that lead to the activation of anti-oxidant genes form a third line of defence for maintaining redox homeostasis. Sensor proteins are typically transcription factors or transcription factor inhibitors that contain reactive cysteine or cytoskeletal targets that are directly modified by ROS. Here we focus on the disulfide stress sensor protein RsrA from Streptomyces coelicolor, which, in its resting state, blocks binding of the sigma factor $\sigma$ to RNA polymerase (Fig. 1). RsrA is a zinc-binding anti-sigma factor (ZAS) protein, the prototypical member of a family of inhibitors of extracytoplasmic function (ECF) sigma factors that regulate bacterial responses to diverse stresses. As yet, no molecular mechanism has been described for the stress-induced inactivation of any ZAS protein. We detail the mechanism by which RsrA responds to oxidation, releasing $\sigma$ to mount the cellular anti-oxidant response.

ZAS proteins were originally identified by their His$_{XX}$Cy$_{YY}$Cys sequence motifs. They share <30% sequence identity, but are readily identified in bacterial genomes by their genomic location, downstream of an ECF (group IV) sigma factor. ZAS proteins are further sub-divided by the identity of the fourth zinc coordination site, which is either a cysteine or histidine residue 23–26 amino acids N-terminal to the His$_{XX}$Cy$_{YY}$Cys motif (hereafter these two types of ZAS motifs are denoted as CHCC or HHCC, respectively), and if they contain an additional domain or transmembrane region. RsrA is a soluble, single domain, CHCC-type ZAS motif protein, while its parologue ChrR from the photosynthetic bacterium Rhodobacter sphaeroides is a HHCC-type ZAS motif protein, which has an additional cupin-like domain. ZAS proteins respond to different stimuli, inducing them to release their cognate sigma factor to activate regulons that respond to the stress. Homologues of the RsrA–$\sigma$ complex are found throughout the actinomycetes, including Mycobacterium tuberculosis, where the system has been shown to be important for pathogenesis, and Corynebacterium diphtheriae. In the case of S. coelicolor, $\sigma$ is a global transcriptional regulator, activating a regulon of >100 genes that includes anti-oxidant genes (Fig. 1). ChrR by contrast senses singlet oxygen stress, a toxic ROS by-product of photosynthesis. Release of its sigma factor, $\sigma^R$ (also known as RpoE), results in the increased production of carotenoids that quench the singlet oxygen radical. RsIW from Bacillus subtilis is a membrane-bound ZAS protein that is proteolytically degraded following cell envelope stress through the action of antibiotics such as vancomycin, releasing its ECF sigma factor to activate a regulon for the detoxification of and protection against antimicrobials. Structures for two ZAS proteins bound to their cognate ECF sigma factors have been reported, the intact ChrR–$\sigma^E$ complex and the isolated ZAS domain of RsA from M. tuberculosis bound to one of the two domains of $\sigma^F$ (ref. 22). No structure has yet been reported for any ZAS protein in the absence of its sigma factor or in an inactivated state following stress-induced dissociation.

RsrA is a 105 amino-acid protein that contains seven cysteines. Three of the cysteines, Cys11, Cys41 and Cys44, contribute to the CHCC ZAS motif and are essential for redox sensing in vivo and in vitro. Zdanowski et al. showed using extended X-ray absorption fine structure spectroscopy that all three cysteines, along with His37, also within the ZAS motif, coordinate a single zinc ion in both RsrA and the RsrA–$\sigma$ complex. Oxidation of RsrA is known to result in the loss of zinc and formation of a degenerate trigger disulfide bond, formed between Cys11 and either of Cys41 or Cys44, which blocks $\sigma$ binding. However, the involvement of the metal ion in redox sensing remains enigmatic. Here we uncover this role and its structural basis. As well as revealing a new mechanism by which an oxidative stress sensor protein responds to the changes in cellular redox status, this study also lays the foundations for understanding how ZAS proteins function as generic stress sensors.

**Results**

**Stoichiometry of zinc binding to RsrA.** We first re-assessed the stoichiometry of zinc binding to the wild-type protein and a mutant in which the four non-essential cysteines (Cys3, Cys31, Cys32 and Cys34) were replaced with alanine. We found that the wild-type protein binds two zinc ions, while the mutant binds one. This suggests that the stabilising effect of the additional zinc ions is essential for the protein’s ability to sense redox changes.

**Figure 1 | Scheme showing redox homeostasis loop for the RsrA–$\sigma$ complex.** The figure highlights the zinc coordination residues in reduced RsrA (RsrA$\text{red}$, $\text{Zn}^{2+}$) from Streptomyces coelicolor. Disulfide stress results in the loss of zinc and formation of a degenerate trigger disulfide bond in RsrA$\text{red}$, formed by the same zinc-binding residues. The transcribed regulon of $\sigma$ includes anti-oxidant genes that re-establish redox homeostasis and the genes for sigR and rsrA (not shown), which amplify the response. Not shown is an additional layer of regulation involving a form of $\sigma$ with an N-terminal extension that also binds RsrA, but is rapidly degraded by proteolysis. Shaded panels denote NMR structures of RsrA reported in the present work.
Cys61 and Cys62) were mutated to alanine (RsrA*). See the Methods section for details. Although multiple zinc ions can bind to reduced RsrA and RsrA*, only a single zinc stabilizes the protein fold (Supplementary Fig. 1) and, as detailed below, modulates redox activity. We refer to this form as RsrA\(_{\text{red}}\).Zn\(^{2+}\).

**Redox potential of RsrA\(_{\text{red}}\).Zn\(^{2+}\).** A key question for an oxidative stress sensor is its redox potential, as this will govern its reactivity towards oxidants. No such measurements have been reported for any ZAS protein. We therefore determined the redox potential for RsrA\(_{\text{red}}\).Zn\(^{2+}\) in complex with \(\sigma^R\) in reference to a glutathione redox couple. The status of the complex in these experiments was monitored by tryptophan emission fluorescence spectroscopy, where we exploited a significant change in \(\sigma^R\) fluorescence that occurs on forming its complex with RsrA (Fig. 2a). The oxidation status of RsrA\(_{\text{red}}\).Zn\(^{2+}\) was determined spectrophotometrically by the stoichiometric release of zinc using 4-(2-pyridylazo) resorcinol (PAR; see Methods). The two data sets were in excellent agreement (Fig. 2b) and showed the redox potential for RsrA\(_{\text{red}}\).Zn\(^{2+}\) in complex with \(\sigma^R\) to be \(-193.04 \pm 2.01\) mV. Our data show that the redox potential of RsrA\(_{\text{red}}\).Zn\(^{2+}\) is ideally poised to act as a redox sensor. Its redox potential is close to that estimated for the bacterial cytoplasm (Fig. 2c), rendering it sensitive to small changes in the redox status of the cell.

**Role of zinc in modulating \(\sigma^R\) binding and oxidation of RsrA.** Using isothermal titration calorimetry (ITC), we determined that RsrA\(_{\text{red}}\).Zn\(^{2+}\) binds \(\sigma^R\) with sub-nanomolar affinity (Fig. 3a), whereas RsrA devoid of zinc-bound \(\sigma^R\) 100-fold more weakly (Fig. 3b). Zinc-associated RsrA\(^*\)-bound \(\sigma^R\) with a similar affinity to wild-type RsrA, while mutation of any zinc-binding cysteine residue decreased \(\sigma^R\) binding by >100-fold (Supplementary Table 1). We conclude that the RsrA\(_{\text{red}}\).Zn\(^{2+}\)–\(\sigma^R\) complex has a much higher affinity than previously reported\(^{11,28}\), which is dependent on zinc being bound at the ZAS motif and explains why zinc limitation activates the \(\sigma^R\) regulon in *S. coelicolor*\(^{29}\).

We next determined the kinetic basis for zinc stabilization of the complex. Stopped-flow tryptophan fluorescence under pseudo-first-order conditions showed that the association rate constant for the RsrA\(_{\text{red}}\).Zn\(^{2+}\) complex was only marginally affected by bound zinc, in contrast to the dissociation rate constant that was accelerated 400-fold when zinc was removed (Fig. 3c,d). Importantly, the kinetically derived \(K_d\) for the RsrA\(_{\text{red}}\).Zn\(^{2+}\)–\(\sigma^R\) complex at 35°C closely matched that obtained by ITC (Supplementary Table 2), demonstrating that the kinetic mechanism can simply be described by single association and dissociation rate constants. The kinetically derived \(K_d\) at 25°C (for which a value could not be obtained by ITC) was approximately twofold lower than that at 35°C, with zinc having very similar effects on the kinetics of binding (Supplementary Table 2). We conclude that the single ZAS motif zinc ion of RsrA stabilizes the high-affinity complex with \(\sigma^R\) by slowing the dissociation rate of the complex.

RsrA is thought to be primarily a sensor of deleterious disulfide bond formation within the *S. coelicolor* cytoplasm, since oxidation by the thiol-specific oxidizing agent diamide is a stronger inducer of \(\sigma^R\)-dependent promoters than hydrogen peroxide\(^{10}\). We therefore determined pre-steady-state oxidation rates of...
RsrA^{red}\cdot Zn^{2+}\) in complex with \(\sigma^R\) to probe the kinetic basis for oxidation, initially using diamide to induce disulfide bond formation within RsrA (Fig. 4a,b; see Methods). We developed a stopped-flow spectrophotometric assay to follow RsrA^{red}\cdot Zn^{2+}\) oxidation, albeit indirectly, by exploiting the absorption changes of diamide on reduction to hydrazine (Supplementary Fig. 2a)\(^3^0\). Zinc release was monitored using the PAR assay (Fig. 4a). These data showed that the bimolecular rate constant for zinc release is the same as that of diamide reduction (\(\sim 190 \text{ M}^{-1}\text{ s}^{-1}\)), suggesting that zinc release limits the rate of oxidation. This was confirmed by removing zinc from the protein, which accelerated the oxidation rate fivefold (Fig. 4a). Identical results were obtained for RsrA^{red}\cdot Zn^{2+}\) in the absence of \(\sigma^R\), demonstrating that complex formation does not influence the kinetics of oxidation (Supplementary Fig. 2c). Moreover, the release of zinc with increasing diamide concentration showed the oxidant formed a weak intermediate complex (\(K_i \sim 0.7 \text{ mM}\); Fig. 4b), most likely the sulfenyl hydrazine (Supplementary Fig. 2a)\(^3^0\).
Figure 4 | Zinc release is the rate-limiting step in RsrA oxidation. Experiments were conducted at 25°C in 50 mM Tris (pH 7.5) buffer containing 100 mM NaCl. (a) Oxidation of the RsrA-red.Zn2+ complex on treatment with diamide under second-order conditions (25 μM). The fraction of reduced RsrA was determined by the change in diamide absorbance at 320 nm (Supplementary Fig. 2a) in the presence and absence of bound zinc. Zinc release was monitored at 500 nm using the PAR assay. The two methods showed good agreement for the bimolecular rate constant for diamide-induced oxidation of RsrA-red.Zn2+ (0.39 ± 0.15 s⁻¹, 183 ± 6 and 195 ± 11 M⁻¹s⁻¹, respectively). (b) Zinc release from the RsrA-red.Zn2+–red.Zn2+ complex (2 μM) on treatment with increasing concentrations of diamide under pseudo-first-order conditions monitored by the PAR assay; 25 μM (filled diamonds), 50, 100, 150 and 200 μM (open diamonds). Inset shows variation of kobs with diamide concentration (with associated error bars). Data were fitted to the Michaelis–Menten equation, with fitted parameters of Kₘ = 0.7 mM and kₗ = 0.15 s⁻¹ (Supplementary Fig. 2b). The corresponding bimolecular rate constant (kₒ/kₗ = 214 M⁻¹s⁻¹) is in reasonable agreement with values obtained in a. (c) Oxidation-induced dissociation of the RsrA-red.Zn2+–red.Zn2+ complex (2 μM) monitored by tryptophan fluorescence on treatment with H₂O₂ under pseudo-first-order conditions. Three H₂O₂ concentrations are shown as follows: 1 mM (triangles), 2 mM (open circles) and 6 mM (closed circles). Inset, pseudo-first-order plot (with associated error bars) from which the bimolecular rate constant for H₂O₂-induced dissociation of the complex was obtained (0.39 ± 0.08 M⁻¹s⁻¹). (d) Zinc release from the RsrA-red.Zn2+–red.Zn2+ complex (2 μM) on treatment with increasing concentrations of H₂O₂ (2–10 mM) under pseudo-first-order conditions. Inset: pseudo-first-order plot (with associated error bars) from which the bimolecular rate constant for the H₂O₂-induced zinc dissociation was obtained (0.32 ± 0.06 M⁻¹s⁻¹).

Fig. 2a,b), before formation of the trigger disulfide bond. Importantly, the kinetic analysis showed that the first-order rate constant (kₒ) for decomposition of this intermediate complex is 0.15 s⁻¹ at 25°C. This rate approaches the intrinsic dissociation rate of the RsrA–σR complex in the absence of zinc under the same conditions (~0.3 s⁻¹; Supplementary Table 2), which is consistent with oxidation increasing the dissociation rate of the complex by driving out bound zinc.

The absorbance of diamide precluded monitoring dissociation of the RsrA–σR complex directly by fluorescence spectroscopy. This was however possible using H₂O₂ as an oxidant, where the release of zinc could also be monitored (Fig. 4c,d). Although the bimolecular rate constant for oxidation by H₂O₂ was 500-fold slower than that of diamide (emphasizing that RsrA is a sensor of disulfide rather than peroxide stress) here again zinc release was rate limiting for oxidation and complex dissociation. Importantly, excess zinc had no effect on the rates of oxidation either by diamide or H₂O₂ (Supplementary Fig. 3b,c), demonstrating that additional zinc ions beyond that bound in the ZAS motif play no role in redox sensing by RsrA. In conclusion, our kinetic data demonstrate that stoichiometric zinc release is the rate-limiting step for RsrA oxidation by different oxidants, which leads to accelerated dissociation of the RsrA–σR complex and formation of the trigger disulfide bond (a full kinetic scheme is shown in Supplementary Fig. 3d).

Cys11 is essential for redox sensing in RsrA. Heo et al. have suggested that the differences in redox sensitivity of different ZAS proteins are due to the differences in electronegative residues and binding of alternative zinc ions. However, as our data above illustrate, additional zinc ions play little or no role in the redox-sensing ability of RsrA. A simpler explanation for whether ZAS proteins react to disulfide stress is whether the N-terminal zinc-coordinating residue is a cysteine, as in the case of RsrA where Cys11 forms the trigger disulfide with either Cys41 or Cys44. HHCC-type ZAS proteins such as ChrR and RsiW do not have this additional cysteine and do not sense disulfide stress. This hypothesis is confounded however by the recent study of Thakur et al. who reported that on oxidation with hydrogen peroxide, the HHCC ZAS protein RslA from M. tuberculosis forms a disulfide bond, expelling the bound zinc and increasing the dissociation rate of the RslA–σR complex. We therefore set out to determine how different zinc ligation chemistries influence oxidative stress sensing, using the RsrA–σR complex as a model. We generated four cysteine mutants in RsrA*: RsrA* Cys11His Cys41His Cys44His, in which each of the zinc-coordinating cysteines was individually mutated to histidine and RsrA* Cys11His Cys41His Cys44His, in which all the coordinating cysteines were simultaneously replaced with histidine. The latter mutant was used as a non-oxidative control. The Cys-to-His mutations all bound σR with lower
Figure 5 | Cys11 is essential for redox sensing in RsrA. Zinc ligation for each RsrA construct used is shown as a schematic. (a) Comparing the intrinsic dissociation rates of $\sigma^R$ complexes with RsrA$^{\text{red}, \text{Zn}}^2+$ (open triangles; $k_{\text{on}} = 0.0027 \text{s}^{-1}$) and RsrA$^{\text{red}, \text{Zn}}^2+$ Cys11His (closed triangles; $k_{\text{on}} = 0.064 \text{s}^{-1}$) obtained by competition stopped-flow tryptophan emission fluorescence using a 10-fold excess of $\sigma^R$ Trp88Ile Trp119Ile. All experiments were conducted at 25 °C in 50 mM Tris (pH 7.5) buffer containing 100 mM NaCl. Inset: dissociation data in the presence (closed circles) and absence (open circles) of zinc for $\sigma^R$ in complex with triple mutant RsrA$^*$ Cys11His Cys41His Cys44His (Fig. 5a; Supplementary Table 1). (b) Comparison of H$_2$O$_2$ (10 mM)-induced dissociation of the RsrA$^{\text{red}, \text{Zn}}^2+$ Cys11His$\sigma^R$ complex (open circles) with RsrA$^{\text{red}, \text{Zn}}^2+$ Cys11His$\sigma^R$ complex (open circles) and RsrA$^{\text{red}, \text{Zn}}^2+$ Cys11His Cys41His Cys44His (closed triangles) complexes. Data for complexes of RsrA$^*$ Cys41His and RsrA$^*$ Cys44His, shown in Supplementary Fig. 4, were essentially identical to RsrA$^*$. The absence of Cys11 renders RsrA insensitive to oxidation-induced dissociation from its complex with $\sigma^R$. (c) Upper panel: native state mass spectrometry data for RsrA$^{\text{red}, \text{Zn}}^2+$ Cys11His in complex with $\sigma^R$ showing the predominance of the zinc-bound species (theoretical/observed mass, 40,128.08/40,061.96 ± 0.28 Da, respectively). The minor species was the reduced complex without zinc bound (theoretical/observed mass, 40,062.69/40,061.96 ± 0.28 Da, respectively). Lower panel: the same complex following treatment with 10 mM H$_2$O$_2$. Although RsrA$^*$ Cys11His remains in complex with $\sigma^R$ the metal ion has dissociated and the remaining cysteines (Cys41 and Cys44) have formed a disulfide bond, as deduced by the ~2 Da reduction in mass (theoretical/observed mass, 40,062.70/40,061.96 ± 0.28 Da, respectively).

We further analysed RsrA$^*$ Cys11His (equivalent to a HHCC motif ZAS protein) in complex with $\sigma^R$ using high-resolution native state nanoelectrospray mass spectrometry to determine the oxidation state of this HHCC-type ZAS protein (Fig. 5c). Under reducing conditions, the RsrA$^*$ Cys11His$\sigma^R$ complex bound 1 equiv. of zinc with only a small fraction of apo-complex present. Upon H$_2$O$_2$-induced oxidation of the complex, a mass shift to lower $m/z$ indicated both the loss of zinc and formation of a disulfide bond between Cys41 and Cys44. However, this did not result in oxidation-induced dissociation of the complex (Fig. 5c). These data suggest that oxidative dissociation of a ZAS protein from its target sigma factor requires an N-terminal zinc-coordinating cysteine residue within the ZAS motif (CHCC).
RsrA exposes its hydrophobic core to bind σR. To understand how RsrA associates with σR, we determined the structure of zinc-bound RsrA in its reduced state (RsrAred.Zn2+) and compared this with a homology model of the RsrAox.Zn2+–σR complex. A modelling approach was employed because repeated attempts at crystallization of the RsrAred.Zn2+–σR complex failed to yield diffracting crystals and solution spectra of 2H, 13C, 15N-labelled RsrAred.Zn2+–σR complex were poorly resolved. The model was based on previous structures of anti-sigma factor–sigma factor complex and constrained by bifunctional lysine–specific crosslinking data (Supplementary Fig. 5). Following testing of RsrA mutants for optimal spectral resolution (including RsrA*), the nuclear magnetic resonance (NMR) solution structure of RsrAred.Zn2+–σR complex was obtained using the mutant RsrA* Cys41Ser (which contains both Cys11 and Cys44) bound to 1 equiv. of zinc. As for the wild-type protein, 1H–15N-HSQC (heteronuclear single quantum coherence) NMR spectra showed that this mutant required stoichiometric zinc for its stabilization (Supplementary Fig. 6a). Although RsrA* Cys41Ser likely binds zinc more weakly than wild-type RsrA, leading to weakened σR binding (Supplementary Table 1), at the protein and zinc concentrations used for NMR structure determination (≈mM) the protein is folded and bound to zinc. Only residues 45–47 in RsrA* Cys41Ser could not be assigned by heteronuclear NMR experiments. In the final structure, the N and C termini, a loop between residues 63 and 72, and residues 42 and 50 were poorly resolved, all other residues (8–86) were well defined. Heavy-atom root mean square deviations (r.m.s.d.’s) for backbone atoms of the secondary structure elements in the 10 overlays of RsrAred.Zn2+ shown in Fig. 6b was 0.48 Å. NMR structure statistics are shown in Table 1.

RsrAred.Zn2+ (Fig. 6b) forms a loosely packed four-helix bundle composed of two sets of roughly parallel helices (I–II, residues 11–23 and 29–39; and III–IV, residues 51–60 and 71–84) connected by loops. The two N-terminal helices are tilted ~45° relative to the C-terminal helices. His37 of the conserved ZAS metal-binding motif is presented from the C-terminal end of helix II, while Cys41Ser and Cys44 are part of the long loop connecting helices II and III. Cys11, the fourth metal ligand, is at the N-terminal end of helix I. The co-localization of the four metal ligands was confirmed by the observation of inter-residue nuclear overhauser effect (NOEs); distance restraints were used during initial structure calculations and restraints specifying the tetrahedral Zn2+ ligation geometry were introduced in the latter stages of refinement (Methods).

On binding σR RsrAred.Zn2+ adopts a characteristic anti-sigma-binding domain (ASD) fold, which was first described for the ChrR–σE complex13 (Fig. 7b). The two key features of the modelled complex are the binding of RsrAred.Zn2+ between the two domains of σR (σ2 and σ4) and the embrace of the sigma factor by the C-terminal helix (helix IV) of RsrAred.Zn2+. Our crosslinking data suggest however that helix IV does not adopt a single conformation as in the ChrR–σE complex, but can likely contact both σ2 and σ4 domains of σR (Supplementary Fig. 5c). For the purposes of the following analysis, we focus only on the form of the complex in which helix IV is docked onto the σ2 domain.

Comparison of the structure of free RsrAred.Zn2+ with the σR-bound state reveals significant structural reorganization of the anti-sigma factor while maintaining its zinc coordination geometry (compare Fig. 7a,b). (1) The four-helix bundle structure of RsrAred.Zn2+ converts to the three-helix ASD fold. This involves helix III changing its orientation by ~90°, helix II by ~30° relative to helix I and helix IV dissociating from the main body of the protein. (2) Helix III in RsrAred.Zn2+ approximately doubles in length. The residues comprising this extension were originally the long loop connecting helices II and III in RsrAred.Zn2+. As a consequence, Cys44 of the ZAS motif becomes part of helix III, while Cys41 sits between helices II and III. The other ZAS motif residues remain within their original secondary structure elements. The orientation of the extended helix III is dictated by Cys44’s role in zinc coordination, emphasizing the importance of zinc in σR binding as it allows

Figure 6 | Solution structures of RsrAred.Zn2+ and RsrAox. (a) Comparison of 1H–15N-HSQC spectra of reduced RsrA* Cys41Ser (RsrAred–Zn2+, blue peaks), in 20 mM Tris buffer (pH 7.1) containing 5 mM DTT and 2 mM ZnCl2, with RsrAox (red peaks), which is the same protein in the same buffer but in the absence of reductant and metal ions. (b) Overlay of the 10 lowest-energy structures for RsrAred.Zn2+ (residues Glu8–Gln86; left-hand figure) and a ribbon diagram of the lowest-energy structure (right-hand figure), showing the location of the zinc-binding residues (His37, blue; Cys41Ser, cyan; Cys11 and Cys44, yellow) and the zinc atom (blue). (c) Overlay of the 10 lowest-energy structures for RsrAox (residues Glu8–Gln86; left-hand figure) and a ribbon diagram of the lowest-energy structure (right-hand figure), showing the location of the disulfide bond and disruption of the metal site following oxidation (residues and helices are coloured as in b).
the hydrophobic core in the free RsrAred.Zn2
(Leu50, Ala53, Val54 and Leu57), which were originally part of Exposed hydrophobic residues in the C-terminal half of helix III with
the body of RsrA allowing the anti-sigma factor to wrap around The telescopic extension of helix III projects helix IV away from hydrophobic core of RsrARed-Zn2
that were part of the loop between helices II and III in the
residues in RsrARed-Zn2
Val75 and Leu79, which were peripheral hydrophobic core in its
state, now make contact with the sigma
RsrA sequesters its \(\sigma^R\)-contacting residues on oxidation. We next determined the structure of oxidized RsrA (RsrAox) to understand how this blocks \(\sigma^R\) binding. We first ascertained which of the two oxidized forms of the trigger disulfide predominate at equilibrium (Supplementary Figs 6 and 7). These experiments focused on RsrA*, which behaves as a redox sensor \textit{in vivo}\(^{14}\) and \textit{in vitro} (Fig. 5b). RsrA* Cys11–Cys44 was found to be the most populated oxidized state. We therefore determined the solution structure of oxidized RsrA* containing the Cys11–Cys44 disulfide bond. Cys41 was mutated to serine in this construct to remove the potential for mixed disulfide bond formation (Methods). As with RsrAred.Zn2\(^{2+}\), the N termini of RsrAox were disordered and the loop between residues 63 and 72 poorly defined. Heavy-atom r.m.s.d.'s for the top 10 solution structures of residues 8–86 in RsrAox were 0.38 Å (Fig. 6c; Table 1 for structure statistics).

\(^1\)H-\(^{15}\)N-HSQC spectra of RsrAred.Zn2\(^{2+}\) and RsrAox are substantially different suggestive of distinct folds (Fig. 6a). This is confirmed by the structure of RsrAox, which is more helical than RsrAred.Zn2\(^{2+}\) (Fig. 6b,c). RsrAox is also more compact than RsrAred.Zn2\(^{2+}\), with 13% less solvent accessible surface area (RsrAred.Zn2\(^{2+}\), 2,608 ± 28 Å\(^2\) and RsrAox, 2,270 ± 33 Å\(^2\) for residues 8–86. Helices I (residues 13–24) and II (residues 31–40) are pulled closer together by the disulfide between Cys11 and Cys44, and helices III\(^C\) (50–60) and IV (70–84) reorient to become roughly parallel to those of helices I and II. An additional short helix (helix III\(^P\); residues 42–48), perpendicular to the other

### Table 1 | NMR and refinement statistics for the structures of RsrAox and RsrAred.Zn2\(^{2+}\).

|                  | RsrAox  | RsrAred.Zn2\(^{2+}\) |
|------------------|---------|----------------------|
| **NMR distance and dihedral constraints** |         |                      |
| Distance constraints |       |                      |
| Total NOE         | 1,532  | 1,033                |
| Intra-residue     | 749    | 577                  |
| Inter-residue     | 660    | 422                  |
| Sequential \(\langle | 333    | 227                  |
| Medium range \(1< | 218    | 108                  |
| Long range \(|>5|) | 109    | 87                   |
| Hydrogen bonds    | 0      | 0                    |
| RDC-based restraints | 40    | 29                   |
| Total dihedral angle restraints \(\phi/\psi\) | 100   | 104                  |
| **Structure statistics** |       |                      |
| Violations        |         |                      |
| Distance constraints \(>0.5\) Å | 0      | 0                    |
| Dihedral angle constraints \(>5\)° | 0      | 0                    |
| Max. dihedral angle violation \(°\) | 4.7    | 4.6                  |
| Max. distance constraint violation (Å) | 0.44   | 0.46                 |
| Deviations from idealized geometry (mean and s.d.) |         |                      |
| Bond lengths (Å)  | 0.005 ± 0.0001 | 0.004 ± 0.00007    |
| Bond angles (°)   | 0.63 ± 0.01  | 0.49 ± 0.01          |
| Average pairwise r.m.s. deviation \(\star\) (Å) | 0.84 ± 0.11 | 0.82 ± 0.10          |
| Heavy             | 0.34 ± 0.07  | 0.40 ± 0.14          |
| Backbone          |         |                      |
| **Ramachandran statistics** |       |                      |
| Residues in mostfavoured regions/additional | 93.5%  | 97.7%                |
| Residues in generously allowed regions | 5.3%   | 2.1%                 |
| Residues in disallowed regions | 1.0% †  | 0                    |

RDC, residual dipolar coupling.
* Averaged over secondary structure of 10 lowest-energy structures.
† None were well-defined residues.

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**Note:** The text is a summary of the research findings related to the structure and function of RsrA, focusing on the structural changes induced by oxidation and the role of hydrophobic residues in sigma factor binding and release.
helices and stabilized by the disulfide, takes the place of the ZAS metal-binding site. Indeed, residues comprising helix III in RsrAox are equivalent to those in the σR-bound state of RsrA, but now the helix is broken into two segments, a 90° one-residue-turn connecting helices IIIa and IIIc (Fig. 7b–d).

These changes have three major consequences. (1) The zinc-binding site of RsrAred.Zn2+ is obliterated; this is most readily appreciated by the distance between NE2 atom of His37 and the S atom of Cys11 (15 Å; Fig. 6b,c). (2) The movement of ZAS ligands away from the metal-binding site is brought about by a change in register of helix II relative to the other helices due to a rotation around the helix axis. (3) The trigger disulfide bond constrains the orientations of helices I and II along with IIIa and IIIc, resulting in wholesale repacking of its hydrophobic core.

The remodelling of the hydrophobic core is exemplified by changes associated with the reorientation of His37 (Fig. 7a–c). In RsrAred.Zn2+, zinc ligation by His37 necessitates rotation of Ph38 out of the hydrophobic core. In RsrAox, Ph38 (helix II) interacts with Val54 (helix IIIc), and Leu18 and Phe21 (helix I) within the hydrophobic core of the protein, which keeps helix IIIc packed onto helices I and II, and so blocking RsrA’s ability to interact with σR. Moreover, Val75 in helix IV also forms hydrophobic contacts with residues in RsrAox (Leu18, Val54 and Leu57). Hence, formation of the trigger disulfide between Cys11 and Cys44 propagates collapse of the helix III and release of helix IV. The consequence of these structural changes is that σR-contacting residues are sequestered back into RsrA’s hydrophobic core.

Figure 7 | RsrAred.Zn2+ uses hydrophobic core residues to bind σR that are sequestered to the RsrAox interior following oxidation. (a) Solution structure of RsrAred.Zn2+. RsrA helices are coloured N to C as in the sequence alignment in d. Zinc is shown as a blue sphere and zinc ligands coloured by atom type. Conserved or conservatively substituted hydrophobic residues that contribute to RsrA’s hydrophobic core in all three of its structural states (α-α-α) are coloured green, while those that also interact with σR are coloured red. (b) Homology model of the RsrAred.Zn2+–σR complex validated by homobifunctional lysine-specific crosslinking (Supplementary Fig. 5). The structure of RsrA red.Zn2+ changes markedly to embrace σR. (c) Solution structure of RsrAox where the trigger disulfide is formed between residues Cys11 and Cys44, expelling bound zinc and repacking the hydrophobic core. (d) Sequence alignment of RsrA and other ZAS proteins (ChrR, RshA and RslA). Zinc ligands in each protein are underlined. Helices in all three structural forms of RsrA are coloured as in a–c. Vertical green shading shows conserved hydrophobic residues that contribute to the hydrophobic core of RsrA in all three structural states (RsrAred.Zn2+, RsrAred.Zn2+–σR complex and RsrAox). Vertical red shading shows conserved hydrophobic residues in RsrA that contribute to the hydrophobic cores of RsrAred.Zn2+ and RsrAox, but also contribute to the protein–protein interface in the RsrAred.Zn2+–σR complex.

The structure of RsrAox explains why the Cys41–Cys44 disulfide does not cause dissociation of the complex. Whereas a disulfide between Cys11 and Cys44 pins helices I and IIIa together, stabilizing the hydrophobic core and sequestering key hydrophobic residues away from σR, a disulfide between Cys41 and Cys44 places no constraints on helix I. Hence, RsrA with the Cys41–Cys44 disulfide is still able to expose its hydrophobic core in order to bind σR. This in turn explains why Cys11 is required for redox sensing. Finally, the structure of RsrAox explains why the trigger disulfide is degenerate since the side chains of Cys41Ser and Cys44 are presented to Cys11 from consecutive turns of helix IIIc such that they can both form a disulfide bond (Fig. 7c). The similarity of the HSQC spectra of the two oxidized forms of RsrA (Cys11–Cys41 and Cys11–Cys44; Supplementary Fig. 6b) further suggest their structures are likely to be similar.

Discussion

The mechanisms by which sensor proteins respond to oxidative stress in bacteria are varied but fall broadly into two groups, those that contain metal centres such as the transcriptional repressor PerR52 and chaperone Hsp33 (ref. 33), and those that have reactive cysteines, such as the transcription factors OxyR34 and OhrR35. Oxidation of tetrameric OxyR by hydrogen peroxide induces disulfide bond formation within OxyR monomers, the resulting conformational changes converting it from a transcriptional repressor into an activator56. The OhrR family of dimeric transcriptional repressors are derepressed by organic hydroperoxides following oxidation either of a single reactive
cysteine or through intersubunit disulide bond formation \(^{35,37}\). The dimeric transcriptional repressor PerR contains two metal centres, a structural Zn\(^{2+}\) site containing histidine residues \(^{35}\), and a regulatory site that in its Fe\(^{2+}\) -bound state is responsive to oxidative stress. Derepression of PerR by hydrogen peroxide occurs through oxidation of metal-binding histidine residues following the generation of HO\(^-\) by Fenton chemistry at the Fe\(^{2+}\) site. Hsp33 is a heat-shock protein that becomes activated during oxidative stress. A single zinc ion is coordinated by four cysteines in the C-terminal domain of Hsp33. Following oxidation with H\(_2\)O\(_2\), intramolecular disulide bonds form between the zinc-ligating cysteines, expelling bound zinc and forming a dimeric chromoprotein \(^{38}\). Zinc is also expelled from RsRA\(^{red}\) -Zn\(^{2+}\) on oxidation to RsRA\(^{ox}\), but in this instance release of zinc accelerates the dissociation rate of its complex with G before formation of the trigger disulide bond between its zinc-coordinating cysteine residues. Intriguingly, RsRA\(^{red}\) -Zn\(^{2+}\) has the same redox potential as OxyR\(^{39}\) even though the two proteins share no structural similarity and sense different oxidative stresses by complex different mechanisms. Finally, RsRA is the first example of an oxidative stress sensor that responds to oxidation by sequestering hydrophobic residues required to stabilize the protein–protein interaction with its cognate transcription factor back into its own hydrophobic core. The same residues are involved in stabilizing three distinct structural states of the anti-sigma factor, RsRA\(^{red}\) -Zn\(^{2+}\), RsRA\(^{red}\) -Zn\(^{2+}\) -\(\sigma^R\) complex and RsRA\(^{ox}\) (Fig. 7).

RsRA\(^{red}\) -Zn\(^{2+}\) and RsRA\(^{red}\) -Zn\(^{2+}\) -\(\sigma^R\) complex are equally reactive towards oxidants however given the high affinity of the complex \((K_D \sim 0.7 \text{ nM})\) and the co-expression of their genes in \(S. coelicolor\) it is likely that the complex is the redox sensor. The distance between the sulfur atoms of Cys11 and Cys44 in the S. coelicolor S. coelicolor the same redox potential as OxyR\(^{39}\) even though the two proteins bound states of anti-sigma factors. The structure of RsRA\(^{red}\) -Zn\(^{2+}\) from the present work that this fold pertains only to the sigma-zinc-binding and non-zinc-binding anti-sigma factors. It is clear that multiple zinc ions bind to RsRA to modulate its redox reactivity \(^{31}\). We re-assessed the stoichiometry of zinc binding before dissecting the redox-sensing mechanism. Using both wild-type RsRA and RsRA*, in which the four non-essential cysteines (Cys3, Cys31, Cys61 and Cys62) were mutated to alanine, native state nanoelectrospray mass spectrometry showed that both proteins in the reduced state bind multiple zinc ions (Supplementary Fig. 1a) shows data for the wild-type protein). Zinc titrations using RsRA* in circular dichroism and mass spectrometry experiments indicated that only a single zinc ion is required to stabilize the RsRA fold (Supplementary Fig. 1a–d). Previous estimates of the affinity of RsRA for zinc documented an equilibrium dissociation constant \((K_D)\) of \(\sim 0.1 \text{ M} (26)\), which is reasonable to assume pertains to this structural metal ion. We refer to this reduced, zinc-bound state of RsRA as RsRA\(^{red}\) -Zn\(^{2+}\). This form of RsRA dissolves in 50 mM Tris (pH 7.5), 100 mM NaCl and 1 mM dithiothreitol (DTT)) was obtained by treating all preparations of the protein with EDTA (1 mM, 15-min incubation), followed by desalting on a HiTrap column (5 ml) and, unless specified, stoichiometric zinc (Alfa Aesar, 99.9% purity) added back to the protein.

**Fluorescence spectroscopy.** Changes in the intrinsic tryptophan fluorescence emission of \(\sigma^R\) on binding RsRA\(^{red}\) -Zn\(^{2+}\) were monitored using a Horiba Fluoromax fluorescence spectrometer, bound containing 100 mM NaCl and 2 mM DTT at 25 °C. Samples were excited at 295 nm and emission spectra recorded from 310 to 450 nm. Titrations were conducted using 3 nmol stock solutions. \(\sigma^R\) was kept at 1 \text{ mM} and the concentration of RsRA varied from 0.1 to 2 \text{ mM} from which the fractional change in fluorescence change was determined.

**Circular dichroism spectroscopy.** Apo-RsRA was prepared by incubating the protein with 1 mM EDTA for 15 min at 4 °C before buffer exchanging to the
experimental buffer using a HiTrap column. Samples were incubated in 50 mM Tris (pH 7.5) buffer containing 100 mM NaCl and 2 mM DTT before desalting in 10 mM Tris (pH 7.5) buffer using a HiTrap column. Experiments were conducted immediately on a Jasco-J815 CD spectrometer at 20 °C using a 1-mm path length cuvette. Data collected as ellipticity in millidegrees were converted to mean residue ellipticity, (θ; degrees cm² per dmol residue).

**Native state mass spectrometry of protein complexes.** Intact mass spectrometry measurements were performed on a Waters SYNAPT G2 HDMS modified for high mass transmission (ref. 41). Samples were buffer exchanged into 25 mM ammonium acetate using BioSpin 6 (Bio-Rad) columns before mass spectrometry (MS) analysis. Typically, each measurement was performed by loading 3 μl of protein sample into gold-coated nanospray capillaries prepared in-house42 and loaded into a static nanospray block providing the spray voltage. Electrospray was induced by applying a potential of 2.5 kV to the capillary of a sample cup containing 50 μl of water to capture the charged droplets, with a source backing pressure of 5.5 mbar. The instrument was operated in time-of-flight mode (no ion mass spectrometry separation), with the trap and transfer collision cells held at an acceleration voltage of 10 and 5 V, respectively, (with an argon pressure of 2 × 10⁻² mbar). Data were processed using the Masslynx software. For high-resolution MS measurement, a Thermo Scientific Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer was used to measure the presence/absence of the disulfide in the RsrA* Cys11His44 complex. The instrument was modified for high mass measurement as described elsewhere43 and optimized for retention of non-covalent interactions. Hardware alterations included increasing the high-frequency radio-frequency power applied to the selection region and a gas line allowing higher pressures to be achieved in the higher-energy collisional dissociation (HCD) cell. The instrument was operated in ‘native mode’, where both the RF frequencies applied to transfer optics and Orbitrap voltages were optimized for high mass species. Ions were generated in the positive ion mode from a static nanospray source using gold-coated capillaries prepared in-house, then passed through a temperature-controlled transfer tube (set to 20 °C, backread 31 °C), RF lens, injection flatplate and bent flatplate. After traversing the selection quadrupole, which was operated with a wide selection width (1,000–10,000 m/z), ions were trapped in the HCD cell before being transferred into the C-trap and Orbitrap mass analyser for detection. Transient times were 128 ms (35,000 at M200) and automatic gain control (AGC) target was 1 × 106 with a maximum fill time of 100 ms. No additional HCD or in-source activation was applied. Argon was used as the collision gas and the pressure in the HCD cell was maintained at around 1.3 × 10⁻⁵ mbar. To achieve both representative mass measurement and good separation of the bound species, ~100 μM of each microwebs were analyzed, and averaged in Thermo Scientific Xcalibur 2.1. Masses were calculated using the three most abundant charge states of the protein sample. Calibration was performed before mass measurement up to 11,300 m/z using clusters of Cs⁺.

**Redox potential measurements.** The redox potential of the RsrAred.Zn²⁺–R complex was determined with reference to a GSH–GSSG (glutathione–glutathione disulfide) redox couple and monitoring the change in tryptophan fluorescence emission at 343 nm (excitation at 295 nm) of the complex (see above) and at 500 nm using the PAR assay in a spectrophotometer to determine the fraction of dissociated zinc (see below). Glutathione and glutathione disulfide were from Sigma-Aldrich. Fluorescence titration experiments were carried out in 50 mM Tris (pH 7.5) buffer and 100 mM NaCl at 25 °C. All buffers were degassed and purged with N₂ gas before the use. In all experiments, GSH was kept constant at 0.5 mM, while GSSG was varied from 100 μM to 100 mM. The RsrAred.Zn²⁺–R complex was at 2 μM. All samples were incubated with the redox couple for 2 h before data collection. Two hours was deemed as having reached equilibrium since longer incubations had no effect. Data were corrected for the inner-filter effect, as described44. Inclusion of the PAR reagent does not influence redox titration experiments, since its affinity for zinc is several orders of magnitude lower than that of RsrA. Positive and negative controls were collected at each redox couple concentration. Positive controls had 1 equiv. Zn²⁺ along with the PAR reagent and the GSH couple. Negative controls had the couple and PAR. The difference between the positive and the negative control gave the total signal change for that concentration of redox couple. Ratio of the experiment over the total signal change gave the ratio of zinc released. The fraction of RsrA₄GSH₄Zn²⁺–R complex dissociated and the fraction of zinc released were used to determine the fraction of reduced RsrA (R). The variation of reduced RsrA against 1/[GSSG] was fitted to equation (1) to determine K₉₀ in M units. K₉₀ was obtained in the Neernt equation (equation 2) to calculate RsrA’s redox potential.

\[
R = \frac{GSH}{GSSG} \cdot K_{90} + \frac{GSH}{GSSG}
\]

\[
g_{RsrA} = g_{RsrA}^{red} \cdot \frac{GSH}{GSSG} \cdot \frac{RT}{\Delta G} \ln K_{90}
\]

where \(g_{RsrA}^{red}\) is the redox potential of RsrA, \(g_{RsrA}^{red} = GSH/GSSG\) is the redox potential of glutathione, which is = -240 mV at 25 °C, pH 7.5 (ref. 47). R is the gas constant 8.314 J/K·mol⁻¹ and F is the Faraday constant, 96,485 J·mol⁻¹·mol⁻¹. The total signal change gave the ratio of zinc released. The fraction of RsrAred.Zn²⁺–R and PAR. The difference between the positive and the negative control gave the total disulfide redox couple and monitoring the change in tryptophan fluorescence. All experimental conditions were as described above. The dissociation trace was fitted to a single-exponential rate equation by nonlinear least square regression on the manufacturer’s software. Values of \(k_{diss}\) were then plotted against RsrA concentration to determine the bimolecular association rate constant (k₉₀). Data presented are averages of three traces in each stopped-flow experiment and each experiment was performed three times. Quoted errors are the s.d. from the three repeats. Apo-RsrA was prepared by incubating the protein with 1 mM EDTA for 15 min at 4 °C before buffer exchanging to the experimental buffer on a HiTrap column.

**Association and dissociation kinetics of the RsrA–R complex.** Association kinetics. Stopped-flow fluorescence experiments were performed on an Applied Photophysics SX20MV instrument set-up for 1:1 single mixing and thermostating using a circulating water bath. An excitation wavelength of 295 nm was used for the excitation of α₂’s two tryptophans (RsrA does not contain tryptophan), while a 320 nm laser was used to collect fluorescence emissions. Excitation and emission slits were set to 2 nm (bandpass = 645 mnM/mmn). Experiments were carried out at 25 or 35 °C in 50 mM Tris (pH 7.5) buffer containing 100 mM NaCl and 2 mM DTT. RsrA was preincubated in 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM DTT, 1 mM ZnCl₂ and buffer exchanged using a 5-mI HiTrap column into 50 mM Tris (pH 7.5), 100 mM NaCl and 2 mM DTT. Zinc could not be included in the cell due to the precipitation of Zn²⁺ during the titrations. All experiments were carried out immediately after buffer exchange and in triplicate. Averages and s.d.’s of the obtained parameters are reported from triplicate experiments. Data were analysed using the manufacturer’s software assuming a single binding site model. Competition ITC titrations were performed at the same temperature and in the same buffer conditions by injecting 100 μM RsrA into 10 mM GSH containing 50 μM RsrA* Cys11Ser. Binding isotherms were analyzed using the manufacturer’s software for a competitive binding mode45.

**Oxidation kinetics of the RsrA–R complex.** All experiments were conducted at 25 °C in 30 mM Tris (pH 7.5) buffer containing 100 mM NaCl. Buffers were thoroughly degassed and purged with N₂ gas before use. Oxidation by diamide. The reaction of diamide with thiols can be followed spectrophotometrically at 320 nm. Each diamide molecule oxidizes two thiols forming a disulfide and in the process diamide is converted from its diazene to hydrazine form (Supplementary Fig. 2). Only the diazene form absorbs at 320 nm (Fig. 3a). For second-order experiments, the reaction could be determined by mixing RsrA (154 nM) with 25 μM RsrA–Zn²⁺–R complex (25 μM), prepared by incubating EDTA-treated complex with 1 equiv. Zn²⁺. Oxidation of the complex (± Zn²⁺) was followed by the change in absorbance at 320 nm for 1.2 s using an Applied Photophysics SX20MV stopped-flow apparatus. The concentration of diamide at various time points was computed by determining the ratio of diamide consumed relative to a control in which 25 μM diamide was converted to the hydrazine form using 500 μM DTT. Raw data were then linearized by plotting the variation of 1/[diamide] against time from which the second-order rate constant for diamide-induced oxidation was determined. Zinc release from the RsrAred.Zn²⁺–R complex was the oxidized with diamide measured by incubating 100 μM RsrA, which absorbs at 500 nm on zinc binding. The second-order rate of diamide-induced release of Zn²⁺ was determined as described above using the PAR assay. For pseudo-first-order experiments, only zinc release was monitored from the RsrAred.Zn²⁺–R complex (2 μM), where diamide was varied from 25 μM to 20 mM. Traces were fitted to a single-exponential rate equation to obtain the observed Zn²⁺ release rate, \(k_{diss}\). Plots of \(k_{diss}\) versus diamide concentration were hyperbolic, the data fitted to the Michaelis–Menten equation from which values for
Ks and ks were extracted (Supplementary Fig. 2b). The average and s.d.’s reported are from triplicate experiments.

**Oxidation by H2O2.** Buffer and experimental conditions were as described above. Only pseudo-first-order experiments were conducted with H2O2. Zinc release was monitored in a stopped-flow apparatus using the PAR assay where the RsrAox–αβδ–σ70 complex (2 μM) was incubated with varying concentrations of H2O2 (1–10 mM). PAR was kept at 100 μM. Observed rates of Zn2+ release were plotted against the concentration of H2O2 from which the bimolecular rate constant was obtained as above. The change in intrinsic tryptophan fluorescence of αδ on complex formation was also exploited to follow the rate of complex dissociation on treatment with H2O2. RsrAox–αβδ–σ70 complex (2 μM) was incubated with varying concentrations of H2O2 (1–10 mM) and the extent of complex dissociation determined by fluorescence spectroscopy (excitation wavelength, 295 nm and emission wavelength, 343 nm) using a Fluoromax4 spectrometer and 10-mm path length quartz cuvettes. The average and s.d.’s reported are from triplicate experiments.

**Crosslinking-based homology modelling of the RsrAred-Zn2+–αδ complex.**

Crosslinking was used to constrain homology models of the complex. A concentration of 5 μM purified RsrAαβδ–Zn2+–σ70 complex in 20 mM Hepes (pH 7.5) buffer containing 50 mM NaCl and 2 mM dTT was incubated with 2 mM of 1:1 mixture of BS2G–dbs and BS2G–db (bis(sulfosuccinimidyl) 2,2′,4,4′-tetra)glutamate) or 1:1 mixture of BS3–dbs and BS3–db. Crosslinking reactions were allowed to take place at room temperature for 30 min, and then quenched with Tris–HCl (pH 7.5) to a final concentration of 100 mM. The samples were then filtered on 15% Silica gel in chloroform/methanol (1:1 v/v) and the extent of complex dissociation determined by fluorescence spectroscopy (excitation wavelength, 295 nm and emission wavelength, 343 nm) using a Fluoromax4 spectrometer and 10-mm path length quartz cuvettes. The average and s.d.’s reported are from triplicate experiments.

**NMR Spectroscopy.** RsrA Cys41Ser concentrations in all NMR samples were 1–1.5 mM. Oxidized samples were prepared in 20 mM Tris–HCl (pH 7.1) and 93% H2O/7% D2O. For tryptophan scans 350–1,800 resolution spectra were acquired in 20 mM Tris-HCl (pH 7.1), 5 mM DTT, 2 mM ZnCl2 and 93% H2O/7% D2O. Where necessary (for example, for acquisition of homonuclear two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) experiments), deuterated Tris and DTT (Cambridge Isotope Laboratories) were used. A mixture of 1H-, 2H-, and 13C-nuclei (TXI, Bruker Biospin) probe equipped with gradient coils, running TopSpin (Bruker BioSpin) software and belonging to the University of York Centre for Magnetic Resonance. All experiments were performed with H2O as the alignment medium for both the zinc-bound and oxidized samples. The data were processed with NMRPipe55 and were further analysed with Sparky60 using the built-in peak fitting program CNS62 (Table 1). The Ser42–Pro43 peptide bond was modelled based on the presence of an NOE between the H2 atoms of Ser42 and Pro43 and between Hα and Hδ protons of Ser42 and Pro43 (ref. 54) and R (ref. 22) and 2 or 3 Hα–Hδ NOEs that would be characteristic of a β-strand conformation. In RsrAox, two additional NOEs were observed between Pro43 and Hα of Ser42 Hδ and on the lack of NOEs between Ser42 Hα and Pro43 Hδ. In addition, Salters was generated for inclusion of the zinc ligand and modification of the zinc ligands (Cys11, His37, Cys41Ser and Cys44) in RsrAred-Zn. Estimates of backbone dihedral angles were obtained using TALOS57 only for residues that had ‘good’ predictions were included. Accessible molecular surface areas on a per residue basis (averaged over the 10 lowest-energies structures for RsrAox–αβδ–σ70 and RsrAox) were calculated using WHAT IF64. Structure coordinates of RsrAox and RsrAred-Zn2+ have been deposited in the Protein Data Bank (5fhi and 5fri, respectively).

**Residual dipolar coupling.** The H1–H2 NOE RDC were used to determine the conformations of the complex. A mixture of BS3-Gly–dbs and BS2G–db in 20 mM Tris–HCl (pH 7.1) and 93% H2O/7% D2O. Residues were trapped on an in-house packed guard column (75 μm inner diameter, 1.8 μm, 120 Å, Acetonitrile solvent A (0.1% formic acid in water) at a pressure of 500 bar and then fractionated using an EASY-spray Acclaim PepMap analytical column (75 μm inner diameter × 500 mm, RSLC C18, 2 μm, 100 Å) eluted with a linear gradient (7–31% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 200 nl min−1. Full-scan MS spectra were acquired initially filtered by E-value (EMBO J. 2017, 41, 1–13 (2019). 2. Malhotra, J. D. & Kaufman, R. J. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid. Redox Signal. 9, 2277–2293 (2007).

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Author contributions

K.V.R. conducted the kinetic, thermodynamic and oxidation experiments, modelled the complex and prepared mutants and crosslinked samples. K.Z., I.P., J.M.W. and J.R.P.
determined NMR structures of RsrA. J.Y., J.T.S.H., S.M. and C.V.R. conducted the mass spectrometry experiments and associated analysis. M.-L.R.F. contributed to kinetic analysis and with C.Sc. prepared the samples. C.Sh. and K.V.R. conducted the bioinformatics analysis. C.K. was the principal investigator and, along with K.V.R., prepared the manuscript.

Additional information
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