

The Role of Zinc in the Disulphide Stress-regulated Anti-sigma Factor RsrA from *Streptomyces coelicolor*

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The regulation of disulphide stress in actinomycetes such as *Streptomyces coelicolor* is known to involve the zinc-containing anti-sigma factor RsrA that binds and inactivates the redox-regulated sigma factor σ^R . However, it is not known how RsrA senses disulphide stress nor what role the metal ion plays. Using *in vitro* assays, we show that while zinc is not required for σ^R binding it is required for functional anti-sigma factor activity, and that it plays a critical role in modulating the reactivity of RsrA cysteine thiol groups towards oxidation. Apo-RsrA is easily oxidised and, while the Zn-bound form is relatively resistant, the metal ion is readily expelled when the protein is treated with strong oxidants such as diamide. We also show, using a combination of proteolysis and mass spectrometry, that the first critical disulphide to form in RsrA involves Cys11 and one of either Cys41 or Cys44, all previously implicated in metal binding. Circular dichroism spectroscopy was used to follow structural changes during oxidation of RsrA, which indicated that concomitant with formation of this critical disulphide bond is a major restructuring of the protein where its α -helical content increases. Our data demonstrate that RsrA can only bind σ^R in the reduced state and that this state is stabilised by zinc. Redox stress induces disulphide bond formation amongst zinc-ligating residues, expelling the metal ion and stabilising a structure incapable of binding the sigma factor.

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Introduction

Cells maintain a reducing cytoplasm. This avoids deleterious disulphide bond formation and minimises damage to macromolecules from reactive oxygen species, generated as a consequence of

aerobic metabolism and implicated in degenerative disease and ageing.^{1,2} Re-establishing redox homeostasis following oxidative stress is therefore critical for cell survival. In the case of disulphide stress,³ conserved oxidoreductases such as thioredoxin and glutaredoxin are used to reduce unwanted disulphide bonds through thiol-disulphide exchange reactions that are ultimately driven by the reducing potential of NADPH.^{4,5} Oxidised thioredoxin is reduced by NADPH-dependent thioredoxin reductase, while glutaredoxin is reduced by the cysteine-containing tripeptide glutathione, which is in turn reduced by NADPH-dependent glutathione reductase.

The mechanisms by which bacterial cells sense and respond to oxidative (including disulphide) stress are beginning to emerge.⁶ In *Escherichia coli*, the transcription factor OxyR is inactive in the reduced state but activated by H₂O₂.⁷ This induces the formation of an intramolecular disulphide

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Abbreviations used: ZAS, zinc-binding anti-sigma factor; IAN, iodoacetamide; PAR, 4-(2-pyridylazo)resorcinol; SELDI-MS, surface-enhanced laser desorption/ionisation mass spectrometry; MALDI-ToF, matrix-assisted laser desorption/ionisation time-of-flight; CAM, carboxamidomethyl; NIPIA, *n*-isopropylidoacetamide.

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bond *via* an intermediate sulfenic acid, causing significant restructuring of the protein.^{8,9} In the oxidised state OxyR activates the transcription of many redox-regulated genes including glutaredoxin 1 and glutathione reductase. Activated OxyR is itself a substrate for reduction by glutaredoxin 1 thereby creating a redox homeostasis loop.⁸ This simple picture has been challenged, however, as a more complex regulatory mechanism appears to be at work. A single, hyper-reactive cysteine of OxyR, involved in disulphide bond formation, has been proposed by Kim *et al.*¹⁰ to be post-translationally modified, with different reactive oxygen and nitrogen species each generating an OxyR protein differing in structure, DNA binding and transcriptional activity.¹¹

Control of a transcription factor by reversible disulphide bond formation is also the mechanism by which *Streptomyces coelicolor* A3(2) initiates a global response to cytoplasmic disulphide stress. In this case, the thiol-specific oxidant diamide induces the RNA polymerase sigma factor σ^R whose regulon of >30 genes includes the *trxBA* operon encoding thioredoxin and thioredoxin reductase.^{12–14} σ^R also activates transcription of its own gene thereby amplifying the response to the disulphide stress. Diamide induction of σ^R -dependent promoters *in vivo* is transient (basal levels of transcription are re-established within 60 minutes), probably the result of elevated concentrations of cellular reductants that reverse the activation of σ^R and consume diamide. While this is consistent with the known σ^R regulon it does not explain how the transcription factor senses disulphide stress; unlike OxyR, σ^R contains no cysteine residues and *in vitro* experiments demonstrate that its transcriptional activity is not responsive to oxidative stress implying that an additional component must be acting as the disulphide stress sensor.¹²

Kang *et al.*¹³ identified this component as RsrA (regulator of *sigR*), a protein of 105 amino acid residues that contains seven cysteine residues. RsrA is a redox-sensitive, anti-sigma factor encoded by a gene lying immediately downstream of *sigR*. Under reducing conditions, RsrA binds to an N-terminal domain of σ^R encompassing region 2 of the sigma factor, thereby preventing its association with RNA polymerase.¹⁵ RsrA forms up to three disulphide bonds *in vitro* under oxidising conditions and can no longer bind σ^R nor inhibit its transcriptional activity.¹³ Moreover, oxidised RsrA can be re-reduced by *S. coelicolor* thioredoxin (TrxA) *in vitro*, suggesting that *in vivo* this system likely forms a homeostatic loop whereby thioredoxin-reduced RsrA can re-associate with σ^R and so block its transcriptional activity.¹³ Orthologues of the σ^R -RsrA system are found in other actinomycetes such as *Corynebacterium diphtheriae* and has been implicated in microbial pathogenesis. In *Mycobacterium tuberculosis*, for example, the σ^R orthologue σ^H regulates the oxidative stress response, with the RshA protein likely acting as

its anti-sigma factor.^{16,17} Kaushal *et al.*¹⁸ demonstrated that this system is important for *M. tuberculosis* virulence, since a *sigH* deletion strain, while still able to infect mice, does not induce disease or cause lethality.

Paget *et al.*¹⁹ have shown that of the seven cysteine residues in RsrA only three are essential for anti-sigma factor activity, Cys11, Cys41 and Cys44. RsrA containing only these cysteine residues retained anti-sigma factor activity and was responsive to disulphide stress *in vivo*. Two of the cysteine residues (Cys41 and Cys44) are part of an invariant sequence motif (Hx₃Cx₂C) present in RsrA-like proteins in other bacteria, while Cys11 is only conserved in some RsrA homologues. Interestingly, mutation of the invariant histidine also abolished anti-sigma factor activity of RsrA *in vivo* (M.S.B.P., unpublished results). Paget *et al.*¹⁹ also found that RsrA is a metalloprotein, since it binds a single ion of zinc. The same is true of ChrR, an RsrA homologue from *Rhodobacter sphaeroides*,²⁰ both proteins now recognised as members of a larger group of ECF anti-sigma factors known as the zinc-binding anti-sigma factor (ZAS) family.¹⁹ The available evidence points toward the conserved Hx₃Cx₂C motif, together with an additional cysteine (C11 in RsrA) or histidine residue, as the likely zinc-coordinating residues in the ZAS family, although this is still to be confirmed.^{19,20}

Here, we address the role of zinc in the redox-sensing and σ^R -binding activities of RsrA, characterise spectroscopically the structural changes that accompany the formation of the first disulphide bond and map its location. We present a model for redox-regulation of σ^R activity by RsrA, of relevance to regulation of other ECF sigma factors by other ZAS proteins, and compare this to other prokaryotic and eukaryotic systems in which zinc plays a critical role in modulating thiol-disulphide reactivity.

Results

Zinc is not required for σ^R binding by RsrA

In order to evaluate the role of zinc in σ^R binding by RsrA we prepared reduced apo-RsrA, Zn-RsrA and RsrA in which all seven thiol groups were irreversibly alkylated with iodoacetamide (IAN; 7CAM-RsrA). The different RsrA preparations were incubated with σ^R and their mobility on native gel compared (Figure 1). Previous work has shown that σ^R -RsrA complexes can be followed conveniently by native gel electrophoresis where the complex migrates at a position intermediate between that of the isolated protomers.¹³ Both Zn-RsrA and apo-RsrA were able to form complexes with σ^R . More surprisingly, we found that 7CAM-RsrA was also able to form a complex with σ^R (Figure 1, lanes 6 and 7), an observation

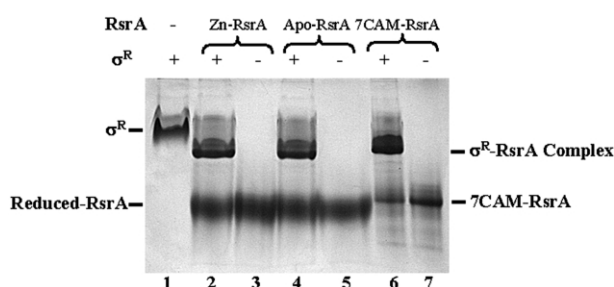


Figure 1. RsrA binding σ^R in the presence and absence of zinc. Different RsrA species were incubated with σ^R (0.2 nmol) in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl and at 30 °C for 30 minutes prior to being analysed by native 12% PAGE at 4 °C with 10 mM DTT in the running buffer (see Materials and Methods for details). Lane 1, σ^R ; lane 2, σ^R and Zn-RsrA (0.5 nmol); lane 3, Zn-RsrA (0.5 nmol); lane 4, σ^R and apo-RsrA (0.5 nmol); lane 5, apo-RsrA (0.5 nmol); lane 6, σ^R and 7CAM-RsrA (0.3 nmol); lane 7, 7CAM-RsrA (0.3 nmol).

confirmed by gel filtration chromatography (data not shown).

Although it is clear that apo and 7CAM-RsrA can bind σ^R *in vitro* this does not mean that they are effective in suppressing σ^R transcriptional activity. In order to begin addressing this issue we compared the ability of 7CAM-RsrA to inhibit σ^R -dependent transcription with Zn-RsrA (Figure 2). The data show that alkylation of RsrA abolishes its ability to inhibit σ^R -dependent transcriptional activity under these conditions. Since 7CAM-RsrA is still able to bind to the sigma factor (Figure 1), these data suggest that fully alkylated RsrA may

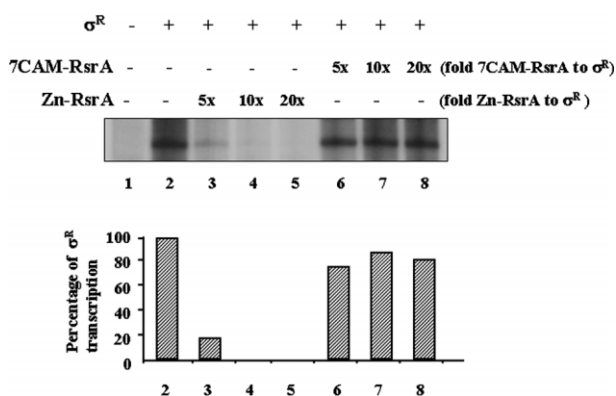


Figure 2. Comparing the anti- σ^R activities of Zn-RsrA and 7CAM-RsrA in transcription run-off assays from a σ^R -specific promoter. Lane 1, no σ^R control; lanes 2–8, all contain σ^R (3.6 pmol); lanes 3–5, addition of excess Zn-RsrA; lanes 6–8, lanes 3–5, addition of excess 7CAM-RsrA. All lanes contain core RNA polymerase (1.5 pmol) and the other components of the transcription assay. The amount of transcript in each lane was quantified by densitometry and plotted as the percentage of σ^R -dependent transcription observed in lane 2. The experiment shows that Zn-RsrA inhibits σ^R -transcriptional activity (lanes 3–5) while 7CAM-RsrA is inactive in this assay (lanes 6–8).

bind more weakly to σ^R than Zn-RsrA and so does not compete effectively with core RNA polymerase in the *in vitro* transcription assay. Whether this is a consequence of alkylation or simply the loss of metal ion is the focus of ongoing work.

Zinc controls RsrA thiol-disulphide reactivity and is expelled during oxidation

In the original identification and preliminary characterisation by Kang *et al.*¹³ it was not known that RsrA was a metalloprotein and so no account was taken of the role of the bound metal ion in oxidation. Therefore, we determined the effect of metal-binding on the ability of disulphide bonds to form in RsrA by comparing the rate of air-oxidation, following the removal of reductant by gel-filtration chromatography, with and without bound zinc (Figure 3). Oxidised and reduced RsrA were distinguished by non-reducing SDS-PAGE, following alkylation of free thiols by IAN, and the extent of oxidation determined by densitometric

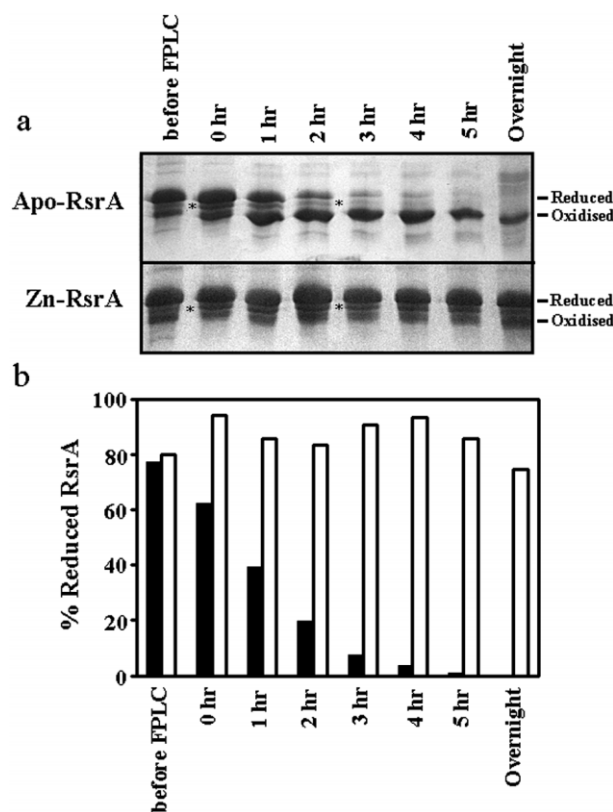


Figure 3. Effect of zinc on the rate of RsrA oxidation. (a) Non-reducing SDS/polyacrylamide gels (15%) showing the time-course of RsrA oxidation with (Zn-RsrA) and without (apo-RsrA) bound metal ion following a desalting step (by FPLC gel filtration) to remove excess reductant. An asterisk (*) indicates RsrA breakdown product that also undergoes oxidation. (b) Percentage of reduced RsrA as determined by laser densitometric scanning of non-reducing SDS/polyacrylamide gels and plotted as a function of the time of elution from the gel filtration column. Filled bar, Apo-RsrA; open bar, Zn-RsrA.

scanning of Coomassie-stained gels. The experiment demonstrated that the metal status of RsrA has a strong influence on its rate of oxidation. Zn-bound RsrA remained largely reduced even after overnight incubation in the absence of any reductant while apo-RsrA was completely oxidised in less than five hours (Figure 3); the oxidation half-life at pH 7.5 and 30 °C was about one hour ($k_{\text{oxid}} \sim 2 \times 10^{-4} \text{ s}^{-1}$). In separate experiments, the rate of air-oxidation for Zn-RsrA incubated with 5 mM EDTA was similar to that of apo-RsrA (data not shown). These observations suggest that RsrA oxidation is accelerated by zinc dissociation but is not rate-limited by it.

Zn-RsrA is relatively insensitive to air oxidation, whereas apo-protein is very sensitive, implying that the metal ion influences the reactivity of the protein thiol groups. The next question we addressed was whether more powerful oxidising agents could induce disulphide bonds in zinc-containing RsrA and whether this caused the expulsion of the bound metal ion. Zn-RsrA was incubated with 0.1 mM diamide and the extent of zinc release monitored spectrophotometrically at 500 nm using the zinc-specific dye 4-(2-pyridylazo) resorcinol (PAR; see Materials and Methods). In a parallel experiment, samples were removed and quenched with IAN in order to visualise the degree of oxidation of the protein during incubation with diamide. The data in Figure 4 demonstrate that a stoichiometric amount of zinc is released in less than five minutes, during which time the protein becomes almost completely oxidised, as deduced

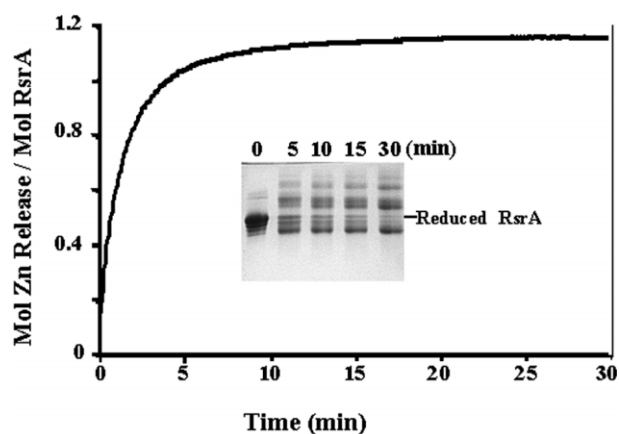


Figure 4. Diamide-induced zinc release from Zn-RsrA. Diamide (0.1 mM final) was added to 1 ml of RsrA (2.6 μM) in 50 mM TEA buffer (pH 7.5) containing 200 mM NaCl and 200 μM 4-(2-pyridylazo) resorcinol (PAR). The absorbance change at 500 nm was monitored spectrophotometrically and the amount of zinc released calculated using a standard curve obtained by titrating ZnCl_2 into the same buffer. Inset, non-reducing SDS-PAGE showing the oxidation time-course of a parallel RsrA sample treated with the same concentration of diamide. Samples were alkylated with IAN prior to their separation on the gel as described in the legend to Figure 2 and in Materials and Methods.

by the loss of the reduced RsrA species. Zinc was also released when the protein was treated with 0.5 mM H_2O_2 but the kinetics were slower by approximately an order of magnitude (data not shown). We also found that the kinetics of diamide-induced zinc release from Zn-RsrA were not affected by complex formation with σ^{R} (data not shown). Hence, zinc plays an important role in modulating the reactivity of the RsrA thiol groups, wherein it dampens their potential for oxidation under mild conditions, but is readily expelled under strongly oxidising conditions with the concomitant formation of disulphide bond(s). It is also noteworthy that the effects of diamide on the kinetics of zinc release from RsrA *in vitro*, where all the metal is lost in about five minutes at 30 °C, are similar to the transcriptional changes that have been measured for the σ^{R} -dependent promoter *trxB*A for *S. coelicolor* grown at 30 °C where induction peaks in less than ten minutes.¹²

A single disulphide bond in RsrA abolishes binding to σ^{R}

Kang *et al.*¹³ reported that up to three disulphide bonds can form amongst the seven cysteine residues of RsrA and that oxidised protein is unable to bind σ^{R} . However, it is not known if there is a single, critical disulphide bond that forms first which might act as a redox sensor. In order to address this issue we devised conditions whereby RsrA with a single disulphide bond was isolated and characterised. Strong oxidants such as diamide were not used to generate oxidised RsrA because the kinetics of oxidation were very rapid, resulting in protein containing more than one disulphide (data not shown) and with evidence of higher-order aggregates (Figure 4). Instead, air-oxidation of EDTA-treated Zn-RsrA was used, since this allowed for slower, more controlled oxidation of the protein akin to that of apo-RsrA in air (Figure 3), with little evidence of aggregation. In these experiments, Zn-RsrA was incubated at 30 °C, in the absence of reductant but in the presence of 5 mM EDTA for up to four hours. Under these conditions, the majority of RsrA was oxidised with little or no appearance of RsrA with more than one disulphide bond. Oxidised RsrA was alkylated with IAN to prevent further oxidation and purified by gel-filtration chromatography. The resulting chromatogram yielded two major peaks, analysed by non-reducing SDS-PAGE (Figure 5(a)), and their molecular masses obtained using surface-enhanced laser desorption/ionisation mass spectrometry (SELDI-MS; Figure 5(b)); the faster eluting peak (Figure 5(a), lane 1) corresponded to reduced, fully alkylated RsrA (7CAM-RsrA) while the slower eluting peak (Figure 5(a), lanes 3 and 4) was 5CAM-RsrA, where the protein contained a single disulphide bond.

The electrophoretic mobility of 7CAM-RsrA and 5CAM-RsrA and their ability to bind σ^{R} were compared using gel electrophoresis (Figure 6). The

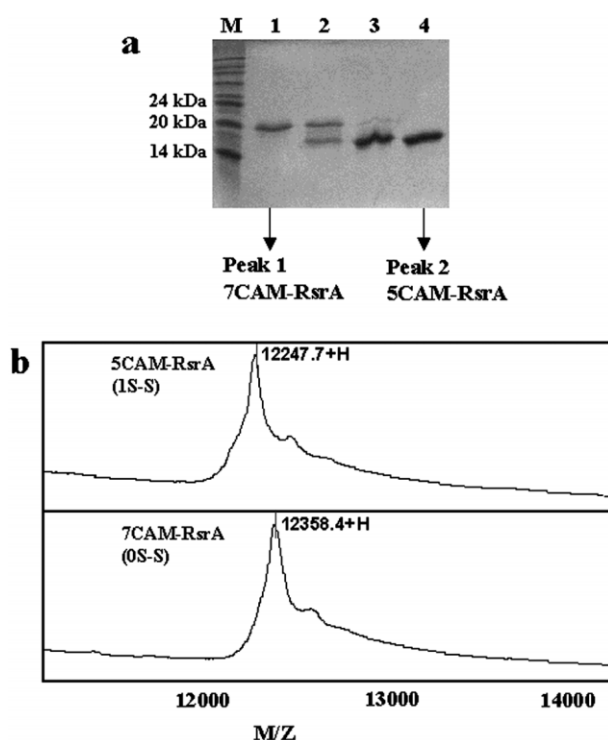


Figure 5. Preparation of RsrA with a single disulphide bond. (a) Non-reducing SDS/polyacrylamide gel of RsrA fractions from a gel-filtration column following EDTA (5 mM) treatment of Zn-RsrA (~1 mg), in 50 mM Tris-HCl (pH 7.5) and containing 200 mM NaCl, in air for about four hours and then alkylated with IAN. Carboxamidomethylated RsrA was separated on a Superdex-S75 column and fractions from the two eluting peaks analysed by gel electrophoresis. (b) SELDI mass spectra of each RsrA peak. Peak 1 (lower spectrum) corresponded to 7CAM-RsrA ($M_{r,obs}$ 12,361(\pm 3) (n = 3); $M_{r,calc}$ 12,361.4), the slower eluting peak 2 (lanes 3 and 4, (a)) corresponded to 5CAM-RsrA containing a single disulphide bond ($M_{r,obs}$ 12,247(\pm 3) (n = 3); $M_{r,calc}$ 12,247.4).

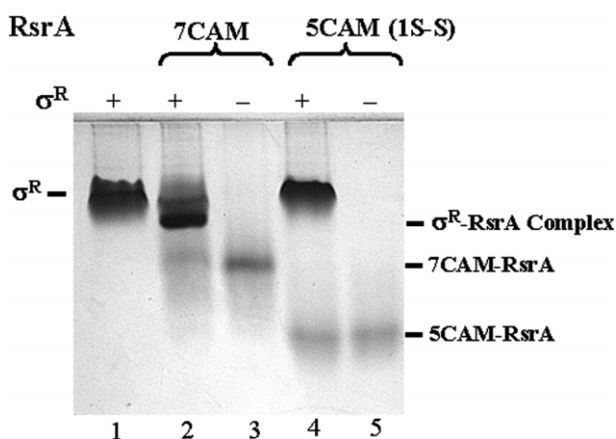


Figure 6. Comparing 7CAM-RsrA and 5CAM-RsrA binding to σ^R . σ^R (0.2 nmol) in 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl was incubated with either 7CAM-RsrA or 5CAM-RsrA (0.3 nmol and 0.2 nmol, respectively) and complex formation monitored by native PAGE (running conditions as shown in the legend to Figure 1).

introduction of a single disulphide clearly affected the electrophoretic mobility of the protein, since 5CAM-RsrA migrated much further down a native gel compared to 7CAM-RsrA (Figure 6, lanes 3 and 5), the same being true in non-reducing SDS/polyacrylamide gels (Figure 5(a)). Since 7CAM and reduced RsrA have similar electrophoretic mobilities on native gels (Figure 1), this implies that 5CAM-RsrA has a more compact structure than the reduced state, consistent with the presence of an intramolecular cross-link in the oxidised protein. These differences in gross conformation were similarly reflected by the behaviour of the proteins on gel-filtration chromatography (Figure 5). Strikingly, whereas fully alkylated RsrA, a mimic of the reduced protein, was still able to bind σ^R even with seven covalently attached carboxamidomethyl groups, 5CAM-RsrA, with a single disulphide bond and less covalent modification, was unable to form a complex with σ^R . Hence, the formation of a single disulphide bond in RsrA is sufficient to completely abolish sigma factor binding. Subsequent disulphide bonds have no additional effects, since RsrA with two disulphide bonds (3CAM-RsrA) was also incapable of binding to σ^R and its mobility on native gels is identical with that of 5CAM-RsrA (data not shown).

Major structural changes accompany disulphide bond formation in RsrA

Far-UV circular dichroism (CD) was used to investigate if disulphide bond formation in RsrA resulted in gross structural changes in the protein. The CD spectra of reduced, apo and Zn-RsrA were indicative of folded proteins each having two minima at 205 nm and 222 nm and a positive band ~190–195 nm (Figure 7(a)). The spectra are similar but with a distinct difference in the intensity of the low UV minimum at 205 nm, suggesting that the proteins are likely to be similar but not identical in structure. The spectrum of Zn-RsrA did not change when left overnight in the cuvette exposed to air. By contrast, when reduced, apo-RsrA was left in the cuvette, a significant, time-dependent change in CD spectrum occurred as the protein oxidised (Figure 7(b)), indicating that it became more structured relative to the reduced state. There was also a shift in the position of the first minimum from 205 nm to 208 nm on oxidation of RsrA, the resulting spectrum resembling a classic $\alpha + \beta$ protein.²¹ Calculations of the secondary structure content for apo and Zn-RsrA indicate they each comprised approximately 23% α -helix and 30% β -sheet whilst oxidised RsrA (after overnight incubation) contained ~30% α -helix and ~20% β -sheet. CD spectra of 7CAM and 5CAM-RsrA showed similar differences in helical content to those induced by air-oxidation of apo-RsrA (19% and 27% for 7CAM-RsrA and 5CAM-RsrA, respectively; data not shown) consistent with the increase in RsrA helicity being associated with formation of the first disulphide bond.

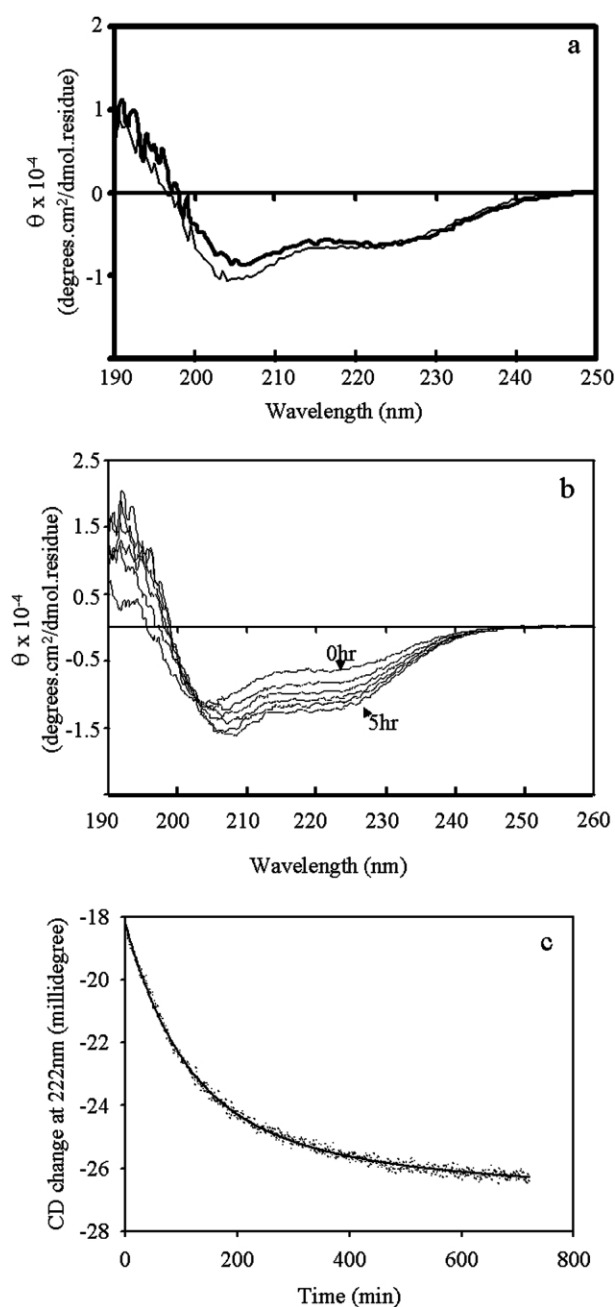


Figure 7. Circular dichroism spectroscopy of RsrA at 30 °C in 30 mM Tris-SO₄ (pH 7.4). (a) CD spectra of apo-RsrA (thin line) and Zn-RsrA (bold line), with five scans averaged for each spectrum. Proteins were prepared as described in Materials and Methods. (b) Oxidation-induced changes in the CD spectrum of apo-RsrA. Freshly prepared reduced, apo-RsrA was desalted to remove reductant and the CD spectrum taken immediately (0 hour). The sample was then left to air-oxidise in the spectropolarimeter with spectra taken every hour, up to five hours. The resulting changes indicate a substantial increase in helical content (see the text for details). (c) Kinetics of air-oxidation for apo-RsrA followed by the increase in helical signal at 222 nm. Data were acquired overnight and have been fitted to a double exponential rate equation using Sigma Plot. From two independent experiments, $k_1 = 2.4(\pm 0.6) \times 10^{-4} \text{ s}^{-1}$ and $k_2 = 0.5(\pm 0.1) \times 10^{-4} \text{ s}^{-1}$.

The changes in secondary structure provided a convenient measure of the oxidation kinetics of RsrA (Figure 7(c)). CD data were recorded over time at 222 nm, the wavelength at which the protein acquires more helical structure as it oxidises in air (Figure 7(b)). The resulting spectral changes gave good fits to a double exponential rate equation with values for $k_1 = 2.4 \times 10^{-4} \text{ s}^{-1}$ and $k_2 = 0.5 \times 10^{-4} \text{ s}^{-1}$. k_1 is very similar to the rate constant k_{oxid} ($2 \times 10^{-4} \text{ s}^{-1}$) obtained from the disulphide-trapping experiments (Figure 3) in which 5CAM-RsrA was the predominant species (Figure 5) and so clearly reflects the conformational changes in RsrA that result from formation of this first disulphide bond. The slower rate k_2 likely represents other structural changes, such as the formation of a second disulphide. From these data we conclude that the first, inactivating disulphide bond in RsrA induces a large conformational change in the protein in which it becomes more compact and significantly more helical.

Mapping the first disulphide bond in RsrA

The position of the first inactivating disulphide bond in oxidised RsrA was determined through a combination of proteolytic digestion and mass spectrometry. Since the first air-catalysed disulphide bond in RsrA forms with a half-life of about one hour at pH 7.5 and 30 °C, oxidation reactions were kept to one hour in order to minimise the possibility of disulphide-exchange (similar conditions were used by Barbirz *et al.*²² in their identification of the intramolecular disulphides that form in the zinc-containing molecular chaperone Hsp33). EDTA-treated Zn-RsrA was then alkylated with IAN resulting in a mixture of 7CAM and 5CAM-RsrA (determined by SELDI-MS) and this mixture digested with chymotrypsin. The peptides were analysed initially by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry. Eight major chymotryptic fragments were readily assigned for 7CAM-RsrA-derived peptides along with the locations of the carbamidomethylated cysteine residues (data not shown). In order to identify peptides involved in disulphide bond formation, MALDI-ToF spectra of the same chymotryptic digest were analysed before and after reduction with DTT. From this comparison we identified only two species (which were partial digests of each other) where peaks disappeared as a result of reduction and where new species appeared at lower molecular mass (Figure 8). Peptide A, the most abundant, corresponded to peptides 2–19 and 35–48 containing two carboxamidomethyl (CAM) groups ($M_{\text{r,obs}} = 3908.4$; $M_{\text{r,calc}} = 3908.6$). Peptide B corresponded to the same peptides and number of CAM substitutions but where the remnants of the thrombin cleavage site (Gly-Ser-His) and N-terminal methionine were retained on the protein ($M_{\text{r,obs}} = 4320.6$; $M_{\text{r,calc}} = 4320.7$). Since each of the peptides involved in forming the

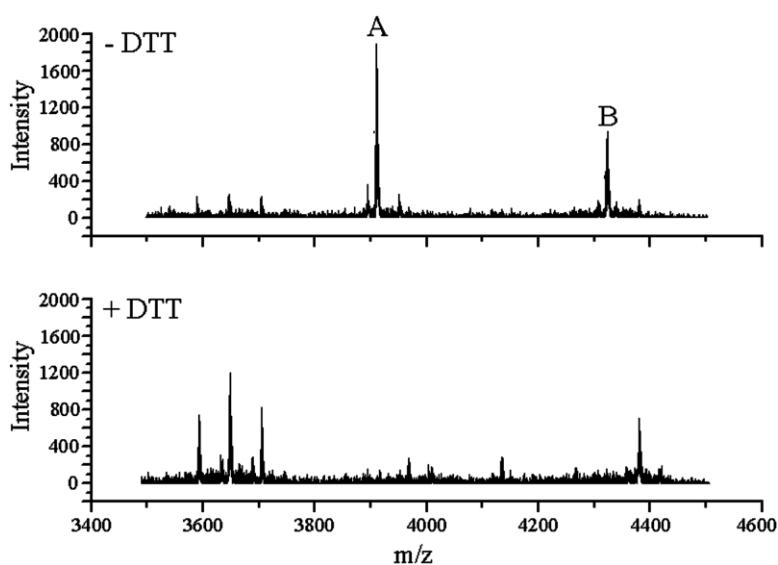


Figure 8. MALDI-ToF mass spectra (m/z range, 3500–4000) of oxidised RsrA chymotryptic digest in the absence (top) and presence (bottom) of 10 mM DTT (see Materials and Methods). Peptide A corresponds to a disulphide between residues 2–19 and 35–48 while peptide B corresponds to a disulphide between residues 1–19 (and including the N-terminal linker GSH) and 35–48 (see the text for details). Both A and B disappeared when DTT was added, confirming the presence of the disulphide bond, with three new smaller peptides (peptides C–E) appearing at lower mass range (not shown; see the text for details).

disulphide contain two cysteine residues this is consistent with each having a single CAM moiety and one protected cysteine residue. This was confirmed by the identities of the three new species (peptides C–E) generated from the reduction of peaks A and B (data not shown). Each corresponded to one half of the cysteine linkage with a single CAM modification: peptide C corresponded to residues 35–48 ($M_{r,obs} = 1806.8$; $M_{r,calc} = 1806.7$); peptide D corresponded to residues 2–19 ($M_{r,obs} = 2103.9$; $M_{r,calc} = 2103.9$); peptide E corresponded to residues 1–19 with the additional GSH sequence ($M_{r,obs} = 2516.0$; $M_{r,calc} = 2516.0$). These data indicate therefore that a single disulphide bond existed in 5CAM-RsrA between amino acid residues 2–19 and 35–48. Tandem mass spectrometry (Q-ToF) was utilised subsequently to determine which of the two cysteine residues in peptides C and D was involved in forming this disulphide.

The tandem mass spectrum of D (Figure 9(a)) confirmed that this peptide corresponded to residues 2–19, and that Cys3 was carbamidomethylated while Cys11 was not. This demonstrated unequivocally that Cys11 was the sole residue protected during the alkylation step and thus involved in disulphide bridging. The tandem mass spectrum of C (not shown) proved to be more difficult to interpret. It was evident from the generated sequence that this peptide corresponded to residues 35–48. However, since all of the crucial fragment ion peaks overlapped each other in the tandem mass spectrum, it was not possible to make unequivocal assignments as to whether there was modification of Cys41, Cys44 or a mixture of both.

To overcome this problem, the oxidised RsrA chymotryptic digest was first reduced with DTT and then further alkylated with *n*-isopropylidodoacetamide (NIPIA) in order to modify all of the previously protected cysteine residues with a corresponding shift in mass. Addition of NIPIA gave the expected mass shift in the MALDI-ToF mass

spectrum for peptide 35–48, from $M_{r,obs} = 1806.8$ (peptide C) to $M_{r,obs} = 1905.8$ ($M_{r,calc} = 1905.8$), indicating that one cysteine was carbamidomethyl modified and another *n*-isopropylcarbamidomethylated (data not shown). Figure 9(b) shows the tandem mass spectrum of this species in the m/z range 700–1400. The data highlight that the peptide was a mixture of two populations, some carrying CAM modification on Cys41 and NIPIA modification on Cys44 and others with the inverse. A comparison of the fragment ion intensities showed that the major component (at least in the gas-phase and by a proportion of 3:1) was the peptide in which Cys44 was unprotected, and hence involved in disulphide bonding, and Cys41 was CAM-modified. In summary, 5CAM-RsrA contained a single disulphide bond formed between Cys11 and either of Cys41 or Cys44, the latter being the major component in the mass spectrum.

Discussion

RsrA responds to redox imbalance in the antibiotic-producing soil organism *S. coelicolor* and in so doing regulates the transcriptional activity of its cognate sigma factor, σ^R . Although only recently described, it is clear that RsrA is a member of a larger class of zinc-binding anti-sigma factors known as the ZAS family. Members of this family are present in several bacterial species,¹⁹ although not all are likely to be redox sensitive. For example, in *R. sphaeroides* regulation of σ^E by the ZAS anti-sigma factor ChrR is not responsive to diamide.²⁰ In *Bacillus subtilis*, σ^W activity, which is controlled by a ZAS protein, is not induced by diamide and no members of the σ^W regulon are connected with thiol-disulphide metabolism.^{23,24} What differentiates a redox-sensitive ZAS protein from one that does not respond to redox change has yet to be determined.

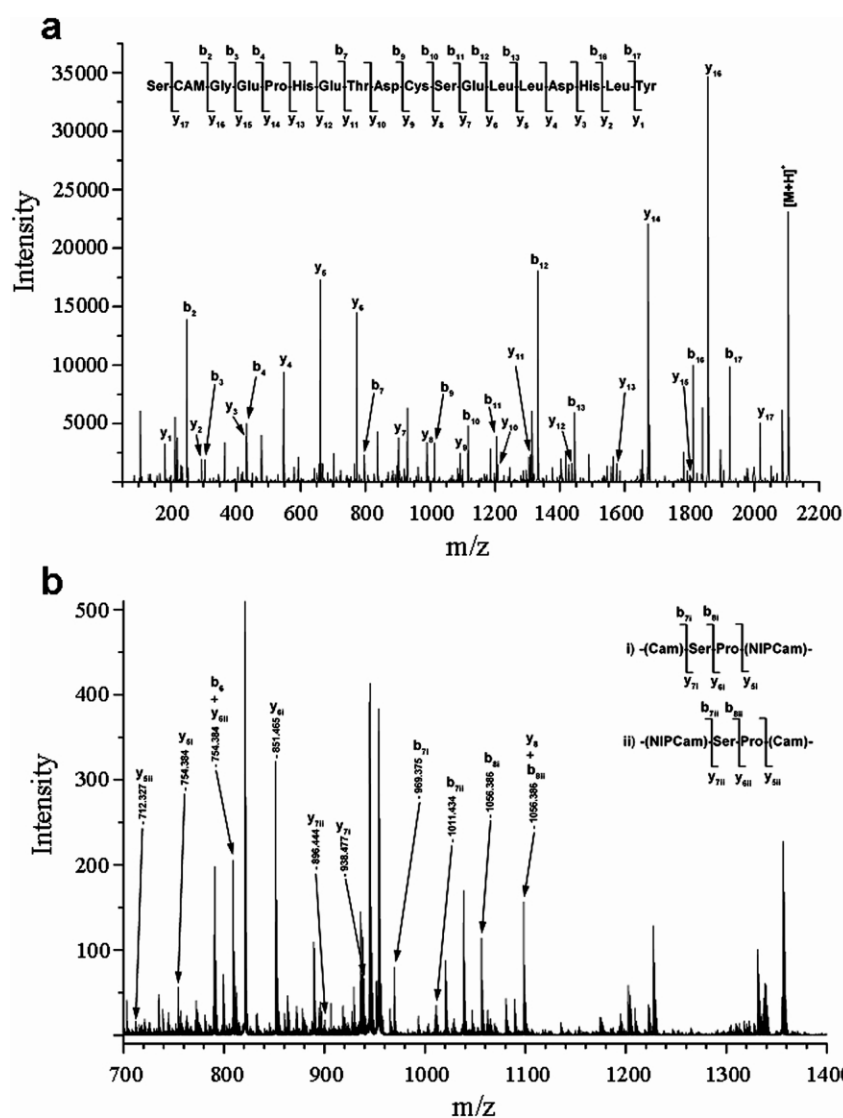


Figure 9. Tandem mass spectra of RsrA chymotryptic fragments. (a) Dominant, singly charged b_n and y_n fragment ion series³⁵ of the precursor ion at m/z 702.2831 (3+) for peptide D (residues 2–19), generated from the reduction of peptide A in Figure 7. (b) Dominant, singly charged b_n and y_n fragment ion series of the precursor ion at m/z 953.928 (2+) for peptide C (residues 35–48), generated from the reduction of peptide A and following the addition of the alkylating agent NIPIA (see Materials and Methods and the text for details). The Figure highlights m/z range 700–1400 where residue-specific assignments could be made for the sequence Cys41-Ser42-Pro43-Cys44. Both Cys41 and Cys44 are involved in disulphide bond formation, since fragment ions are observed which both carry CAM and NIPIA modification; for example, y_{5i} and y_{5ii} , and b_{7i} and b_{7ii} . CAM, carbamidomethyl modified cysteine. NIPICam, *n*-isopropyl-carbamidomethyl modified cysteine.

We address the role of zinc in the σ^R -binding activity and redox-sensing mechanism of RsrA. We find that removal of the bound metal ion has only modest effects on secondary structure (Figure 7(a)) and does not affect the overall topology of the protein, as deduced by its migration properties in native gels (Figure 1). Moreover, zinc is not essential for sigma factor binding, since apo-RsrA and 7CAM-RsrA are still able to bind σ^R (Figure 1). However, alkylated RsrA loses its ability to out-compete core RNA polymerase for σ^R (Figure 2), suggesting that it binds σ^R more weakly than does Zn-RsrA. The importance of zinc to ZAS anti-sigma activity has been shown for ChrR, and this was attributed to a structural role of the metal ion.²⁰ Based on the present work, zinc has a relatively minor role in maintaining the structural integrity of RsrA, but seems to be required for functional anti-sigma factor activity.

Zinc has a profound influence on the reactivity of the RsrA cysteine thiol groups, since its removal increases markedly its susceptibility toward air oxidation (Figure 3). Zinc-bound RsrA, on the

other hand, is very stable in air (Figure 3). Such zinc protection of protein thiolate groups has been observed in many other proteins, most notably in metallothioneine²⁵ and in zinc-finger transcription factors.²⁶ In the case of metallothioneine, seven Zn ions are bound by 20 coordinating cysteine residues with every thiolate engaged in metal binding.²⁷ This is not the case in Zn-RsrA, since the single zinc ion is likely coordinated by only three of the seven protein cysteine residues (Figure 10). This leads us to conclude that the Zn-bound form of RsrA adopts a structure in which ligating and non-ligating cysteine residues are held in a conformation in which they cannot engage in disulphide bond formation without a major structural rearrangement.

Treatment of Zn-RsrA with strong oxidants results in the expulsion of the bound metal ion and the formation of a single intramolecular disulphide bond (Figure 4). Since Zn-RsrA is more susceptible to oxidation by diamide than H_2O_2 , this implies that RsrA is primarily a sensor of disulphide rather than peroxide stress in *S. coelicolor*

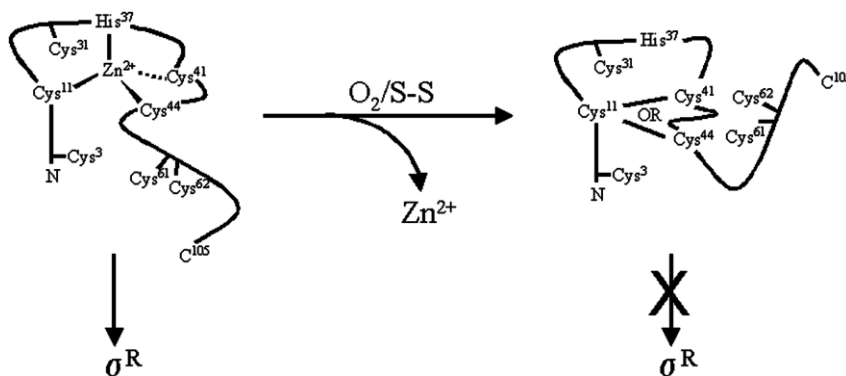


Figure 10. Hypothetical scheme for the oxidation of Zn-RsrA in *S. coelicolor*. The zinc-coordination scheme for Zn-RsrA in the resting state is based on previous *in vitro* and *in vivo* data (see the text for details).¹⁹ On exposure to oxidants such as oxygen or diamide, an initial disulphide bond occurs between Cys11 and one of either Cys41 or Cys44. Concomitant with its formation, the bound zinc is expelled and the protein undergoes a large conformational change in which its helicity increases. The resulting oxidised RsrA is unable to bind its target sigma factor σ^R , releasing it to activate the expression of many genes whose products are required to re-establish redox homeostasis.

and this concurs with the known σ^R regulon.¹⁴ The oxidative release of zinc in concert with disulphide bond formation has been documented for other redox-sensitive zinc metalloproteins such as metallothionein and the molecular chaperone Hsp33.^{28–30} These studies point to a linkage between cellular zinc metabolism and thiol-disulphide redox status, a link that has been emphasised in mammals by the work of Maret & Vallee on metallothionein but is only just emerging in bacteria.^{28,31,32} Gaballa & Helmann, for example, have found that zinc uptake in *B. subtilis* plays a critical role in the resistance to both disulphide and peroxide stress.³³

Two intramolecular disulphide bonds are formed in Hsp33 during oxidative stress, resulting in a large structural rearrangement and the induction of chaperone activity. RsrA similarly undergoes a dramatic change in protein conformation during oxidation in which its helical content increases (Figure 7). The kinetics of this air-induced structural change are the same as those for the formation of the first disulphide bond in air (Figures 3 and 7) implying that they occur together. The resulting protein becomes more compact and unable to bind σ^R .

The identities of the zinc-ligating residues in RsrA have yet to be identified definitively. However, studies on both RsrA and the non-redox active ChrR provide a firm basis for their assignment. Newman *et al.* found that the two conserved cysteine residues present within the HX₃CX₂C motif are both required for zinc binding and anti-sigma factor activity in ChrR.²⁰ Paget *et al.*¹⁹ demonstrated that the same residues are essential for RsrA function *in vivo* and *in vitro*, with the same being true of the conserved histidine, albeit *in vivo* (M.S.B.P., unpublished results). In the same study, Cys11 was found to be essential for RsrA function. Hence, it is reasonable to assume that Cys11, His37, Cys41 and Cys44 constitute the zinc-binding site in RsrA. It is striking then

that the first, critical disulphide bond should involve the three cysteine residues implicated in zinc binding (Figure 10); Cys11 forms one half of the cysteine linkage, with either Cys41 or Cys44 able to form the other half. Cys11 is not found in some ZAS proteins such as ChrR, whereas the other cysteine residues are invariant. This may explain why some ZAS proteins are redox-active while others are redox-insensitive.

It is intriguing that both Cys41 and Cys44 are involved in forming the first disulphide bond with Cys11, an observation that is consistent with two possible mechanisms (Figure 10). In the first, a single disulphide species forms between either Cys11 and Cys41, or Cys11 and Cys44, followed by thiol-disulphide exchange to leave a mixture of the two. Alternatively, disulphide bond formation in RsrA is naturally degenerate. In both scenarios, Cys11 must be close enough to its partner cysteine residues in three-dimensional space in order to form a mutually exclusive, inactivating disulphide with either. This could be achieved if Cys41 and Cys44 were presented to Cys11 from an α -helix, the intervening two amino acid residues placing them on the same side of the helix, and this would be consistent with the increase in RsrA helicity during oxidation. A model for how RsrA might function in redox sensing along these lines is depicted in Figure 10. We propose that zinc binding by RsrA in the reduced state coordinates the redox-sensing cysteine residues in a conformation that inhibits facile oxidation of the protein. The formation of an inactivating disulphide during oxidative stress requires loss of the metal ion (which, for example, could occur through the formation of a mixed disulphide with one of the cysteine residues coordinating the metal ion and an oxidised protein substrate). Concomitant with its loss, a major restructuring of the protein occurs wherein Cys41 and Cys44 are brought into close proximity with Cys11 through a newly

formed α -helix. The resulting disulphide bond stabilises the new conformation of RsrA that cannot now bind σ^R .

Materials and Methods

Protein purification and quantitation

RsrA and σ^R were purified and protein concentrations determined as described.^{12,13,15} In the case of RsrA, the protein was expressed with an N-terminal histidine tag that was removed subsequently by thrombin digestion, leaving three additional residues at the N terminus (Gly-Ser-His). Apo-RsrA was generated by treating the protein either with a chelating agent (5 mM EDTA for five hours in the presence of 10 mM DTT), or a strong thiol oxidant (2 mM diamide for one hour), followed by extensive dialysis against 50 mM triethanolamine (TEA) buffer (Tris, EDTA, etc. pH 7.5) containing 200 mM NaCl. Metal analysis of treated RsrA by ICP-OES (VISTA-PRO; School of Environmental Sciences, University of East Anglia) indicated that residual zinc was typically ~ 0.1 mol Zn/mol RsrA.

Alkylation of RsrA

Iodoacetamide (IAN, Sigma; 0.5 M stock in water), final concentration 40 mM, was added to RsrA (typically ~ 50 – 200 μ M) in 50 mM TEA buffer (pH 7.5) containing 200 mM NaCl. The reaction was kept at room temperature in the dark for 30 minutes and then stopped by the addition of cold trichloroacetic acid (TCA; 14% (w/v) final concentration). After one hour incubation on ice, the precipitated protein was pelleted (15 minutes in a bench-top centrifuge) and resuspended in 50 mM Tris-HCl buffer (pH 8.4). The extent of cysteine alkylation was subsequently determined by SELDI-MS using protocols essentially as described.¹⁵ Fully alkylated protein, containing seven carboxamidomethyl groups (7CAM-RsrA), yielded a relative molecular mass ($M_{r,obs}$) of $12,361(\pm 3)$ ($M_{r,calc}$ 12,361) while RsrA containing one disulphide bond, and hence containing five carboxamidomethyl groups (5CAM-RsrA), had an $M_{r,obs}$ value of $12,247(\pm 3)$ ($M_{r,calc}$ 12,247).

When alkylated proteins (5CAM and 7CAM-RsrA) were to be used in σ^R binding and transcription run-off assays or for CD measurements, the alkylation protocol was modified so that the TCA precipitation step was omitted. In these cases, excess IAN was removed by dialysis against 20 mM Tris-HCl (pH 7.5) in the dark at 4 °C.

Monitoring σ^R -RsrA complexes

Native PAGE (12% polyacrylamide (w/v)) was used to follow σ^R -RsrA complex formation as described by Kang *et al.*¹³ Protein concentrations were typically 20–30 μ M and buffer conditions 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl. The running buffer contained 0.06% (w/v) Tris base and 0.29% (w/v) glycine (pH 8.0), and included 10 mM DTT were indicated. Gels were run at 4 mA for four to five hours at 4 °C.

In vitro transcription run-off assays

In vitro transcription assays used the σ^R -specific pro-

motor *sigRp2* as the DNA template and were performed as described by Li *et al.*¹⁵ Briefly, the effects of Zn-RsrA and 7CAM-RsrA on this activity were monitored by pre-incubating these proteins with the sigma factor at 30 °C for 30 minutes prior to addition of *E. coli* core RNA polymerase, transcription buffer, NTP mix and [α -³²P]CTP in a final volume of 40 μ l. Radiolabelled transcripts were analysed on a 6% polyacrylamide gel containing 7 M urea.

Time-course of disulphide bond formation in RsrA

Apo-RsrA (1.2 mg/ml; 100 μ M) in 50 mM TEA buffer (pH 7.5) containing 200 mM NaCl was incubated with either 10 mM DTT or 10 mM DTT containing 500 μ M ZnCl₂ at 30 °C for one hour before desalting on a Superdex-75 analytical gel filtration column or a NAP10 column. Desalted RsrA was incubated at 30 °C and quenched with iodoacetamide (IAN) at various time-points up to five hours after eluting from the gel-filtration column in order to trap disulphide bonds forming in the protein through air oxidation. For comparison, the starting material (apo-RsrA \pm Zn treatment) was also alkylated. Following TCA precipitation, samples were separated by non-reducing SDS-PAGE (15%) where oxidised and reduced RsrA could be distinguished by differences in migration. The percentage of reduced RsrA was then estimated by laser densitometry of Coomassie-stained gels.

PAR assay

Oxidant-induced zinc release was monitored using 4-(2-pyridylazo) resorcinol (PAR) as described by Hunt *et al.*³⁴ Diamide (100 μ M) was added to 1 ml of RsrA (2.6 μ M) in 50 mM TEA buffer containing 200 mM NaCl and 200 μ M PAR, and the change in absorbance at 500 nm monitored spectrophotometrically over 30–60 minutes. The amount of Zn release was obtained using a standard curve by titrating ZnCl₂ into the same buffer. The extent of oxidation in a parallel protein sample was monitored simultaneously using non-reducing SDS-PAGE as described above.

Circular dichroism spectroscopy

CD spectra were obtained using a JASCO 810 spectropolarimeter using a 0.5 mm cuvette equilibrated at 30 °C. Apo-RsrA in 50 mM Tris-HCl buffer (pH 7.5) was reduced with 10 mM DTT in the presence (for Zn-RsrA) or absence (for apo-RsrA) of 500 μ M ZnSO₄ at room temperature for one hour before desalting and buffer exchange on an NAP10 column (Amersham BioScience) pre-equilibrated with 30 mM Tris-SO₄ (pH 7.4). CD spectra of eluted samples of RsrA (generally 0.5–0.7 mg/ml after the desalting step) were then measured at 30 °C in a 0.5 mm cuvette. Oxidation of apo-RsrA was allowed to proceed in the cuvette and spectra measured periodically for up to 12 hours (no further changes in CD were observed after this time). In all cases, an aliquot was removed for determining the protein concentration. Generally, five scans were averaged and spectra corrected for concentration. Secondary structure content was predicted using CD Spectra Deconvolution programme (CDNN, version 2.1, Gerald Böhm, 1997). Oxidation kinetics were monitored at 222 nm in the CD spectropolarimeter and traces fitted to double-exponential rate equations using Sigma Plot.

Generating RsrA with a single disulphide bond

Zn-RsrA in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl was oxidised by incubating with 5 mM EDTA at 30 °C for about four hours and then alkylated with IAN as described above. Alkylated protein was fractionated on a Superdex-75 gel-filtration column in the same buffer in order to separate 7CAM-RsrA (fully reduced) from 5CAM-RsrA (RsrA containing one disulphide bond). The extent of alkylation and hence disulphide bonding of purified fractions was confirmed by SELDI-MS, as described above.

Mapping the first disulphide bond in RsrA

Zn-RsrA in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl was air-oxidised for one hour at 30 °C by incubating with 5 mM EDTA, alkylated with IAN (see above), digested with chymotrypsin (2% (w/w)) at room temperature for 1.5 hours and the reaction stopped using chymotrypsin inhibitor. Initial peptide fingerprinting of RsrA chymotryptic fragments was carried out using MALDI-TOF where samples were prepared using one of two matrix solutions consisting of either saturated 3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Poole, UK) or α -cyano-4-hydroxycinnamic acid (Bruker UK Ltd, Coventry, UK) in acetone, made up and centrifuged to remove any particulates. The matrix solution was mixed 1:1 with the peptide samples, and then 0.5 μ l aliquots of each of the resulting matrix/peptide mixtures were spotted onto separate positions of a stainless steel target plate and allowed to dry. RsrA peptides were analysed on a Reflex III MALDI-ToF mass spectrometer (Bruker UK Ltd, Coventry, UK), equipped with Scout 384 ion-source, and using a nitrogen laser ($\lambda = 337$ nm) to desorb/ionise the matrix/analyte material from the sample substrate. Ions generated in this way were allowed to drift for a short delayed extraction time, before being accelerated by a potential of +25 kV. Spectra were acquired at a microchannel plate detector located after the reflectron ion-mirror. Calibration of the spectra was carried out by using a set of peptide standards (angiotensin II, angiotensin I, bombesin, adrenocorticotrophic hormone clip 1-17, adrenocorticotrophic hormone clip 18-39, somatostatin 28) on a spot adjacent to the sample. The calibrated spectra were searched against the NCBI non-redundant database (National Center for Biotechnology Information, Bethesda, MD) using the MASCOT search engine (Matrix Science, London, UK).

Peptide sequence data were obtained using quadrupole time-of-flight mass (Q-ToF) spectrometry. Chymotryptic peptides of RsrA were loaded at high flow rate (30 μ l/minute, 5% (v/v) acetonitrile, 0.1% (v/v) formic acid) onto a C18 reverse-phase trapping column (0.3 mm i.d. \times 1 mm, with 5 μ m C18 100 Å PepMap packing, LC Packings, Netherlands) using the tertiary pump of an HPLC (CapLC, Waters Corporation) for three minutes. After the initial loading time, a binary gradient consisting of 5%–80% solvent B (100% acetonitrile, 0.1% formic acid) was developed ($t = 0$ minutes, 5% B; $t = 3$ minutes, 5% B; $t = 3.5$ minutes, 15% B; $t = 15$ minutes, 80% B) and the peptides eluted through a C18 reverse-phase capillary column (75 μ m i.d. \times 50 mm column, with 5 μ m C18 100 Å PepMap packing, LC Packings, Netherlands) and directly into the nano-electrospray ion-source of a Q-ToF 2 mass spectrometer (Micromass, Manchester, UK). Spectra

were acquired in the data-dependent mode throughout the HPLC gradient, switching between a full survey mass spectrum (m/z range 400–1600; 1.2 seconds scan time; MS to MS/MS switch above threshold intensity of seven counts/second) and one MS/MS spectrum (m/z range 50–3000; 1.2 seconds scan time; charge state recognition for 2+, 3+ and 4+ ions; argon collision gas; switch back to MS survey after 3.6 seconds or below threshold of two counts/second; dynamic exclusion time of 25 seconds) recorded sequentially on the most abundant ion present in the survey mass spectrum.

In some instances, oxidised peptide mixtures were reduced with dithiothreitol (10 mM) for 30 minutes at room temperature prior to mass spectrometric analysis or alkylated with *N*-isopropylidiodoacetamide. In the latter experiments, a solution of *N*-isopropylidiodoacetamide (Molecular Probes, Leiden, Netherlands) in methanol (BDH Laboratory Supplies, Poole, UK) was added to the dithiothreitol-treated chymotrypsin digest to a final concentration of 100 mM. Alkylation proceeded for 20 minutes at room temperature in the dark after which samples were taken immediately for mass analysis.

All MS/MS spectra recorded for each sample were searched against the NCBI non-redundant database using the MASCOT search engine. MS/MS assignments were checked using the PepSeq *de novo* sequencing tool (Micromass, Manchester, UK).

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