RsrA, an anti-sigma factor regulated by redox change

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SigR (σ^R) is a sigma factor responsible for inducing the thioredoxin system in response to oxidative stress in the antibiotic-producing, Gram-positive bacterium *Streptomyces coelicolor* A3(2). Here we identify a redox-sensitive, σ^R -specific anti-sigma factor, RsrA, which binds σ^R and inhibits σ^R -directed transcription *in vitro* only under reducing conditions. Exposure to H_2O_2 or to the thiol-specific oxidant diamide caused the dissociation of the σ^R -RsrA complex, thereby allowing σ^R -dependent transcription. This correlated with intramolecular disulfide bond formation in RsrA. Thioredoxin was able to reduce oxidized RsrA, suggesting that σ^R , RsrA and the thioredoxin system comprise a novel feedback homeostasis loop that senses and responds to changes in the intracellular thiol-disulfide redox balance.

Keywords: anti-sigma factor/disulfide bond formation/
oxidative stress/redox regulation/thioredoxin

Introduction

Although the reducing environment of the cytoplasm seldom permits the formation of stable protein disulfide bonds (Gilbert, 1990; Hwang *et al.*, 1992; Dukan and Nyström, 1998), living cells have evolved several highly conserved pathways to deal with the potentially lethal consequences of thiol oxidation (Raina and Missiakas, 1997; Rietsch and Beckwith, 1998). These include the thioredoxin and the glutathione/glutaredoxin pathways (Holmgren, 1989; Åslund *et al.*, 1997; Prinz *et al.*, 1997). Both pathways use the reducing potential of NADPH ultimately to reduce the small thiol-disulfide oxidoreductases, thioredoxin and glutaredoxin, which are then able to undergo thiol-disulfide exchange with disulfide bonds in other proteins. Whereas thioredoxin is reduced by an NADPH-dependent thioredoxin reductase, glutaredoxin is reduced by the cysteine-containing tripeptide glutathione, a reaction that renders the glutathione oxidized. Glutathione is then regenerated by the action of an

NADPH-dependent glutathione reductase. The mechanisms by which organisms regulate these pathways in response to changes in intracellular thiol-disulfide redox balance are only just starting to emerge.

In *Escherichia coli* at least two components of the glutathione/glutaredoxin pathway (glutaredoxin 1 and glutathione reductase) are regulated by the oxidative stress response transcription factor OxyR (Christman *et al.*, 1985; Tao, 1997; Zheng *et al.*, 1998). It was recently shown that the activity of OxyR is modulated by reversible disulfide bond formation in OxyR itself (Zheng *et al.*, 1998), thus establishing an important link between sensing and responding to changes in intracellular thiol-disulfide redox balance.

Recently we described a novel extracytoplasmic function (ECF) sigma factor, σ^R , that is required for induction of the thioredoxin reductase/thioredoxin operon (*trxBA*) of *Streptomyces coelicolor* in response to various oxidants, including the thiol-specific oxidant diamide (Kang *et al.*, 1997; Paget *et al.*, 1998). σ^R also directs diamide-inducible transcription from *sigRp2*, one of the promoters of its own gene, thereby establishing a positive feedback loop for its own synthesis (Paget *et al.*, 1998). The transient induction *in vivo* of *trxBA* transcription upon diamide treatment suggested a model in which σ^R induces expression of the thioredoxin system in response to cytoplasmic disulfide bond formation (Paget *et al.*, 1998). Upon re-establishment of normal thiol levels, σ^R activity is switched off, resulting in down-regulation of *trxBA* and *sigR*. However, since σ^R itself contains no cysteines, there must be another component in the system to explain how σ^R activity responds to thiol oxidation and reduction. Here we identify that component and show that it is a σ^R -specific anti-sigma factor whose activity appears to be directly modulated by reversible disulfide bond formation, and which is itself a substrate for the thioredoxin system.

Results

Identification of RsrA

Analysis of the DNA sequence downstream of *sigR* revealed a gene (*rsrA* = Regulator of Sigma R) whose start codon overlaps the *sigR* stop codon (Figure 1A). *rsrA* encodes a polypeptide of 105 amino acids with M_r 11 680. Although searches against the entire protein database failed to identify obvious RsrA homologues, a search against the individual *Mycobacterium tuberculosis* genome database revealed an RsrA-related protein, here designated RshA (32% identity) (Figure 1B). RshA is encoded by a gene that lies downstream of *sigH*, which in turn encodes the closest relative of σ^R (68% identity; Paget *et al.*, 1998). *rshA* was not identified in the complete genome sequence of *M. tuberculosis* (Cole *et al.*, 1998) due to a frameshift in database submission Z95120 (at

Fig. 1. Genetic organization of the *sigR* and *rsrA* coding region and alignment of the amino acid sequences of RsrA and RsrA-related proteins encoded by ORFs lying immediately downstream of *sigR*-like genes in other bacteria. (A) The coding regions of *sigR*, *rsrA* and flanking ORFs are indicated by thick arrows. Transcription start sites are marked by thin arrows. The DDBJ/EMBL/GenBank accession No. is AJ010320. (B) The deduced amino acid sequence of RsrA was aligned with those of the products of uncharacterized ORFs lying immediately downstream of *M. tuberculosis sigH* (accession number Z95120; MtuRshA), *sigL* (Rv0736; MtuRslA), and *sigE* (Rv1222; MtuRseA); *M. xanthus rpoE1* (AF049107; MxaOrfE); *B. subtilis ylaC* (Z97025; BsuYlaD) and *sigW* (BsuYbbM), and *S. coelicolor sigT* (AJ007313; ScoRstA). The positions of conserved amino acids are marked with asterisks and colons for identical and similar matches, respectively. Dots indicate positions where only one residue deviates. Positions where more than half of the residues match similarly are shaded. Cysteine residues are highlighted in bold letters. The number of total amino acid residues as well as those aligned in the figure are shown at the end.

nucleotide 17 742; J.Parkhill, personal communication). Interestingly, *rshA* is separated from *sigH* by a 0.6 kb transposase-like pseudogene, also not recognized in database submission Z95120. The transposase-like pseudogene lies in the opposite orientation to *sigH* and *rshA*, and contains numerous stop codons.

Analysis of sequences downstream of further ECF sigma factor genes revealed several more RsrA-related proteins, including one in *S. coelicolor* itself, two more in *M. tuberculosis*, two in *Bacillus subtilis*, and one in the Gram-negative bacterium *Myxococcus xanthus* (Figure 1B). Each of these RsrA-like proteins contains several conserved residues, mostly in the N-terminal region, including the motif HXXXCXXC (Figure 1B). In addition to those shown in Figure 1B, the products of genes lying downstream of ECF sigma factor genes in *Mycobacterium smegmatis* (U87307) and *Mycobacterium avium* (U87308) also retain similar conserved residues.

RsrA inhibits σ^R -directed transcription in a redox-dependent manner

RsrA was overproduced in *E. coli* and purified from the soluble fraction of cell extracts. We examined the effect of RsrA on σ^R -directed transcription in reactions containing purified σ^R (Paget *et al.*, 1998), a *sigRp2* promoter template and *S. coelicolor* core RNA polymerase. In a control experiment we replaced σ^R with σ^{HrdB} , the principal sigma factor of *S. coelicolor*, and substituted *sigRp2* with a DNA template containing the σ^{HrdB} -dependent promoter *rrnDp2*. RsrA inhibited σ^R -directed transcription, but had no effect on σ^{HrdB} -directed transcription (Figure 2A), suggesting that RsrA might function as a σ^R -specific anti-sigma factor (a possibility confirmed below).

Given that RsrA contains seven cysteine residues, some of which are conserved among the other RsrA-related proteins (Figure 1B), and that σ^R activity responds to thiol

Fig. 2. Redox-dependent changes in anti-transcriptional activity of RsrA. (A) Inhibition of σ^R -directed transcription by purified recombinant RsrA under reducing conditions. Purified RsrA (100 or 200 nM final concentration) was added to the transcription buffer containing σ^R or σ^{HrdB} (100 nM) and DNA templates (5 nM) containing *sigRp2* (for σ^R) or *rrnDp2* (for σ^{HrdB}). The buffer contained 0.1, 1 or 5 mM DTT. Transcripts were synthesized and analysed as described in Materials and methods. (B) RsrA inhibition of σ^R -directed transcription is reversible by redox change. *In vitro* transcription assays were performed as in (A), using N_2 -saturated transcription buffer. Purified (oxidized) RsrA (240 nM) or reduced RsrA (RsrA^{red}, 240 nM) was incubated with σ^R (60 nM) in transcription buffer containing no DTT (lanes 1 and 6) or varying amounts of DTT (0.1, 0.25, 0.5 or 1 mM; lanes 2–5), H_2O_2 (1 mM, lane 7) or diamide (1 mM, lane 8).

oxidation *in vivo* (Paget *et al.*, 1998), we postulated that RsrA might regulate σ^R -directed transcription in response to changes in thiol oxidation and reduction. We therefore tested the effect of the thiol reductant dithiothreitol (DTT)

on transcription (Figure 2A). When the DTT concentration was 0.1 mM, RsrA failed to inhibit σ^R -directed transcription from the *sigRp2* promoter. In contrast, RsrA did inhibit σ^R -directed transcription when the transcription buffer contained 1 or 5 mM DTT. All the other components of the transcription system (core RNA polymerase, σ^R , and σ^{HrdB}) were insensitive to DTT concentration (Figure 2A). These data suggested that the anti-sigma factor activity of RsrA is modulated via the oxidation and reduction of cysteine residues in RsrA.

To see whether the activation of RsrA was reversible, oxidized (inactive) RsrA was reduced by treatment with 10 mM DTT and the DTT was subsequently removed under anaerobic conditions. This material (RsrA^{red}) was active in inhibiting σ^R -dependent transcription in the absence of DTT but was inactivated by exposure to either H₂O₂ or diamide (Figure 2B), indicating that RsrA can be activated and inactivated reversibly by thiol oxidation and reduction.

RsrA is an anti-sigma factor, binding specifically to σ^R only in the reduced state

The *in vitro* transcription data were consistent with the idea that RsrA functions as a redox-sensitive, σ^R -specific anti-sigma factor. To test this hypothesis, purified RsrA and σ^R were incubated together in the presence of 1 mM DTT and run on a native polyacrylamide gel. As a control, RsrA was also incubated with σ^{HrdB} . As shown in Figure 3A, a σ^R -RsrA complex was detected as a new band (lanes 2–4), whereas no complexes were detected between RsrA and σ^{HrdB} (lane 7). When DTT was omitted from the binding buffer, no complexes were detected (lane 1), suggesting that the redox state of RsrA affects its ability to bind σ^R , as predicted from the transcription assay. At a molar ratio of ~1:1, complex formation reached maximal levels and free σ^R disappeared completely. Reduced RsrA from which DTT had been removed (RsrA^{red}) also formed complexes with σ^R (lane 5), indicating that DTT itself was not directly involved in σ^R -RsrA interaction. When RsrA^{red} was treated with diamide, it lost its ability to bind σ^R and its mobility on native polyacrylamide gels changed from a slow-moving to a fast-moving form (lane 6).

The direct interaction of σ^R and RsrA was further analysed by surface plasmon resonance (SPR) using a BIAcore optical biosensor as described by Seok *et al.* (1997). When purified σ^R was exposed to immobilized RsrA, no interaction was detected in the absence of DTT in the running buffer (Figure 3B). In contrast, when 1 mM DTT was present, σ^R bound to immobilized RsrA. The binding parameters were estimated from the changes in resonance at four different concentrations of purified σ^R (1, 3, 4 and 5 $\mu\text{g/ml}$). Assuming interaction between monomeric forms of both σ^R and RsrA, the dissociation constant (K_D) for the σ^R -RsrA interaction was estimated to be 1.1×10^{-8} M. Addition of H₂O₂ abolished the interaction between σ^R and RsrA, which was restored by subsequent addition of 1 mM DTT to the running buffer, confirming the reversible modulation of RsrA anti-sigma factor activity (data not shown).

Taken together, these data suggested that the σ^R -binding activity of RsrA might be governed by the formation of reversible intramolecular disulfide bond(s), consistent with

Fig. 3. (A) Redox-dependent formation of the RsrA- σ^R complex monitored by native PAGE. σ^R (4 μM) and RsrA (2–8 μM , lanes 2–4) were incubated in 25 μl of N₂-saturated binding buffer as described in Materials and methods. In a control reaction, DTT was omitted from the binding mixture (lane 1). Reduced RsrA (RsrA^{red}, 8 μM) was incubated with σ^R (4 μM) in the binding buffer without DTT (lane 5) or with added diamide (DA; 1 mM) (lane 6). In parallel reactions, σ^{HrdB} (4 μM) was incubated with or without RsrA (8 μM) in the presence of 1 mM DTT (lanes 7 and 8). Samples were separated by electrophoresis on a native 10% polyacrylamide gel and visualized by Coomassie Blue staining. The positions of σ^R , the reduced and oxidized forms of RsrA (RsrA^{red}, RsrA^{ox}) and the σ^R -RsrA complex, are indicated. (B) Direct interaction between RsrA and σ^R measured by changes in SPR. RsrA was immobilized on the carboxymethylated dextran surface of a CM5 sensor chip as described in Materials and methods. σ^R (at the concentrations indicated on the curves in $\mu\text{g/ml}$) in HBS containing 1 mM DTT was allowed to flow over an immobilized RsrA surface to determine the kinetic parameters for the interaction between σ^R and RsrA. A control sensorgram is also shown (-DTT) in which σ^R (4 $\mu\text{g/ml}$) was passed over immobilized RsrA in the absence of DTT.

the induction of σ^R -dependent promoters *in vivo* following diamide treatment (Paget *et al.*, 1998).

RsrA forms intramolecular disulfide bonds upon oxidation

A protein containing an intramolecular disulfide bond usually migrates more quickly during SDS-PAGE than when it is fully reduced, because of a decrease in chain flexibility and hydrodynamic volume (Creighton, 1989; Loferer *et al.*, 1995; Gostick *et al.*, 1998). When purified RsrA (5.9 μM) was incubated with increasing concentrations of DTT, it migrated as multiple bands from 14 to

20 kDa apparent molecular weight on SDS–polyacrylamide gels. Given the calculated mass of 11.96 kDa for RsrA, this suggests that intramolecular, not intermolecular, disulfide bonds are formed under oxidizing conditions at the concentration of RsrA tested (data not shown). We treated RsrA with various reducing agents and analysed the effect on its mobility during SDS–PAGE (Figure 4). In addition to DTT, β -mercaptoethanol (5 mM) also decreased the electrophoretic mobility of RsrA, whereas other thiols, such as glutathione, *N*-acetylcysteine and cysteine, retarded mobility to a lesser extent. Non-thiol electron donors such as ascorbate and NADPH did not change the mobility of RsrA at all. We also examined the effect of these reducing agents on the inhibitory activity of RsrA in an *in vitro* transcription assay. Only β -mercaptoethanol was as effective as DTT; glutathione, *N*-acetylcysteine and cysteine all failed to potentiate the inhibitory activity of RsrA (data not shown).

The ability of RsrA to form intramolecular disulfide bonds in the absence of DTT was demonstrated directly using electrospray mass spectrometry (ES-MS). Free thiols present in either oxidized or reduced RsrA were alkylated with iodoacetamide, and the number of free thiols modified was determined by mass analysis, each modification causing an increase in molecular mass of 57 Da. In the

presence of 10 mM DTT, the major species detected had a molecular mass of 12 362 Da, corresponding to alkylation of all seven cysteines present in RsrA (Figure 5A). After 8 h dialysis against buffer lacking DTT, the two major species detected had molecular masses of 12 132 Da and 12 247 Da corresponding to RsrA molecules having two and one intramolecular disulfide bonds, respectively (Figure 5B). After 36 h dialysis against buffer lacking DTT, a species of 12 019 Da, corresponding to RsrA having three intramolecular disulfide bonds, was readily apparent (Figure 5C). In all these experiments, additional species were detected which suggested that a non-cysteine residue in RsrA was capable of being alkylated with reasonable efficiency (for example the 12 421 Da species in Figure 5A). The data shown in Figure 5 were generated following the reduction of any disulfide bonds in the starting material with DTT. If this reduction step was not performed, species were detected with molecular masses 6, 4 and 2 Da smaller, respectively, than the 12 019, 12 132 and 12 247 species, confirming the presence of intramolecular disulfide bonds in the oxidized material (data not shown).

Oxidized RsrA is a direct substrate for thioredoxin

In *S.coelicolor*, transcription from the *pI* promoter of the *trxB* operon is induced at least 50-fold within 10 min of exposure to diamide in a σ^R -dependent manner. However, *trxBpI* transcription falls back to uninduced levels within 40–50 min, suggesting possible negative feedback regulation of σ^R activity (Paget *et al.*, 1998). This could be explained if oxidized RsrA were a direct substrate for the thioredoxin system. To test this hypothesis, *S.coelicolor* thioredoxin was overproduced in *E.coli*, purified and reduced with DTT. When reduced *S.coelicolor* thioredoxin was used to replace DTT in the binding buffer, it allowed the formation of a complex between RsrA and σ^R (Figure 6, lanes 4 and 5). Reduced *E.coli* thioredoxin stimulated the σ^R -binding activity of RsrA to the same extent (Figure 6, lane 6), but other reducing agents such as *N*-acetylcysteine

Fig. 4. Effect of reducing agents on the electrophoretic mobility of RsrA. RsrA protein (5.9 μ M) was incubated with various reducing agents [5 mM each of *N*-acetylcysteine (NAC), L-ascorbic acid (ASC), L-cysteine (CYS), NADPH, glutathione (GSH), β -mercaptoethanol (β -ME) and DTT] or triple-distilled water (TDW) for 10 min at room temperature. Samples were run on a 15% polyacrylamide gel containing 0.1% SDS. Proteins were visualized by staining with Coomassie Brilliant Blue. Lane M contains molecular weight markers.

Fig. 5. Detection of disulfide bond formation in RsrA by ES-MS. The molecular mass of RsrA was determined after alkylation of free thiols using iodoacetamide. Alkylation was performed on RsrA that was either fully reduced in buffer containing 10 mM DTT (A) or had been dialysed to remove the DTT for 8 h (B) or 36 h (C). The theoretical molecular masses for the various redox states of RsrA after modification are 12 361 Da (fully reduced), 12 247 Da (one disulfide bond), 12 133 Da (two disulfide bonds) and 12 019 Da (three disulfide bonds). The 12 421 Da species seen in (A) and the 12 190 Da species seen in (C) probably represent species that have been modified at an additional non-thiol residue.

Fig. 6. Reduction of RsrA by thioredoxin. Reduced *S.coelicolor* thioredoxin (Trx^S; 4, 12, 24 μ M; lanes 3–5), reduced *E.coli* thioredoxin (Trx^E; 18 μ M; lane 6) or 1 mM DTT (lane 7) were incubated with RsrA (12 μ M) and σ^R (4 μ M) in the binding buffer as described in the legend to Figure 4A. As negative controls, σ^R (20 μ M) was incubated with either RsrA in the absence of any reductant (lane 1) or with *S.coelicolor* thioredoxin (lane 2). The generation of reduced thioredoxin and all subsequent steps were carried out under anaerobic (N_2 -flushed) conditions.

and glutathione did not stimulate the σ^R -binding activity of RsrA (data not shown). Reduced *E.coli* thioredoxin also caused RsrA to inhibit σ^R -directed transcription in the absence of DTT, confirming the above result (data not shown).

Discussion

We have shown that RsrA is a σ^R -specific anti-sigma factor, that loss of anti-sigma factor activity correlates with the formation of one or more intramolecular disulfide bonds and that oxidized RsrA is a substrate for the thioredoxin system *in vitro*. These findings lead us to propose the model shown in Figure 7, in which σ^R activity is modulated in response to oxidation and reduction of RsrA. Under unstressed conditions, RsrA exists in its reduced form. Exposure to oxidative stress induces the formation of one or more intramolecular disulfide bonds in RsrA, which cause it to lose its affinity for σ^R , thereby allowing σ^R to bind core RNA polymerase and induce transcription of the *trxB* operon. Expression of the thioredoxin system in turn leads to reduction of RsrA to its active state in which it re-binds σ^R , thereby shutting off σ^R -dependent transcription and completing the feedback regulatory loop. The post-translational regulation of σ^R ensures a rapid and effective response to oxidative stress. In addition, σ^R positively autoregulates its own expression via the *sigRp2* promoter (Paget *et al.*, 1998). As a consequence, oxidative stress not only activates σ^R post-translationally, but also induces its *de novo* synthesis. Although RsrA can form up to three intramolecular disulfide bonds *in vitro*, it is not yet known whether all three can form *in vivo*, or whether the number of disulfide bonds in RsrA affects its anti-sigma activity. Consistent with the biochemical demonstration that RsrA is a σ^R -specific anti-sigma factor, we have recently shown that a constructed *rsrA* null mutant substantially overexpresses the thioredoxin system (M.S.B.Paget and M.J.Buttner, unpublished data).

There is a striking comparison to be made between RsrA and OxyR of *E.coli*. OxyR is a positive regulator

Fig. 7. Model for a feedback regulatory loop that modulates expression of the thioredoxin system in response to oxidative stress. Under unstressed conditions, σ^R is sequestered by binding to the reduced form of RsrA [RsrA-(SH)₂]. Upon oxidative stress, RsrA is inactivated by the formation of intramolecular disulfide bond(s) (RsrA-S₂), releasing σ^R . σ^R then binds core RNA polymerase and directs transcription of its own operon (*sigR-rsrA*) and the thioredoxin (TRX)/thioredoxin reductase (TR) genes (*trxB*). The induction of the thioredoxin system shifts the intracellular thiol–disulfide balance and reduces RsrA to its active state in which it rebinds σ^R , thereby returning the system to the pre-stimulus state.

of the oxidative stress response which controls the induction of two components of the glutaredoxin system, glutaredoxin 1 and glutathione reductase, and is activated by the reversible formation of an intramolecular disulfide bond in OxyR itself (Dempsey, 1998; Zheng *et al.*, 1998). Whereas OxyR is a positive regulator and the oxidized form of the protein is active, RsrA is a negative regulator (anti-sigma factor) and the reduced form of the protein is active. In addition, glutaredoxin 1, in the presence of glutathione, can catalyse the reduction of OxyR (Zheng *et al.*, 1998), thereby creating a feedback homeostasis loop for its own expression analogous to the one proposed here for RsrA and the thioredoxin system in *S.coelicolor*. Streptomycetes lack glutathione. Instead, they possess an abundant low-molecular-weight sugar-containing monothiol called mycothiol [2-(*N*-acetylcysteinyl) amido-2-deoxy- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-myo-inositol], the biosynthetic genes for which have yet to be identified (Aharonowitz *et al.*, 1993; Cohen *et al.*, 1993; Newton *et al.*, 1996). There is no published information about glutaredoxin-like proteins in streptomycetes.

In addition to OxyR, two other proteins have recently been described that are activated by intramolecular disulfide bond formation. Flp is a homodimeric transcription factor of the CRP-FNR family, found in *Lactobacillus casei* (Gostick *et al.*, 1998). Although the cellular targets of Flp are unknown, a consensus binding sequence has been determined, and its ability to bind this site has been shown to require the formation of an intra-subunit disulfide bond (Gostick *et al.*, 1998). Similarly, Jakob *et al.* (1999) have shown that *E.coli* Hsp33, a member of a newly discovered family of heat shock proteins, is a potent molecular chaperone that is activated by intramolecular disulfide bond formation. The mechanisms that control the activities of OxyR, Flp, Hsp33 and RsrA suggest that thiol–disulfide exchange might play a much more widespread role in regulating cytoplasmic proteins than had previously been anticipated (Åslund and Beckwith, 1999).

The occurrence of *rsrA*-related genes downstream of ECF sigma factor genes in several bacterial genera

(Figure 1B) suggests that RsrA might be the first example of a family of anti-sigma factors regulated by related mechanisms. As noted previously (Paget *et al.*, 1998), there is a gene encoding a σ^R -like sigma factor in *M.tuberculosis* (*sigH*; Cole *et al.*, 1998), and an *rsrA* homologue is found downstream (*MtuRshA*; Figure 1B). In addition, a sequence upstream of the *M.tuberculosis* *trxBA* operon shows striking similarity to the σ^R -dependent *trxBp1* promoter of *S.coelicolor* (Paget *et al.*, 1998), suggesting that a σ^R -RsrA-like system may modulate expression of the thioredoxin system in mycobacteria. Intriguingly, *M.tuberculosis* *sigH* and *rshA* are separated by a 0.6 kb transposase-like pseudogene, suggesting that their transcription might be uncoupled. These observations may have important implications for the regulation of σ^H activity in this actinomycete pathogen, and hence its ability to withstand oxygen-dependent killing during infection.

Materials and methods

Overproduction and purification of RsrA

RsrA protein was overproduced in *E.coli* from the *rsrA* gene of *S.coelicolor* A3(2) cloned in pET15b (Novagen). The recombinant protein with an N-terminal hexa-histidine sequence was purified through a Ni-NTA column as recommended by the manufacturer (Novagen). It was eluted at 100–200 mM imidazole. Following thrombin treatment to remove the His-tag, recombinant RsrA, containing three additional N-terminal residues (gly-ser-his), was concentrated by ultrafiltration (Amicon membrane, 3 kDa cut-off size), exchanged into TGE buffer [10 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 10% (v/v) glycerol], and further purified by Resource-Q (Pharmacia) chromatography in the same buffer. RsrA preparation eluted at ~0.4 M NaCl in TGE was >90% pure as judged by Coomassie Blue staining of SDS-polyacrylamide gels. Reduced RsrA was prepared by incubating RsrA in 10 mM DTT at 37°C for 5 h, followed by dialysis against N₂-saturated storage buffer [10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 0.1 M KCl, 0.1 mM EDTA, 50% (v/v) glycerol] to remove DTT.

Purification of *S.coelicolor* thioredoxin from *E.coli*

The *S.coelicolor* *trxA* gene was cloned in pET21b (Novagen) and overexpressed in *E.coli* BL21 (DE3). Following induction, cells were resuspended in lysis buffer [20 mM Tris-HCl pH 7.9, 10% (v/v) glycerol, 5 mM EDTA, 1 mM DTT, 10 mM MgCl₂, 0.15 M NaCl] and sonicated. The clarified lysate was loaded directly on to a Q-Sepharose CL 6B column. A gradient of 0.15–0.6 M NaCl in TGE buffer [10 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 20% (v/v) glycerol] was applied at 50 ml/h and thioredoxin activity was assayed using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as substrate as described previously (Sedlak and Lindsay, 1968). Thioredoxin eluted at 0.2 M NaCl was concentrated by ammonium sulfate precipitation (66%) and further purified by Superdex-75 gel chromatography. Peak fractions were treated with 10 mM DTT at 37°C for 5 h, dialysed against storage buffer [10 mM Tris-HCl pH 7.9, 10 mM MgCl₂, 0.1 M KCl, 0.1 mM EDTA, 50% (v/v) glycerol] to eliminate DTT, and tested for their ability to reduce RsrA. All buffers were purged with N₂ gas (99.999%).

In vitro transcription assay

In vitro transcription assays were performed as described previously (Paget *et al.*, 1998). Purified RsrA (100–200 nM final concentration) was added to the transcription buffer containing σ^R or σ^{HrdB} (100 nM), and DNA templates (5 nM) containing *sigRp2* (for σ^R) or *rmDp2* (for σ^{HrdB}) in 12 μ l transcription buffer with varying amounts of DTT. Following incubation at 30°C for 20 min, *S.coelicolor* core RNA polymerase (50 nM) was added and the mixture was incubated for a further 15 min before initiating RNA synthesis with NTP mix (0.4 mM) containing [α -³²P]CTP (400 Ci/mmol) and heparin (0.12 mg/ml) to ensure single-round transcription. Transcripts were analysed by autoradiography after separation on a 5% polyacrylamide gel containing 7 M urea.

Native PAGE analysis of σ^R -RsrA interaction

σ^R (4 μ M) and RsrA (2–8 μ M) were incubated in 25 μ l of N₂-saturated binding buffer [40 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 0.01 mM

EDTA, 20% (v/v) glycerol] in the presence of 1 mM DTT at 30°C for 30 min. Reduced RsrA was incubated with σ^R in the binding buffer without DTT, or with added diamide (1 mM). Samples were separated by electrophoresis on a native 10% polyacrylamide gel at 15 V for 16 h. The acrylamide solution was extensively de-gassed before gel casting and the electrophoresis buffer was saturated with N₂ gas. Proteins were visualized by Coomassie Blue staining.

Measurement of surface plasmon resonance

To probe the real-time interaction of σ^R with RsrA under different conditions, RsrA was immobilized on to the carboxymethylated dextran surface of a CM5 sensor chip by amine coupling according to the manufacturer's instructions (Pharmacia Biosensor AB, Uppsala, Sweden). RsrA solution (10 μ g/ml) in 40 μ l coupling buffer (10 mM sodium acetate pH 4.0) was allowed to flow over a sensor chip at 5 μ l/min to couple the protein to the matrix by a NHS/EDC {a mixture of 0.1 M *N*-hydroxysuccinimide and 0.1 M 1-ethyl-3[(3-dimethylamino)propyl]-carbodiimide} reaction. Unreacted NHS was inactivated by injecting 40 μ l of 1 M ethanolamine-HCl pH 8.0. A blank surface was prepared to examine non-specific protein interactions with the carboxymethylated dextran surface, if any, by activation and inactivation of the sensor chip without any protein immobilization. Assuming that 1000 resonance units (RU) corresponds to a surface concentration of 1 ng/mm², RsrA was immobilized to a surface concentration of 2.5 ng/mm². HBS (10 mM HEPES, pH 7.2 with 150 mM NaCl) was used as a standard running buffer and was introduced at a flow rate of 10 μ l/min. The indicated concentrations of σ^R (in μ g/ml) in the standard running buffer containing 1 mM DTT were allowed to flow over an immobilized RsrA surface for the determination of kinetic parameters for the interaction between σ^R and RsrA. A control sensorgram is also shown (–DTT) in which 4 μ g/ml of σ^R was flown over RsrA surface in the absence of DTT. The sensor surface was regenerated between assays by sequential injections of 5 μ l of 5 mM EDTA and 10 μ l of 0.01% SDS to remove bound analyte. Kinetic parameters for the interaction of σ^R with immobilized RsrA were determined using the BIA-evaluation 2.1 software.

Electrospray ionization mass spectrometry (ES-MS)

Reduced thrombin-cleaved RsrA (500 μ l of 1 mg/ml in 100 mM Tris-HCl, 1 mM EDTA, 10 mM DTT, pH 8.5) was dialysed against 6 l dialysis buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.5) with three changes for either 8 or 36 h. Free thiols in RsrA were alkylated by the addition of 0.5 M iodoacetamide to a final concentration of 100 mM, followed by incubation at 25°C for 30 min. The alkylated samples were extensively dialysed against ice-cold dialysis buffer in the dark, then dialysed against 10 mM Tris-HCl pH 8.5. Electrospray ionization mass spectrometry spectra were obtained on a Micromass VG Platform using 1:1:0.001 CH₃CN:H₂O:HCOOH as the sample solvent and horse heart myoglobin as a standard. Samples were treated with 10 mM DTT for 10 min and then mixed with the sample solvent prior to analysis. Approximately 150–200 pmol of protein was analysed per run. Data were acquired over the *m/z* range 700–17 000 with 10 scans averaged and processed using the supplier's MassLynx software. The data presented are representative of three separate experiments. Control experiments using guanidine-HCl-denatured RsrA indicated that all free thiols were modified.

Acknowledgements

We are grateful to Drs Sa-Ouk Kang and Jae-Yul Kwon for many stimulating discussions, to Joon-Hee Lee, You-Hee Cho, Maureen Bibb and Rick Evans-Gowing for technical assistance, and to David Hopwood, Ray Dixon, Mervyn Bibb and Keith Chater for critical reading of the manuscript. This work was supported by the Korean Science and Engineering Foundation (to J.H.R.) through the Research Center for Molecular Microbiology at Seoul National University, by BBSRC grant GR/J67994 (to M.J.B.), by a Lister Institute research fellowship (to M.J.B.), by a grant-in-aid to the John Innes Centre from the BBSRC and by the UK–Korea (BBSRC–KOSEF) Joint Programme for Actinomycete Research.

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Received May 4, 1999; revised and accepted June 7, 1999