RsmA is an anti-sigma factor that modulates its activity through a [2Fe-2S] cluster cofactor

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The rsmA gene of Streptomyces coelicolor lies directly upstream of the gene encoding the group 3 sigma factor σM. The RsmA protein is a putative member of the HATPase_c family of anti-sigma factors, but is unusual in that it contains seven cysteine residues. Bacterial two-hybrid studies demonstrate that it interacts specifically with σM and in vitro studies of the purified proteins by native PAGE and transcription run offs confirmed that they form a complex. Characterization of RsmA revealed that it binds ATP and that, as isolated, contains significant quantities of iron and inorganic sulfide, in equal proportion, with spectroscopic properties characteristic of a [2Fe-2S] cluster-containing protein. Importantly, the interaction between RsmA and σM is dependent on the presence of the iron-sulfur cluster. We propose a model in which RsmA regulates the activity of σM. Loss of the cluster, in response to an as yet unidentified signal, activates σM by abolishing its interaction with the anti-sigma factor. This represents a major extension of the functional diversity of iron-sulfur cluster proteins.

Iron-sulfur proteins constitute a ubiquitous and hugely functionally diverse family of proteins that contain clusters of iron atoms bridged by acid-labile sulfides. Protein cysteine residue side chains normally complete the tetrahedral coordination of each iron. The capacity to delocalize electrons over both iron and sulfur ions makes iron-sulfur clusters ideally suited to roles in electron transfer pathways such as the respiratory and photosynthetic electron transfer chains and in nitrogen fixation. They are also involved in substrate binding and activation (eg in dehydratases such as aconitase and the radical SAM family of enzymes), sulfur donation (eg biotin synthase), and the regulation of enzyme activity (eg phosphoribosylpyrophosphate amidotransferase (see (1) and references therein).

In addition to these roles, there are also several well-characterized examples of iron-sulfur clusters involved in regulation of gene expression in bacteria. SoxR, which contains a redox active [2Fe-2S] cluster (2), responds to oxidative stress and activates SoxS, another transcription factor that, in turn, activates the transcription of the target genes (3,4). FNR is a CRP-like transcription regulator that controls the expression of genes involved in the adaptation to anoxia (5). The sensory domain (N-terminus) binds a [4Fe-4S] cluster that degrades in the presence of O2 to a [2Fe-2S] cluster via a two-step mechanism involving a [3Fe-4S] cluster intermediate (6), with a concomitant loss of DNA-binding activity (7,8). IscR, a transcription regulator involved in iron-cluster synthesis, requires the presence of a [2Fe-2S] cluster for its activity (9).

Transcriptional regulation also occurs through the composition of RNA polymerase holoenzyme (10). In addition to the principle sigma factor, essential for housekeeping functions, many bacteria contain several alternative sigma factors that are required for cellular responses to various stress signals. These alternative sigma factors are sometimes regulated via specific inhibitor proteins, called anti-sigma factors (11). These proteins respond to a relevant internal signal resulting from a stress condition, which, by a variety of mechanisms, results in dissociation of the bound sigma factor, enabling its interaction with RNA polymerase. Several mechanisms have been characterized to date involving: secretion of the anti-sigma factor out of the cell (as in FlgM (12)); proteolytic degradation of the anti-sigma factor (as in E. coli RseA (13)); partner switching, which necessitates sequestration of the anti-sigma factor by an anti-anti-sigma factor (as in SpoIIB/SpoIIBA and RsbW/RsbV (14) or, inactivation of the anti-sigma factor through stress-induced oxidation. The first example of the latter mechanism was demonstrated for Streptomyces coelicolor RsrA, the archetypal member of a growing family of zinc containing anti-sigma
factors (ZAS) (15). The family is characterized by the presence of a highly conserved Hx6CxxC motif implicated in Zn\(^{2+}\) coordination (16). Under oxidative stress conditions, the Zn\(^{2+}\) ion is expelled, with concomitant formation of a disulfide bond (17). *Rhodobacter sphaeroides* ChrR, another member of the ZAS family, responds to singlet oxygen generated as a by-product of photosynthesis (18), although the mechanism by which this occurs is not yet clear.

Here we report studies of a putative member of the HATPase\(_c\) anti-sigma factor family from *Streptomyces coelicolor*. We demonstrate, using bacterial two-hybrid studies, native PAGE\(^2\) and *in vitro* transcription that the protein interacts specifically with the group 3 sigma factor \(\sigma^{M}\), which has been shown to be a part of the regulatory cascade that is initiated in response to osmotic shock, under the control of \(\sigma^{B}\) (19). The anti-sigma factor, subsequently referred to as RsmA, contains, upon isolation, iron and inorganic sulfide with spectral properties characteristic of a [2Fe-2S] cluster. We show that the presence of the cluster modulates the interaction between RsmA and \(\sigma^{M}\), leading to a model in which the cluster plays a central role in controlling the switch between active and inactive forms of RsmA.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and culture conditions**– Media and culture conditions for *E. coli* were as described previously (20). For plasmid selection in *E. coli* LB broth was supplemented with the appropriate antibiotic at its working concentration: kanamycin (25 \(\mu\)g/ml); ampicillin (100 \(\mu\)g/ml); chloramphenicol (25 \(\mu\)g/ml). The ß-galactosidase assay was carried out in *E. coli* cya strain DHM1 (F' glnV44 (AS) recA1 endA gyrA96 thi-1 hsdR17 spoT1 rfbD1 cya-854) (21).

**DNA manipulation**– Over-expression vectors were generated by using the amplified open reading frame of sigM and rsmA from *S. coelicolor* cosmid 5F8. *NdeI* and *EcoRI* restriction sites were engineered into the 5' and 3' primers, sigMX1 and sigMX2 and rsmAX1 and rsmAX2 (for a list of primers, see Supplementary Data, Table S1), respectively, to maintain unidirectional cloning. The PCR product was blunt ended and cloned into pUC18, generating pAG10 (\(\sigma^{M}\)) and pAG11 (RsmA), respectively, and the inserts verified by sequencing. *NdeI/EcoRI* fragments (containing the sigM and rsmA genes, respectively) were then ligated into pET28a (Novagen). The resulting plasmids, pAG110 (\(\sigma^{M}\)) and pAG111 (RsmA) were introduced into *E. coli* BL21DE3/pLysS (22).

Two-hybrid expression plasmids were generated by PCR-amplification of the genes of interest using primers sigML1 and sigML2 and rsmAL1 and rsmAL2, respectively, and *S. coelicolor* 5F8 cosmid DNA. *EcoRI* and *PstI* restriction sites introduced at the 5' and 3' ends, respectively, were subsequently used for cloning into pKT25 and pUT18 (23). The resulting vectors expressed hybrid proteins such that the proteins of interest were fused to the T25 or T18 part of adenylate cyclase, respectively.

**Over-expression and purification of \(\sigma^{M}\)**– Typically 8 \(\times\) 250 ml LB was inoculated with *E. coli* BL21DE3/pLysS containing pAG110. At an OD\(_{600}\) of 0.6, the cultures were induced with 0.4 mM IPTG and incubated with shaking (200 rpm) at 25 °C for 16 h. Subsequent purification was performed at 4 °C unless otherwise indicated. Cells were harvested by centrifugation at 7000 rpm for 15 min and the pellet resuspended in 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 200 \(\mu\)g/ml lysozyme, 1 mM benzamidine, 0.1 mM PMSF) and sonicated. Cell lysates were centrifuged at 12000 rpm for 20 min and the pellet, containing \(\sigma^{M}\) as inclusion bodies, was twice resuspended in 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 2% sodium deoxycholate and centrifuged at 12000 rpm for 20 min. The washed pellet was solubilised in 50 mM Tris/HCl pH 6.8, 100 mM NaCl, 6 M guanidine hydrochloride, 10% (v/v) glycerol. Following centrifugation at 20000 rpm for 30 min, the supernatant was dialyzed against two changes (2 l) of 50 mM Tris/HCl pH 6.8, 100 mM NaCl, 10% (v/v) glycerol for 6 h. The dialysate was centrifuged to remove any mis-folded protein and further purified by application to a Heparin column (GE Healthcare) followed by elution in a KCl gradient (0 – 1 M) in the same buffer. \(\sigma^{M}\) containing fractions, which eluted at 800 mM KCl, were pooled and desalted/concentrated using a disposable centrifugation concentrator (20 ml, Vivascience) with a 10 kDa cut off.

**Over-expression and purification of RsmA**– Typically 8 \(\times\) 250 ml LB was inoculated with *E. coli* BL21DE3/pLysS containing pAG111. At an OD\(_{600}\) of 0.6, the cultures were induced with 0.1 mM IPTG and incubated with shaking (100 rpm) at 25 °C for 4 h. Cells were lysed and RsmA inclusion bodies washed as described above for \(\sigma^{M}\). Inclusion bodies were solubilised by incubating for 5 min at 50 °C and 15 mins at room temperature in 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 10% (v/v) glycerol, 0.3% (w/v) n-laurylsarcosine, 1 mM DTT.
The solution was centrifuged at 20000 rpm for 30 min to remove any non-solubilised protein. The resulting reddish-brown supernatant was applied to a gel filtration column (PD10, GE Healthcare), equilibrated with 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, to remove any excess detergent. Eluted protein was stored anaerobically below 4 °C. Apo-RsmA was acquired by prolonged exposure to aerobic conditions (typically 16 hours). Protein assays confirmed that little or no protein was lost in the process, whereas iron and sulfide assays on the gel filtered sample verified that no detectable iron or sulfide remained associated with the protein, and bands characteristic of an iron-sulfur cofactor were absent from the UV-visible spectrum.

β-galactosidase assay– Assays were performed on chloroform treated bacterial suspensions using the modified Miller protocol (24). The enzyme activity was expressed as Miller units using Equ. 1, in which T is the reaction time (min) and V is the volume (ml) of culture used in the assay.

\[
\text{MillerUnits} = \frac{1000 \times (OD_{600} - 1.75 \times OD_{500})}{T \times V \times OD_{600}}
\] (1)

Native gels– Native gels (7% acrylamide) were cast horizontally using a gel-bonded assembly in which the upper glass plate had a slit in the middle allowing for a comb to be fitted. Gels were run at 40 mA for the required time at 4 °C in Tris-glycine buffer, pH 8.3.

In vitro transcription assay– In vitro transcription reactions were performed as described previously (25). Primers ctc1 and ctc2 were used to generate a fragment of pCB340, carrying the B. subtilis ctc promoter and coding DNA, which was used as the template in all of the reactions, resulting in a ~250 nucleotide transcript. Purified \( \sigma^M \) (37 nM) was incubated with E. coli RNA polymerase (0.1 unit, Epicentre) in the absence or presence of as isolated RsmA (400 nM) and the DNA template, and the reaction initiated with NTP mix and [\( \alpha^32P \)] CTP (3000 Ci mmol\(^{-1}\); GE Healthcare).

ATP assay– The ATP content of RsmA was analyzed using a bioluminescence luciferase assay (Biaffin). Briefly, D-luciferin and the thermo-stable firefly luciferin were mixed in the proprietary reaction buffer before mixing in 1:1 ratio with the unknown RsmA sample (in 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 1 mM DTT; 10% (v/v) glycerol). Samples were incubated at room temperature for 10 min prior to measurement. A calibration curve was constructed by using ATP solutions of known concentration in place of RsmA samples. Luminescence was recorded on a Bio-tek Instrumentals FLx800 microplate fluorescence reader in bioluminescence mode (emission at 560 nm).

Spectroscopy– UV-visible absorption spectra were recorded using a Jasco V-550 spectrophotometer. CD spectra in the near-UV and visible range (300-800 nm) and far-UV range (190-260 nm) were recorded using a Jasco J-810 spectropolarimeter interfaced to a PC. CD intensity is expressed as molar ellipticity ([\( \theta \)]) in units of deg cm\(^2\) mmol\(^{-1}\) for the far UV region, and as molar CD extinction coefficient (\( \Delta \epsilon \)) in units of M\(^{-1}\) cm\(^{-1}\), with reference to the cluster cofactor concentration, for the UV-visible region. Spectra were corrected for intensity due to the buffer. EPR spectra were measured with a X-band spectrometer (Bruker ER200D with an EPS 3220 computer system) fitted with an ESR9 liquid helium flow cryostat (Oxford Instruments). Spin intensities of paramagnetic samples were estimated by double integration of EPR spectra using 1.25 mM Cu(II), 10 mM EDTA as the standard. Fluorescence emission spectra were recorded using a Perkin Elmer LS55 luminescence spectrophotometer with excitation at 408 nm, and slit widths of 5 nm. Fluorescence titrations of RsmA were carried out using a microsyringe (Hamilton) to add sub-stoichiometric quantities of TNP-ATP or ATP. Analysis of the binding of TNP-ATP, which was complicated by the fact that the unbound nucleotide contributed to emission intensity at 560 nm, was achieved using the program Dynafit (Biokin, CA, USA). Competition binding titration experiments were analyzed in terms of the reaction in Equ. 2.

\[
\text{RsmA-TNP-ATP} + \text{ATP} \rightleftharpoons \text{RsmA-ATP} + \text{TNP-ATP}
\] (2)

For which the exchange constant expression can be written as in Equ. 3.

\[
K_{\text{ex}} = \frac{K_{\delta \text{ATP}}} {K_{\delta \text{TNP-ATP}}} = \frac{[\text{RsmA-ATP}] [\text{TNP-ATP}]} {[\text{RsmA-TNP-ATP}] [\text{ATP}]}
\] (3)

For each point in the titration, each term of the exchange expression was calculated and a value for \( K_{\text{ex}} \) obtained. An average value of \( K_{\text{ex}} \) was determined (titration points at the extremities were omitted from the averaging calculation), and Equ. 3 used to determine the \( K_j \) for ATP binding to RsmA.

Other methods– Protein concentrations were determined using the BCA method (26) using a
Two *B. subtilis* anti-sigma factors, RsbW and SpoIIAB, were used as probes in a BLAST search (30) against *S. coelicolor* proteins. The family of anti-sigma factors to which these proteins belong possesses a nucleotide binding domain (called HATPase_c (31)) that is similar to the ATP binding domain of histidine kinases. Thus, the initial low stringency BLAST search highlighted some two-component histidine kinases. The two families were separated based on differences in size and domain organization. The potential candidates, satisfying the size requirement of approximately 250 residues, and lacking any other obvious domain structure belonging to the histidine kinase family, were further confirmed by iterative PSI-BLAST searches against the available protein database. Sequence alignments were performed against *B. stearothermophilus* SpoIIAB using the ClustalW program (32) and manually refined using the conserved motifs (the N-, G1-, G2 and G3-box) of the GHKL superfamily (33).

**RESULTS**

*Identification of RsmA−* Searches of the *S. coelicolor* proteome revealed 27 putative anti-sigma factors belonging to the RsbW/SpoIIAB-type HATPase_c protein kinase anti-sigma factor family (34). Although the proteins share little overall primary sequence identity, they each contain, to varying degrees, the conserved motifs of the HATPase_c family, which is itself part of the GHKL superfamily of ATPases (33), Fig. 1. Sco7313 (subsequently referred to as *rsmA* (regulator of sigma M $\Delta$) is located immediately upstream of *sigM*, *rsmA* encodes a putative soluble protein containing seven cysteine residues, including a CXXC motif that is found as part of iron-sulfur cluster binding motifs, as well as those of thioredoxin-like proteins and Zn$^{2+}$-binding proteins, and has a high proportion of charged residues. Neither of these are typical characteristics of this anti-sigma factor family.

*RsmA contains a [2Fe-2S] cluster−* RsmA was overproduced with a N-terminal his-tag in *E. coli* and purified from inclusion bodies using n-laurylsarcosine, as described in Experimental Procedures. The near-UV-visible absorption spectrum of aerobically purified RsmA contained major bands at 280 nm, 320 and 420 nm, with shoulders present at 460 nm and 550 nm, Fig. 2A, and was characteristic of that arising from a [2Fe-2S] cluster-containing protein (35).

CD spectra of metal cofactors provide a more detailed insight into differences in cluster properties and can be used to distinguish between different iron-sulfur cluster structures. The near-UV-visible CD spectrum of as isolated RsmA, Fig. 2B, contained negatively signed peaks at 348, 390, 530 and 620 nm, a dominant positive peak at 460 nm and low intensity positive peaks at 315, 370, 600 and 700 nm. These features resemble those of [2Fe-2S] type clusters (36, 37), in which only minor differences in peak maxima, probably reflecting differences in the local protein environment, are usually found.

Protein, iron and labile sulfide analyses revealed sub-stoichiometric, but near equivalent concentrations of iron and sulfide (iron:sulfide = 1:0.91 ± 0.01). The metal to protein ratio varied somewhat from one preparation to another, with an average of ~0.7: 1. Taken together, the data indicate that RsmA contains a [2Fe-2S] cluster and is isolated, on average, as ~35% holo-protein. This is based on the assumption that each RsmA is capable of binding one cluster, a conclusion supported by gel filtration experiments showing that the presence of the cluster does not appear to influence the association state of the protein, see Supplementary Data.

As isolated, the iron-sulfur cluster of RsmA is present in an EPR-silent form, consistent with a cluster oxidation state of [2Fe-2S]$^{2+}$ (diamagnetic, S = 0, not shown). Attempts to reduce RsmA by anaerobic addition of sodium dithionite did not give rise to an EPR signal, consistent with UV-visible spectra which demonstrated loss of the cluster (but not the protein) on addition of this reductant (data not shown). Anaerobic treatment of RsmA with a 20-fold excess of the milder reducing agent sodium ascorbate, followed by incubation at ambient temperature, resulted in a low intensity EPR-active species, with g-values at ~2.047, 2.01 and 1.97, which arise from a rhombic $S = \frac{1}{2}$ species, Fig. 2C. Simulation of the signal was consistent with this, although the central signal at g = 2.01 was of higher intensity than predicted. Radical species commonly give rise to isotropic signals at this g-value and so a minor radical species may be present here. However, such signals normally persist up to room temperature; in this case, the signal exhibited homogeneous temperature dependence and was no longer detected above 40 K (not shown). The $S = \frac{1}{2}$
species was assigned to a minor component of a $[2\text{Fe-2S}]^{1+}$ cluster. Integration versus a Cu$^{2+}$ standard revealed that ~10% of clusters present were in the reduced, EPR-active state. EPR signals arising from $[2\text{Fe-2S}]^{1+}$ clusters exhibit significant variation in symmetry and anisotropy (38) from the rhombic signals of plant ferredoxins (39) and Rieske-type clusters (40) to the axial ones of mammalian and some bacterial ferredoxins (41,42).

A usual feature is that the value of $g_{av}$ is $<2$ (43,44), although there are examples, particularly in the xanthine oxidase family, in which this is not the case (45). Here, the observed $g_{av}$ value is also $>2$, indicating that this is an unusual signal for a $[2\text{Fe-2S}]^{1+}$ cluster.

RsmA is an anti-sigma factor– To characterize an in vivo physical interaction between RsmA and $\sigma^M$, a bacterial two-hybrid system, based on functional complementation of the Bordetella pertussis adenylate cyclase fragments T25 and T18 (23), was utilized. DNA fragments encoding the two proteins of interest were cloned into the pKT25 and pUT18 vectors and were co-transformed into E. coli strain DHM1, along with the empty vectors, in all possible pairwise combinations. The efficiency of the interaction was quantitated by measuring $\beta$-galactosidase activities in liquid cultures, see Experimental Procedures.

The vectors expressing RsmA and $\sigma^M$ fused to either the T25 or T18 fragment of adenylate cyclase gave a substantial complementation signal when co-expressed together, in both configurations. None of the other combinations gave a significant complementation signal, Fig. 3, consistent with the proposal that RsmA and $\sigma^M$ constitute an anti-sigma factor/sigma factor pair. To assess the specificity of the interaction between RsmA and $\sigma^M$, sigB, encoding another S. coelicolor $\sigma$M, was also cloned into pKT25 and pUT18 and tested against the RsmA-encoding vectors described above. Although above baseline levels for both configurations, the observed signals did not indicate a significant interaction (Fig. 3), suggesting that RsmA specifically interacts with $\sigma^E$.

Interactions between RsmA and $\sigma^M$ (also over-produced as a His-tagged protein in E. coli) in vitro were probed by native PAGE. We were unable to resolve both proteins simultaneously using standard vertical electrophoresis; despite a predicted $pI < 7.5$, $\sigma^M$ ran as a basic protein, and although RsmA has a predicted $pI \sim 8.8$, tightly associated n-laurylsarcosine molecules (which remain bound after exchange into non-detergent buffer) imparted an overall negative charge to RsmA at neutral/high pH. Thus, horizontal native PAGE, in which both acidic and basic proteins can be analyzed, was employed. Equimolar concentrations of the sigma and anti-sigma factors were mixed and incubated for 15 minutes at room temperature prior to loading on a 7% acrylamide native gel, along with separate samples of each, Fig. 4A. Lanes in which $\sigma^M$ alone was loaded (lanes 2 and 5) contained two bands running towards the cathode (-), with the faster running band representing a minor species. The origin of this difference is not known, but we note that these two forms of $\sigma^M$ were not resolved by vertical native PAGE (see later). Because RsmA preparations contained a residual amount of the detergent n-laurylsarcosine, $\sigma^M$ was also run in the presence of the detergent to assess the effect on its migration characteristics (lane 1). Apart from a slight smearing of the bands, such that the two forms of $\sigma^M$ are not clearly distinguished, no effect was observed. Mixing RsmA and $\sigma^M$ at equal concentrations gave rise to a band corresponding to unbound $\sigma^M$, but this was substantially weaker than that due to the same concentration of $\sigma^M$ in the absence of RsmA (lane 5), indicating that $\sigma^M$ binds to RsmA and the resulting complex migrates towards the anode, resulting in a more diffuse band than observed for RsmA alone.

That not all of the $\sigma^M$ was recruited by RsmA suggested that either the binding affinity for the $\sigma^M$-RsmA complex is not very high, or that some of the RsmA or $\sigma^M$ present in the mixture was not able to participate in complex formation. To investigate this further, $\sigma^M$ was incubated with a ~4-fold excess of RsmA, and the gel was run under the same conditions as above, Fig. 4B. Unbound $\sigma^M$ was not detected (see lane 3), indicating that there was no sub-fraction of $\sigma^M$ that could not bind RsmA. Furthermore, the complete complexation of $\sigma^M$ at a 4-fold excess of RsmA indicates a tight binding interaction and suggests that, in the equimolar mixture of RsmA-$\sigma^M$, there was a sub-fraction of RsmA that could not bind $\sigma^M$. As isolated RsmA contains only ~35% $[2\text{Fe-2S}]$-containing protein; one possibility is that the iron-sulfur cluster affects binding of RsmA to $\sigma^M$.

Only the $[2\text{Fe-2S}]$ form of RsmA binds to $\sigma^M$– To investigate this further, an entirely apo-sample of RsmA was generated by storing the protein under conditions that favored cluster degradation, see Experimental Procedures. Apo-RsmA (lane 1) was incubated with an equimolar concentration of $\sigma^M$ (lane 2) and loaded onto a 7% native horizontal gel, Fig. 5A. Here, $\sigma^M$ clearly remains unbound and we conclude that apo-RsmA has a significantly reduced capacity to bind $\sigma^M$. 
To further confirm that the iron-sulfur cluster is essential for the observed high affinity interaction between the two proteins, σM was incubated with RsmA containing a decreasing amount of [2Fe-2S] cofactor. The resulting mixtures were analyzed by standard vertical native PAGE, so that σM, which runs towards the cathode, could be easily visualized. Fig. 5B. σM was detected in proportion to the loss of the cluster, such that when [2Fe-2S] RsmA was near equimolar with σM, σM was essentially fully bound (lane 2); consequently, σM was not able to enter the gel and thus was not detected. Decreasing the ratio of [2Fe-2S] RsmA to apo-RsmA resulted in the concomitant appearance of σM on the gel (lanes 3-6).

Far UV circular dichroism spectroscopy was employed to monitor secondary structure changes upon loss of the [2Fe-2S] cluster. Although the initial spectrum resulted from a sample containing both apo- and holo-RsmA, significant changes were observed upon conversion of the RsmA to the fully apo-form, Fig. 5C, possibly resulting from partial unfolding of the protein following loss of the cluster. This presumably results in the release of decreased affinity for σM.

The ability of RsmA to interfere with σM-mediated in vitro transcription was investigated using the B. subtilis Pctc promoter. In vivo, Pctc is a strong σB-dependent promoter (46), which has often been used to test the activity of σB-like sigma factors of S. coelicolor. Purified σB and E. coli core RNA polymerase were sufficient to generate a specific product in an in vitro run-off transcription assay, see Fig. 5D (lane 3). As isolated RsmA (containing an apo-/holo-RsmA mix) or apo-RsmA (both at ~10-fold excess over sigma factor) was incubated with σM for 15 minutes at 25 °C prior to adding core RNA polymerase. While the presence of apo-RsmA did not affect the activity of σM (lane 4), as isolated (cluster-containing) RsmA almost completely inhibited σM-mediated transcription activity (lane 5). Thus, taken together, the above data demonstrate that the loss of cluster abolishes high affinity binding of RsmA to σM.

Characterization of RsmA ATP-binding properties-- RsmA contains a motif involved in ATP binding, Fig. 1. To determine whether or not RsmA is isolated containing ATP, a luciferase bioluminescence ATP assay was used. ATP was not detected in native or heat-treated (100 °C for 15 mins) samples of as isolated RsmA. However, addition of ATP (0.5 µM) to RsmA (8 µM) prior to analysis resulted in detection of ~80% of the added ATP in the heat-treated sample; no ATP was detected in the native sample (data not shown). 100% recovery of ATP was observed in equivalent control reactions with bovine serum albumin (both native and heat-treated). These experiments indicate that RsmA, although isolated containing little or no ATP, binds the nucleotide with reasonably high affinity. To further investigate the interaction of the nucleotide and RsmA, a fluorescent trinitrophenylated analogue of ATP, TNP-ATP, was used to probe the interaction of the nucleotide and RsmA. TNP-ATP has a high affinity for most nucleotide binding proteins, and its absorption and fluorescence characteristics in the visible range alter significantly when protein is bound (47). An excess of the analogue was added (8 µM) to a solution of RsmA (4 µM) and the fluorescence spectrum was measured between 520 and 700 nm. A marked increase in fluorescence intensity was observed, indicative of binding (Fig. 6A), whereas no increase was observed upon addition to a protein control (lysozyme, not shown). Fluorescence titrations of RsmA at two different concentrations (3.2 and 0.4 µM) with TNP-ATP were subsequently carried out, and resulting fluorescence intensities at 560 nm were plotted as a function of TNP-ATP concentration (Fig. 6B shows the higher concentration data). Both datasets were fitted to a simple binding model yielding a $K_d$ of ~0.5 µM and a stoichiometry of binding of ~1:1. Essentially identical data were obtained from similar titrations of apo-RsmA (not shown), indicating that the iron-sulfur cluster is not required for nucleotide binding.

The affinity of RsmA for ATP itself was measured indirectly through competition studies with TNP-ATP. 4 µM TNP-ATP was added to RsmA (3.2 µM) and the complex titrated with ATP. Fluorescence intensity at 560 nm was plotted as a function of ATP concentration, Fig. 6C. From this, the value of the exchange constant, $K_{ex}$, was estimated to be ~0.17, which corresponds to a $K_d$ for ATP binding to RsmA of ~3 µM, i.e. the affinity of RsmA for ATP is ~ 6 fold lower than that for TNP-ATP. Essentially identical data were obtained from titrations of TNP-ATP-apo-RsmA, again indicating that the iron-sulfur cluster does not influence nucleotide binding.

To determine whether the presence of ATP affects the interaction of RsmA with σM, native gel experiments similar to those shown in Fig. 4A and B were carried out, see Fig. 4C. Lanes 1 and 4 showed that σM is entirely complexed in the presence of a 16-fold excess of RsmA (which contained a 3-fold excess of holo-RsmA), but a large fraction of it remained un-complexed with a 16-fold excess of apo-RsmA alone (lane 6). The
presence of a sub-stoichiometric amount of holo-RsmA led to the complexation of a fraction of the available $\sigma^M$ (lane 5). Lanes 2 and 3 confirmed that the holo- and apo-proteins have similar mobilities in the presence of ATP. Therefore, although absolute affinities are not revealed here, the presence of ATP does not significantly affect the affinities of holo- and apo-RsmA for $\sigma^M$.

**DISCUSSION**

RsmA was identified as one of 27 potential *S. coelicolor* anti-sigma factors through a bioinformatic search using *B. subtilis* SpoIIAB and RsbW as probes (Fig. 1). The number of putative anti-sigma factors identified was considerably lower than that reported previously (48), most likely a consequence of the fact that our search criteria excluded putative histidine kinases. RsmA (Sco7313) stood out amongst these proteins, not only because it is encoded by a gene located immediately upstream of $\sigma^M$, which encodes the sigma factor $\sigma^M$, but also because it contains seven cysteine residues which seemed likely to have functional significance.

Bacterial two hybrid experiments provided clear evidence for an interaction between RsmA and $\sigma^M$ (Fig. 3). Importantly, a similar interaction between RsmA and $\sigma^H$, a highly related sigma factor from *S. coelicolor*, was not observed, strongly indicating that the interaction between RsmA and $\sigma^M$ is a specific one. RsmA isolated following over-production in *E. coli* was found to be reddish-brown in color, indicative of the presence of a metallo-cofactor. Analyses revealed the presence of significant quantities of iron (and no other metals), and equivalent concentrations of inorganic sulfide, indicating the presence of an iron-sulfur cluster. UV-visible absorbance and CD spectra contained features that are entirely consistent with those of a [2Fe-2S] cluster (and are quite distinct from those of other cluster types) (Fig. 2).

Native gel and *in vitro* transcription studies with purified RsmA and $\sigma^M$ (Figs. 4 and 5) further demonstrated a strong interaction between the proteins. Importantly, the data also showed that the interaction is modulated by the presence or absence of the [2Fe-2S] cluster, such that only the cluster-containing form of RsmA is able to bind to $\sigma^M$ with high affinity.

These data lead us to propose a model in which the [2Fe-2S] cluster acts as a regulatory switch determining the affinity between the sigma factor-anti-sigma factor pair. The data indicate that the degradation of the cluster, rather than simply a redox process, is required to activate the switch. The large changes in secondary structure content revealed by far UV-CD spectra of RsmA as isolated and the all apo-form (Fig. 5C) suggest that loss of the cluster causes a major conformational change, perhaps leading to partial unfolding of the anti-sigma factor. In IRP-1 the loss of [4Fe-4S] cluster also leads to significant structural changes, opening up the compact protein conformation (49). In this system the extensive structural changes are central to the dual functions of IRP-1 as a translational regulator or enzyme.

The nature of the signal that activates the switch is not yet known. Although exposure to oxygen does lead to the loss of the cluster, this reaction is slow (note that the protein was isolated under aerobic conditions) and is unlikely to be physiologically relevant. Another possibility is that loss of the cluster is *initiated* by a redox process. EPR analysis revealed that, upon isolation, the cluster is present in the diamagnetic, oxidized [2Fe-2S]^{2+} state (with both irons as Fe^{3+}), and attempts to generate a stable reduced form by addition of dithionite were not successful due to the degradation of the cluster. A reduced [2Fe-2S]^{1+} cluster (with unusual properties) could be detected following treatment with a large excess of the milder reductant ascorbate, but the signal represented only ~10% of the clusters present. Hence, it is possible that cluster degradation is triggered by a redox event at the cluster.

The two best characterized examples of anti-sigma factors belonging to the GHKL superfamily of ATPases, *B. subtilis* SpoIIAB and RsbW, are protein kinases that use ATP to phosphorylate and inactivate an antagonist protein (an anti-anti-sigma factor) (50,51). Since RsmA is a member of the same superfamily, and contains a HATPase_c domain that is characteristic of anti-sigma factors regulating group 3 sigma factors, the interaction of RsmA with nucleotides was investigated. Binding studies using a fluorescent ATP analogue revealed high affinity binding to form a 1:1 complex, and competition studies with ATP enabled the $K_d$ for RsmA-ATP binding to be estimated at ~3 $\mu$M, which is similar to that observed for SpoIIAB (~1 $\mu$M), and well within the physiological range of ATP concentration. Importantly, nucleotide binding was not dependent on the [2Fe-2S] cluster. A possible kinase activity has not been probed here because we have not been able to identify a putative substrate (*ie*, an anti-anti-sigma factor): analysis of the region surrounding $\sigma^M$ and $\sigma^M$ failed to
identify an anti-anti-sigma factor, although this does not rule out that one is involved in the regulation of \( \sigma^M \) (and we note that the \( S. \ coelicolor \) genome contains several putative anti-anti-sigma factors). The data presented here, however, indicate that RsmA alone, through modulation of its \( \sigma^M \)-binding activity, is sufficient to regulate \( \sigma^M \) activity, and so, like \( S. \ coelicolor \) \( \sigma^H \) (52), the regulatory mechanism is distinct from those for \( S. \ coelicolor \) \( \sigma^B \) (53) and \( B. \ subtilis \) SpoIIAB and RsbW.

The significance of nucleotide binding remains to be established. However, since it is not sensitive to the status of the [2Fe-2S] cluster, and ATP binding did not significantly affect the interaction of RsmA with \( \sigma^M \), it does not appear to be an essential feature of the switch mechanism. The alignment of putative anti-sigma factors in Fig. 1 showed that there is significant variation in the ATP binding motifs, such that these are, in many cases, only partially conserved. One possibility is that the genes encoding the putative anti-sigma factors arose as a consequence of evolutionary duplication events, followed by divergence as each became adapted to a particular regulatory role. No close homologues of RsmA are present in \( S. \ avermitilis \), which, like \( S. \ coelicolor \), has 9 group 3 sigma factors, although it seems to lack a \( \sigma^M \) orthologue. Recent sequencing projects also revealed no direct orthologue of this sigma-anti-sigma factor pair in \( S. \ scabies \), whereas \( S. \ ambofaciens \) ATCC23877 (54) contains very close homologues of \( \sigma^M \)/RsmA (with 90/76% identity at protein level, respectively). Moreover, the surrounding region also seems to be highly conserved between the two species. This suggests that this sigma-anti-sigma pair may have propagated through the Streptomyces genus by horizontal gene transfer and has a role in adaptation conferring a long term advantage.

\( \sigma^M \) has previously been shown to be induced following growth under high salt conditions (generating osmotic stress), and is itself regulated by \( \sigma^B \) (19). However, growth under high salt conditions was found to up-regulate, in a delayed manner, many responses more usually associated with oxidative stress, leading to the proposal that growth under these conditions leads in some way to oxidative stress. Thus, the exact function of \( \sigma^M \) is not yet clearly established.

Many questions remain concerning RsmA; the most important amongst these is the nature of the signal to which the anti-sigma factor responds. Also unknown at present is which of its seven cysteine residues are involved in ligating the [2Fe-2S] cluster. It is noteworthy that the RsmA homologue of \( S. \ ambofaciens \), despite very high sequence identity, contains only five cysteines, and only four of these align with those of \( S. \ coelicolor \) RsmA (Cys25, 50, 123 and 140, see Supplementary Data, Fig. S1). While the arrangement of cysteine ligands (i.e., the spacings between them) is not characteristic of known iron-sulfur proteins, it is tempting to speculate that these are likely to act as ligands to the cluster. Other non-cysteine ligands cannot be excluded at this stage, however. Also unclear is whether or not RsmA acts as a dimer. This is a common characteristic of known members of the HATPase_c serine kinase family of anti-sigma factors. However, in the bacterial two-hybrid studies reported here a complementation signal was not observed when both vectors expressed rsmA (Fig. 3), suggesting that RsmA doesn’t dimerize. It is noted that recent studies of FNR using the same two-hybrid (Ladant) system did not provide evidence for dimerization until the copy numbers of the bait and target plasmids were adjusted to be equivalent (55); thus dimerization may be difficult to detect.

The mechanisms by which anti-sigma factors achieve regulation of their cognate sigma factors are diverse, and as more systems are discovered, it is highly likely that they will become even more so. One such mechanism, which has been clearly demonstrated for \( S. \ coelicolor \) RsrA, involves sensing disulfide stress. However, even in this well characterized example, the exact nature of the direct signal is not known. Here we have described a system which also appears to respond directly to a stress signal but in a quite different and, to date, unprecedented way: through the presence or absence of an iron-sulfur cluster. It thus represents an expansion of the roles played in biological systems by these inorganic clusters.

**ACKNOWLEDGEMENTS**

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REFERENCES


**FOOTNOTES**

5. The abbreviations used are: CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; ICP-AE, inductively coupled plasma atomic emission; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNP-ATP, trinitrophenol-adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.
FIGURE LEGENDS

Fig. 1. Amino acid sequence alignment of the 27 *S. coelicolor* putative anti-sigma factors with *B. stearothermophilus* SpoIIAB. The alignment is shown for the conserved regions of the GHKL ATPase family (33). Residues involved in ATP binding/kinase activity in SpoIIAB (56) are denoted with an asterisk. Conserved and similar residues are indicated by highlighting in black and grey, respectively. RsmA (Sco7313), underlined, is encoded upstream of the gene encoding $\sigma^M$ (Sco7314), a $\sigma^B$-like sigma factor.

Fig. 2. Spectroscopic analysis of RsmA. (A) UV-visible spectrum of as isolated RsmA (400 $\mu$M), pathlength 1 mm. Inset is the spectrum of the 300-700 nm region of the same sample, pathlength 10 mm. (B) CD spectrum of as isolated RsmA (96 $\mu$M), pathlength 10 mm. (C) The EPR spectrum of RsmA (96 $\mu$M in cluster) following reduction with sodium ascorbate (10 mM). Temperature 10 K, microwave power 6 mW, microwave frequency 9.683 GHz and modulation amplitude 10 G. g-values are indicated on the spectrum. A simulation of the spectrum is indicated by a solid grey line. RsmA was in 50 mM Tris/HCl pH 7.5.

Fig. 3. Bacterial two-hybrid studies of the interaction between RsmA and $\sigma^M$. Bar chart showing $\beta$-galactosidase activity measured for DHM1 cells harboring pKT25sigM (black bars), empty pKT plasmid (dark grey bars), pKT25rsmA (light grey bars), or pKT25sigB (white bar) and the indicated pUT18 plasmid are shown. Each bar represents the mean value of enzyme activities (represented as Miller units calculated as indicated in *Experimental Procedures*) from three independent cultures, each measured in duplicate. The standard deviation of the mean for each is shown as an error bar.

Fig. 4. Native gel studies of the interaction between RsmA and $\sigma^M$. (A) RsmA and/or $\sigma^M$ (as indicated; numbers indicate concentrations in $\mu$M) were run on horizontal native gels at 30 mA for 5 h at 4°C. For experiments in which RsmA and $\sigma^M$ were mixed, samples were incubated at room temperature for 15 minutes prior to loading. The asterisk indicates that $\sigma^M$ was pre-incubated with 0.3% (w/v) n-laurylsarcosine. RsmA concentrations are those of total protein, and the concentration of cluster-containing RsmA was ~35% that of total RsmA. Lanes are numbered for convenient cross-reference with the main text. (B) As in (A), except that mixtures of $\sigma^M$ and RsmA contained an excess of RsmA (as indicated). (C) As in (A), except that RsmA was pre-incubated with ~12 fold excess of ATP for 15 minutes at room temperature. RsmA concentrations are those of total protein. The concentration of cluster-containing RsmA was ~20% that of total RsmA, except for lanes 3 and 6, which are marked ‘apo’ to indicate the absence of the cluster.

Fig. 5. The effect of loss of the [2Fe-2S] cluster on complex formation and folding. (A) Horizontal gel analysis of apo-RsmA and $\sigma^M$ and (B) vertical native gel analysis of $\sigma^M$ and RsmA (concentrations of protein and [2Fe-2S] cluster, in $\mu$M, are as indicated). Gels were run at 40 mA for 5 hours at 4°C. For experiments in which RsmA and $\sigma^M$ were mixed, samples were incubated at room temperature for 15 min prior to loading. (C) Far UV CD spectrum of as isolated RsmA (filled circles) and apo-RsmA (open circles). Proteins (28 $\mu$M) were in 20 mM Tris/HCl pH 7.5, pathlength 1 mm. (D) The ability of RsmA to interfere with $\sigma^M$ directed *in vitro* transcription of the *B. subtilis* P_{csc} promoter was examined. $\sigma^M$ (37 nM in 50 mM Tris/HCl pH 6.8, 100 mM NaCl, 10% (v/v) glycerol) was pre-incubated with as isolated RsmA or apo-RsmA (0.4 $\mu$M in 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 10% (v/v) glycerol, 1 mM DTT) prior to incubation with *E. coli* DNA polymerase core (0.1 Unit in 50 mM Tris/HCl pH 7.5, 250 mM NaCl, 0.1 mM EDTA, 1 mM DTT) and promoter template (~0.2 pmol in deionized water), as indicated. The expected size of the transcript was ~250 bases, as indicated by the marker in lane 1.

Fig. 6. Nucleotide binding to RsmA. (A) Fluorescence emission spectra of RsmA (3.2 $\mu$M), TNP-ATP (8 $\mu$M) and RsmA (4 $\mu$M) following addition of TNP-ATP (8 $\mu$M), all in 50 mM Tris/HCl pH 7.5. Excitation was at 408 nm. (B) Plot of fluorescence emission intensity at 560 nm as a function of TNP-ATP concentration for the titration of RsmA (3.2 $\mu$M) in 50 mM Tris/HCl pH 7.5. The fit to a simple binding model (see *Experimental Procedures*) is indicated by a solid line. (C) Plot of fluorescence emission intensity at 560 nm as a function ATP concentration for the titration of TNP-ATP-RsmA (3.2 $\mu$M) in 50 mM Tris/HCl pH 7.5.
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Fig. 1
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**C**

![Graph showing changes in absorbance over wavelength](image)

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| RsmA | - | - | + | - | + | - | - |
| Apo-RsmA | - | - | + | + | + | + | + |

250 bases
Fig. 6
RsmA is an anti-sigma factor that modulates its activity through a [2Fe 2S] cluster cofactor

Alisa A. Gaskell, Jason C. Crack, Gabriella H. Kelemen, Matthew I. Hutchings and Nick E. Le Brun

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