doi:10.1016/j.jmb.2005.11.045 J. Mol. Biol. (2006) 355, 1026–1036

JMB

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Functional Analysis of the Post-transcriptional Regulator RsmA Reveals a Novel RNA-binding Site

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The RsmA family of RNA-binding proteins are global post-transcriptional regulators that mediate extensive changes in gene expression in bacteria. They bind to, and affect the translation rate of target mRNAs, a function that is further modulated by one or more, small, untranslated competitive regulatory RNAs. To gain new insights into the nature of this protein/RNA interaction, we used X-ray crystallography to solve the structure of the *Yersinia enterocolitica* RsmA homologue. RsmA consists of a dimeric β barrel from which two α helices are projected. From structure-based alignments of the RsmA protein family from diverse bacteria, we identified key amino acid residues likely to be involved in RNA-binding. Site-specific mutagenesis revealed that arginine at position 44, located at the N terminus of the α helix is essential for biological activity *in vivo* and RNA-binding *in vitro*. Mutation of this site affects swarming motility, exoenzyme and secondary metabolite production in the human pathogen *Pseudomonas aeruginosa*, carbon metabolism in *Escherichia coli*, and hydrogen cyanide production in the plant beneficial strain *Pseudomonas fluorescens* CHA0. R44A mutants are also unable to interact with the small untranslated RNA, RsmZ. Thus, although possessing a motif similar to the KH domain of some eukaryotic RNA-binding proteins, RsmA differs substantially and incorporates a novel class of RNA-binding site.

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Keywords: CsrA; post-transcriptional regulation; RNA-binding domain; RsmA

Introduction

Proteins belonging to the RsmA family are small (less than 7 kDa) RNA-binding proteins that play key roles in regulating gene expression in diverse eubacteria. The *Escherichia coli* gene *csrA* (carbon storage regulator) was originally characterised as a negative regulator of glycogen metabolism, which also controls cell size and surface properties.[1] Subsequent studies have revealed that CsrA plays a major role in controlling intracellular carbon flux in *E. coli*, acting as a negative regulator of both glycogen metabolism and glycolysis and as a positive regulator of gluconeogenesis.[2] In addition, it positively regulates motility by modulating the expression of the *flhDC* operon responsible for controlling flagellar biosynthesis.[3] The *Erwinia carotovora* (*Pectobacterium carotovorum*) homologue, RsmA (repressor of secondary metabolites), was identified as a global repressor controlling the production of extracellular enzymes, *N*-acylhomoserine lactone (AHL) quorum sensing signal molecules and pathogenicity.[4,5] The members of the RsmA/CsrA family have subsequently been found to play important roles in global post-transcriptional regulation in many other bacterial genera. In the opportunistic human pathogen *Pseudomonas aeruginosa*, for example, RsmA negatively regulates the production of hydrogen cyanide, pyocyanin, LecA (PA-1L) lectin and AHLs,[6] whereas it positively controls swarming motility, lipase and rhamnolipid production.[7] In the plant beneficial agent *Pseudomonas fluorescens* CHA0, RsmA also controls the production of exoenzymes and antifungal secondary metabolites such as hydrogen cyanide.[8] Members of the RsmA/CsrA family are involved in regulating the expression of

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Abbreviation used: MAD, multiple wavelength anomalous diffraction.

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genes required for host cell interactions and adaptation to the host cell environment in *Salmonella typhimurium*,[9] for the transmissibility, cytotoxicity and efficient macrophage infection in *Legionella pneumophila*,[10] for swarming motility in *Serratia marcescens*,[11] for swarming motility and virulence in *Proteus mirabilis*,[12] and for lipooligosaccharide production in *Haemophilus influenzae*.[13] The importance of this family of post-transcriptional regulators is further highlighted by the fact that it is present in the highly adapted human gastric pathogen *Helicobacter pylori*, which has relatively few transcriptional regulators and where it controls virulence and the stress response.[14]

A number of studies have shown that RsmA homologues act post-transcriptionally by binding to mRNA, so modulating translation.[15,16] In *E. coli*, CsrA binds to multiple sites near the Shine–Dalgarno sequence in the transcripts of *glgC* and *cstA* preventing ribosomal binding.[17,18] In *P. fluorescens*, RsmA represses the production of hydrogen cyanide during exponential growth by reducing the translation rate of *hcnA*, the structural gene coding for the hydrogen cyanide synthase. This repression of *hcnA* translation by RsmA requires a specific sequence near the ribosome-binding site.[8] At the start of the stationary phase, the effects of RsmA/CsrA are abrogated by the induction of a number of small, untranslated regulatory RNAs that sequester multiple copies of the protein.[15,19–24] In *P. aeruginosa*, RsmZ is a regulatory RNA with several predicted binding domains for RsmA.[7] Consequently, these RNA–protein networks facilitate rapid responses to changing environmental conditions.

Although previous studies of RsmA/CsrA have revealed some of the mechanisms of post-transcriptional regulation by these proteins, our understanding of their structure–function relationships is very limited. To establish a platform for the molecular investigation of these ubiquitous protein–RNA interactions we crystallised RsmA from *Yersinia enterocolitica* and found that it has a unique structure compared with other known RNA-binding proteins. Furthermore, using site-directed mutagenesis we identified a key site for RNA recognition that is directly involved in RsmA functionality *in vivo* and *in vitro*.

Results

Structural determination of RsmA

RsmA from *Y. enterocolitica* 8081 could be readily over-expressed in *E. coli* and the purified protein produced well-diffracting crystals. The structure was solved by multiple wavelength anomalous diffraction (MAD) of Se-substituted protein produced in minimal medium supplemented with selenomethionine. The crystal unit contains two protein molecules with the dimer interface burying 1972 $Å^2$ of surface area (Figure 1(a)). The protein behaves as a dimer during size-exclusion chromatography. Cross-linking studies have previously suggested that the *E. coli* CsrA protein is also a homodimer.[17] These data all indicate that the dimer observed in the crystal is the functional unit of RsmA. Each monomer has a β-β-β-β-β-α secondary structure. The RsmA dimer contains two, five-stranded antiparallel β-sheets (Figure 1(a)). The three central strands are contributed by one subunit and are hydrogen bonded in the order 2 3 4 whilst the two peripheral strands originate from the other molecule in the dimer such that the first β-strand in one monomer is hydrogen bonded to the fourth strand in the other monomer while the fifth strand is hydrogen bonded to the second strand in the other monomer. In addition to these interchain hydrogen bonds, the dimeric structure is maintained by extensive contacts between hydrophobic residues in the β-strands, which form a hydrophobic core between the two sheets. The α-helices project out from the β-sheets with only the N-terminal portion interacting with the rest of the protein. This interaction is primarily mediated by the packing of Y48 onto M13 in the second strand of the sheet in the other subunit and the formation of an interchain salt-bridge between E46 and R6 (Figure 1(b)). Comparison of the structure with other known protein structures using the program DALI revealed no significant similarities, indicating that this is a novel fold.

Sequence conservation in the RsmA/CsrA family

Structure-based alignments of RsmA/CsrA sequences are shown in Figure 2. When aligning homologues found in a wide variety of genera, mainly Gram-negative gamma proteobacteria but including also Gram-positive species, only one member of this family (from *Legionella*) has an additional residue in the turn between β-strands 2 and 3 of the β-sheet (Figure 2(a)). This insertion is unlikely to have any major structural consequences. The sequences diverge considerably at the C terminus of the α-helix (Figure 2). However, due to their peripheral location, away from the rest of the protein, they are unlikely to have any significant effect on the structure. Several residues are strictly conserved. These include Arg6 and Glu46, which form the interchain salt-bridge, suggesting that this interaction plays a crucial role in maintaining the structure (Figure 1(c)). Residues Ala36 and Pro37, situated at the end of the fourth β-strand, are also highly conserved. It is likely that they have an important role in directing the polypeptide chain so that the fifth β-strand can form hydrogen bonds with residues on the other subunit. There is a strong preference for glycine or alanine at residue 33, in the middle of the fourth strand of the sheet. The presence of a small amino acid at this position may be important for maintaining the twist of the sheet. Most of the residues whose side-chains form the hydrophobic core of the protein are not so well conserved, but show a strong preference for hydrophobic amino acids in other members of the family (Figure 2). In addition to

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