Structural basis for intrinsic thermosensing by the master virulence regulator RovA of Yersinia

Nick Quade1*, Chriselle Mendonca2*, Katharina Herbst2, Ann Kathrin Heroven2, Christiane Ritter1, Dirk W. Heinz1* & Petra Dersch2*

Running title: Thermosensing by the virulence regulator RovA

From the departments of 1Molecular Structural Biology and 2Molecular Infection Biology, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany

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Background: The Yersinia virulence regulator RovA is an intrinsic thermometer.

Results: Crystal structure of RovA is reported and evidence is given that a thermosensing loop in the dimerization domain controls RovA activity.

Conclusion: Partial unfolding of RovA upon a temperature upshift leads to distortion of the DNA-binding domain and release from the operator sites.

Significance: Minor alterations, reflecting evolutionary changes between homologues, transform the thermotolerant regulator in a thermosensor.

SUMMARY

Pathogens often rely on thermosensing to adjust virulence gene expression. In yersiniae, important virulence-associated traits are under the control of the master regulator RovA which uses an in-built thermosensor to control its activity. Thermal upshifts encountered upon host entry induce conformational changes of the RovA dimer that attenuate DNA-binding and render the protein more susceptible to proteolysis. Here, we report the crystal structure of RovA in the free and DNA-bound form and provide evidence that thermo-induced loss of RovA activity is mainly promoted by a thermosensing loop in the dimerization domain and residues in the adjacent C-terminal helix. These determinants allow partial unfolding of the regulator upon an upshift to 37°C. This structural distortion is transmitted to the flexible DNA-binding domain of RovA. RovA mainly contacts the DNA backbone in a low affinity-binding mode which allows the immediate release of RovA from its operator sites. We also show that SlyA, a close homologue of RovA from Salmonella with a very similar structure, is not a thermosensor and remains active and stable at 37°C. Strikingly, changes in only three amino acids, reflecting evolutionary replacements in SlyA, result in a complete loss of the thermosensing properties of RovA and prevent degradation. In conclusion, only minor alterations can transform a thermotolerant regulator into a thermosensor that allows adjustment of virulence and fitness determinants to their thermal environment.

INTRODUCTION

Microbial pathogens need to adapt rapidly to milieus in warm-blooded hosts after entry from external habitats. This requires a coordinated control of large sets of genes covering a wide range of
controlling virulence-associated functions (1-3). The MarR family constitutes an important class of transcriptional regulators by which prokaryotes sense their surrounding biosphere. They control numerous biological functions important for survival, stress resistance, metabolic adaptation and virulence (4). The SlyA/RovA family represents a subgroup of the MarR-type regulators that participate in the control of virulence factors and are as such crucial for the successful establishment of an infection (5). The Salmonella SlyA protein was shown to control hemolysins, antimicrobial peptides and induces expression of the Salmonella pathogenicity island 2 necessary for survival in macrophages and virulence in mice (6-9). RovA, the SlyA homolog of pathogenic yersiniae coordinates the expression of multiple genes contributing to host colonization and persistence. For instance, in enteropathogenic Yersinia species RovA activates expression of the internalization factor invasin, allowing a more efficient colonization of gut-associated lymphatic follicles, whereas RovA of Y. pestis affects production of the type III secretion system and antiphagocytic Yop effector proteins which are essential for the development of the bubonic plague (10-14). In contrast to all other previously identified MarR homologs, RovA of Y. pseudotuberculosis, which is 100% identical to RovA of Y. pestis, represents an intrinsic proteinaceous thermometer which is able to sense temperature-shifts directly through reversible alterations in its conformation. Thermo-induced conformational changes within the RovA structure modulate its DNA-binding capacity and render the regulator more susceptible to proteolytic degradation by the ATP-dependent protease Lon (11).

Use of an in-built thermosensor in the MarR-type regulator RovA to control DNA-binding activity and protein stability represents a unique regulatory strategy to adjust virulence-associated processes. In order to gain insight into the molecular mechanism how RovA confers its specific biological activities, we solved the crystal structure of free and DNA-bound RovA and identified amino acids implicated in thermosensing and regulated proteolysis.

RESULTS AND DISCUSSION

Structure of the thermosensor RovA. The structure of RovA of Y. pseudotuberculosis alone and in complex with DNA was solved at a resolution of 1.85Å and 2.1Å, respectively. Data collection and refinement statistics are given in Table S1. The overall structure of RovA consists of two subunits comprising six α-helices and two β-sheets and resembles other members of the MarR regulator family (Fig. 1A, S1). The two RovA monomers are tightly intertwined with the three long helices α1, α5 and α6, which form a large dimer interface of 2252.6 Å² and a ΔG of -34.4 kcal/mol. The interface is mainly supported by hydrophobic interactions involving surface located aliphatic and aromatic amino acids in the respective dimerization domains. The DNA-binding domain is formed by the central part of RovA and contains a winged helix-turn-helix (wHTH) DNA-binding motif. It comprises helices α3 (stabilizing helix) and α4 (recognition helix) and two antiparallel β-strands linked by a loop (wing) (Fig. 1A, S1).

Three different RovA dimer variants exist in a crystallographic asymmetric unit which have the same overall structure but vary slightly in their conformation (Fig. 1B). Those differences are mainly due to movements of the DNA-binding domain or the mobility of one of the long helices in the dimerization region. Similar to other structures of MarR-type regulators, the wing of the DNA-binding region was found disordered in the unbound form. This indicates that the unbound form of RovA shows a considerable degree of flexibility, especially in the DNA-binding region.

RovA structure in the DNA-bound state. Two high-affinity RovA binding sites (I, II) were previously detected at diverse positions in the rovA and inv promoter (Fig. S2) to which RovA bound with a similar apparent dissociation constant (Kd 32-46 nM) (10,11). To compare the DNA-binding mode to different target sites, RovA was crystallized in the presence of one binding site of each promoter which contained a short consensus sequence A4/TATTAT3/5T shown to be important for RovA binding (10). RovA bound to both promoter fragments in a similar fashion with an r.m.s.d. of 0.41Å. The asymmetric unit contained one RovA-DNA complex. In contrast to unbound RovA, only one RovA dimer variant is present and the wing region is well-defined. This suggests that RovA
binding to DNA locks the regulatory protein into one conformation (Fig. 1C, D). The superposition of RovA in the free and DNA-bound form shows that RovA performs a twisting motion upon DNA binding (data not shown). This conformational change increases the gap between the two DNA-binding domains in the dimer in order to allow the insertion of the α4 helix into consecutive major grooves of the DNA (Fig. 1C,D). Both, the conformational flexibility of RovA and motions of the DNA binding domains seem crucial to propagate thermo-induced conformational changes into an attenuation of DNA-binding.

The DNA-binding mode of RovA. As expected, RovA bound to the previously identified DNA binding site in the middle of the rovA promoter fragment (Fig. 2A). In case of the inv promoter fragment, however, RovA interacted with the joint ends of the promoter fragments (Fig. 2B). The newly generated RovA binding sequence in the pseudo-continuous DNA strand in the crystal is very similar to the originally detected binding sequence (Fig. S3), indicating that the new site represents a similar or even better target site for the RovA protein. Both RovA subunits make extensive and comparable contacts within the rovA and inv promoter region (Fig. 2). Although, helix α4 of the HTH motif is deeply inserted into the major groove of the DNA, only very few specific interactions are visible between RovA and the DNA bases, namely Gln60, Val64 and Arg86 (Fig. 2, S4). The Gln60 residue of one RovA monomer is able to bind to an adenine located 6 bp from the pseudosymmetrical centre in the rovA fragment (Fig. 2A). RovA binding to the original binding site in the inv fragment would have allowed a similar contact of Gln60 by only one monomer. However, both Gln60 residues of the RovA dimer are able to bind to adenines in the newly generated recognition site, which most probably increased the affinity for this binding sequence (Fig. S3). Notably, the importance of the Gln60 residue for the DNA interactions is also supported by the fact, that a RovA<sub>Q60R</sub> variant had an impaired DNA-binding capacity and a reduced ability to stimulate inv expression (15). The other important residue Val64 makes hydrophobic interactions with a thymidine at position 5 from the centre of pseudo-symmetry in both monomers, and Arg86 of the wing domain interacts with a thymidine residue (position 9) at the end of the pseudopalindromic sequence (Fig. 2, S4).

In addition to helix α4, also helices a2 and a3 as well as the wing make direct contacts to DNA (Fig. 1C,D). However, most of these interactions are unspecific hydrogen bonds between the wing and the deoxyribose-phosphate backbone of the DNA (Fig. 2). This is in line with previous results demonstrating that RovA is a global regulator which directly interacts with many promoter regions of Yersinia (10,13,16). The relatively small number of specific contacts between RovA and DNA allows the virulence regulator to recognize multiple non-palindromic promoter sequences in the Yersinia genome that do not share a highly conserved consensus sequence (10,13,16). Of the nonspecific interactions, binding of amino acid Gln49 seems particularly important since this residue interacts with two adjacent sugar-phosphate units on both sides of the bound DNA. This is supported by a previous study which showed that a Q49R mutation impairs DNA-binding and renders the RovA molecule defective for transcriptional activation (15).

RovA of Yersinia and SlyA of Salmonella display similar DNA-binding properties. The closest homologue of RovA with a solved structure is SlyA of Salmonella enterica serovar Typhimurium, showing an amino acid sequence identity of 76% (Fig. 3, S1) (17). In particular the DNA-binding regions are virtually identical, and both regulators show a similar promiscuity regarding their DNA-binding sites. However, in contrast to RovA, Gln60 was not involved in DNA-binding in the SlyA-DNA structure. On the other hand, Arg65 of SlyA, which is bound to a guanine residue in the SlyA operator site (17), is not visible in the RovA structures in complex with the inv or the rovA promoter fragments. This strongly suggests that RovA and SlyA recognize DNA sequences that are partially incomplete; i.e. do not contain all possible binding partners. The small number of specific contacts of RovA may be important to ensure a DNA-binding strength that allows rapid attenuation of DNA-binding after a thermal upshift and counter-regulation and replacement of other competing global regulatory proteins. Previous studies showed that RovA acts mainly as antisilencer that alleviates transcriptional repression by the small nucleoid protein H-NS which has also little sequence specificity (10,11,18). This is different to
other global regulators with conserved consensus sequences (e.g. the cAMP-receptor protein Crp) which mainly serve to increase levels of promoter occupancy by RNA polymerase. In this case the activator needs to be precisely positioned to ensure optimal interaction with the RNA polymerase.

A flexible loop in the dimerization domain mediates intrinsic thermosensing of RovA. Although the structures of the Yersinia virulence factor RovA in its non-bound form and in complex with DNA share many features with the Salmonella SlyA protein (Fig. 3) (17), major differences have been observed in their biochemical properties. The Yersinia RovA protein itself is a protein thermometer which uses intrinsic thermal sensing to control its DNA binding functions and its degradation (11). In contrast, thermosensing, concomitant with a loss in stability at body temperature, has not been described for Salmonella SlyA. All previous studies on SlyA-mediated control of virulence gene expression in Salmonella were performed and report SlyA production at 37°C (19-22). In fact, when the stabilities of RovA and SlyA were compared in Y. pseudotuberculosis and S. enterica serovar Typhimurium, RovA was rapidly degraded, whereas the Salmonella SlyA protein remained stable at 37°C in both pathogens (Fig. 4A,B). However, at 25°C both the RovA and SlyA protein remained stable. CD spectroscopy of the purified proteins further demonstrated that SlyA, in contrast to RovA (Fig. 4C), does not undergo a temperature-dependent conformational change (Fig. 4D) and does not lose its DNA-binding capacity at body temperature (Fig. S5). This strongly indicated that SlyA is not an intrinsic thermosensor which is subjected to controlled proteolysis, although its overall structure is very similar to RovA (Fig. 3). Thermosensing has only been described for three other bacterial transcriptional regulators. However, these regulators are structurally unrelated to RovA and are most likely controlled by a different mechanism (23-25).

Superposition of the SlyA and RovA structures to identify variations between the two regulatory proteins revealed differences in a small loop between helices α5 and α6 (Fig. 3). This loop, including the amino acid Gly116 in RovA, has a different conformation in SlyA in which position 116 is occupied by an alanine (Fig. 3, S1). We introduced a corresponding substitution into RovA and found that the mutant protein (RovA<sub>G116A</sub>) shows no major thermally induced loss of structural integrity (Fig. 5B) and does not display a thermo-induced reduction of DNA-binding (Fig. 6B). DNA-binding of the RovA<sub>G116A</sub> variant was comparable at 25°C and 37°C. This demonstrated that RovA<sub>G116A</sub> lost most of its intrinsic thermosensitivity observed for wildtype RovA. Consequently, RovA<sub>G116A</sub> does not reduce its DNA-binding property at 37°C, which is concomitant with an enhanced stability of RovA (Fig. 5A). It is likely that the recognition sites for the Lon protease are occluded in the active DNA-bound RovA<sub>G116A</sub> variant even at 37°C. In fact, previous studies with chimeric RovA proteins indicated that amino acids in the vicinity of the DNA-binding region are important for proteolytic susceptibility (11). We conclude that the specific intrinsic thermosensing ability of RovA involves residue Gly116 located in a flexible loop between helices α5 and α6 implicated in RovA dimerization. This represents a novel thermosensing mechanism. Of the three known thermosensing regulators, the heat-sensing domain is only known for CtsR, a master regulator of protein quality control of low-GC, Gram-positive bacteria (25). Strikingly, in CtsR intrinsic heat-sensing occurs through a tetraglycine loop connecting the two β-sheets of its wHTH domain. Although both regions are structurally and functionally unrelated they possess a low thermal stability due to a flexible loop structure.

Additional residues important for thermo-induced degradation of RovA. Although thermosensing and degradation of RovA<sub>G116A</sub> was strongly reduced, degradation was not entirely abolished at 37°C (Fig. 5A). Compared to SlyA slightly less of the RovA protein was detectable 90 min after blockage of protein synthesis. This indicated that additional amino acids contribute to the thermostabilization of SlyA. We replaced other amino acids of RovA against the equivalent amino acids of SlyA and found that a substitution of S127I/G128K in addition to G116A (RovA<sub>G116A/S127I/G128K</sub>) further increased stability of RovA to the level of SlyA (Fig. 7A). DNA-binding studies further showed that RovA<sub>G116A/S127I/G128K</sub> did not bind DNA in a temperature-dependent manner (Fig. 6D), similar to the SlyA protein (Fig. S5). We also included both mutations into RovA without the G116A substitution and found that the RovA<sub>S127I/G128K</sub> protein was more stable than the
RovA wildtype protein, although it was still degraded at 37°C (Fig. 5C). In contrast to RovAG116A, only a slight reduction of the thermosensing capacity was apparent in the RovAS127/G128K protein (Fig. 5D). In agreement with this observation, DNA-binding of this variant was increased at 37°C, but still lower relative to RovAG116A (Fig. 6B, C). Interestingly, thermal denaturation measured by far-UV CD spectroscopy revealed two unfolding transitions for RovA and its variants, while SlyA exhibited only a single transition at a higher temperature (Fig. S6). The midpoints of both unfolding transitions were lowest for RovA, considerably higher for RovAG116A and highest for RovAG116A/S127/G128K (Fig. S6). The in vitro thermal denaturation behaviour is therefore in full agreement with the thermosensing capacities of the variants observed in vivo. To confirm these observations, we also included the reverse mutations into SlyA to determine whether introduction of the reciprocal substitutions leads to its destabilization at body temperature. In fact, a considerable degradation of SlyA A116G/I127S/K128G in contrast to wild type SlyA of Salmonella in the free and DNA-bound form. However, it contains a small number of distinct single amino acid substitutions leading to major changes of the biochemical properties of the regulator. Here, we provide evidence that RovA thermosensing mainly depends on a flexible loop situated between two α-helical structures involved in dimer formation. Intrinsically disordered regions are supported by additional residues, including Gly128 which introduces more conformational flexibility in the adjacent helix α6. It is very likely that these determinants promote partial and reversible unfolding of the intervening helices upon a thermal upshift without disruption of the dimer. This conformational change is transmitted to the flexible DNA-binding domain of RovA mainly contacting the DNA backbone. A low-affinity DNA-binding mode appears to enable the immediate release of RovA from its operator sites and allows rapid degradation of non-functional protein.

We further conclude that even minor changes, which cause only negligible alterations of the overall structure of the RovA/SlyA regulators can provoke significant differences in the biochemical properties which affect their ability to promote transcription and reprogram virulence-associated processes. We posit that similarly small evolutionary changes in master regulators could generate significant phenotypic changes. A clear advantage is that it allows pathogens to respond to external pressures (e.g. temperature) which allows them to adapt rapidly and long-term to changing reservoirs, different hosts or host environments. The fact that thermo-induced conformational changes of RovA are reversible makes this regulator also a useful tool for the development of inducible expression systems for biotechnological applications.

Conclusion. The thermal upshift encountered upon host entry is the most crucial signal for many pathogens to induce and adjust their virulence functions. Frequently thermo-induced structural changes of the DNA (e.g. supercoiling, bending) and mRNAs (thermoswitches) are used to control virulence gene expression and the molecular mechanisms underlying these control systems have been elucidated in several pathogens. Lately, also regulatory proteins have been identified which function as ‘molecular thermometers’ (11,23-25). However, the mechanism of the primary thermosensing event and the structural determinants defining the thermosensitive properties of these regulators are largely unknown. In this study we show that the overall structure of the thermosensitive master regulator RovA of Yersinia resembles the thermotolerant homologous protein SlyA of Salmonella in the free and DNA-bound form.
proteins (15,26). RovA in the non- and DNA-bound form was crystallized in a concentration of 20 mg/ml +/- DNA (8 mg/ml). The crystals of RovA without DNA belong to the space group P2_12_12_1 and a dataset up to 2.4 Å was collected at the ESRF ID14h2. The crystals of RovA in complex with DNA belong to the space group P3 and diffracted up to 1.9 Å. For details about the determination of the structure see supporting information (Text S1). Table S1 summarizes data collection and refinement statistics. The final models were validated using MolProbity (27). All residues of all models fall into the allowed region of the Ramachandran plot (28). Atomic coordinates and structure factors were deposited at the Protein Data Bank (29) under the accession codes: 4aih (RovA), 4aik (RovA bound to inv fragment) and 4aij (RovA bound to rovA fragment).

Analysis of the biochemical properties of RovA and SlyA. CD spectroscopy of the RovA proteins, the DNA retardation assays and the protein stability assays was performed as described in the supporting information or previously (11,30).

SUPPORTING INFORMATION
Supporting information is available online.

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FIGURE LEGENDS

FIGURE 1: Structure of the thermosensing regulator RovA.
(A) Illustration of the RovA dimer. (B) Superposition of three RovA dimers found in the asymmetric unit. The three different types of dimers (AB, CD and EF) are shown in blue, green and red. The RovA monomers could be built despite of the flexible wing domain. Cartoon representation of a RovA dimer in complex with DNA from the front (C) or the side view (D). α-helices, β-sheets and amino acids implicated in thermosensing and RovA stability are indicated. One monomer is shown in red, the other in blue.

FIGURE 2: Interaction of RovA with the inv and rovA promoter.
Schematic drawing of the RovA rovA-DNA (A) or RovA inv-DNA (B) interactions. Direct interactions are symbolized by full arrows, water mediated interactions by dashed arrows. Bases of both DNA strands (chain C; chain D) are represented by rectangles, deoxyribose by pentagons, phosphates by circles. The colours of the RovA residues correspond to the chains (blue: chain A, red: chain B). The pseudosymmetrical centre is indicated by a green oval symbol in the middle of the double strand. Open arrows indicate direct interactions, closed arrows water-mediated interactions; bb: amino acid backbone.

FIGURE 3: Superposition of RovA and SlyA from S. enterica serovar Typhimurium.
Cartoon representation of a RovA dimer in complex with DNA. The SlyA dimer is shown in green, the RovA dimer in blue, the DNA is illustrated in orange (phosphate-sugar backbone) and purple (base pairs). The flexible loop between the helices α5 and α6 is enlarged in a separate part of the figure. Residue Gly116 important for thermosensing and the equivalent amino acid Ala116 of Salmonella SlyA are illustrated.

FIGURE 4: Thermo-induced conformational changes and proteolysis of RovA and SlyA.
Stability of RovA or SlyA was investigated at 25°C and 37°C in the original bacterial strains Y. pseudotuberculosis YPIII and S. enterica serovar Typhimurium (A) or in the isogenic ΔslyA (SCM1) and ΔrovA mutant strains (YP107) containing either the P<sub>lac</sub>::rovA (pCM3) or the P<sub>lac</sub>::slyA (pCM15) plasmid (B). A higher molecular weight protein (c) that reacted with the polyclonal antisera was used as loading control. (C, D) Conformational analysis of RovA and SlyA using CD spectroscopy. CD spectra, mdeg (M⁻¹cm⁻¹) versus wavelength of RovA (0.2 mg/ml) (C) or SlyA (0.2 mg/ml) (D) is shown as function of temperature.

FIGURE 5: Thermo-induced conformational changes and proteolysis of RovA mutant proteins.
Stability and thermo-induced conformational changes of the RovA mutant variants RovAG116A (A) and RovAS127I/G128K (C) were investigated at 37°C. A higher molecular weight protein (c) that reacted with the polyclonal antisera was used as loading control. Thermo-induced conformational changes of RovAG116A (B) and RovAS127I/G128K (D) were detected by CD spectroscopy. CS spectra, De (M⁻¹cm⁻¹) versus wavelength of RovAG116A (0.2 mg/ml) or RovAS127I/G128K (0.2 mg/ml) at 20°C and 37°C are presented.

FIGURE 6: Interaction of RovA, RovAG116A, RovAS127I/G128K or RovAG116A/S127I/G128K with the rovA regulatory region at 25°C or 37°C.
Double-stranded promoter fragments of the rovA regulatory region harbouring the RovA binding site I (10) were incubated without or with increasing amounts (25, 36, 48 and 65 nM) of the different purified RovA variants (A) RovA, (B) RovAG116A, (C) RovAS127I/G128K or (D) RovAG116A/S127I/G128K at 25°C or 37°C. Lanes in which lower concentrations RovA (< 25 nM) were added to the DNA fragments are not shown. A non-specific probe containing an unrelated sequence (csiD promoter of E. coli) was included as negative control. The RovA-DNA complexes are indicated by arrows.

FIGURE 7: Thermo-dependent stability of the RovAG116A/S127I/G128K and SlyA<sub>116G/I127S/K128G</sub> proteins.
Stability of the RovA<sub>G116A/S127I/G128K</sub> produced in <i>Y. pseudotuberculosis</i> YPIII (A) and SlyA<sub>A116G/I127S/K128G</sub> synthesized in <i>S. typhimurium</i> SL1344 (B) was investigated at 37°C. RovA (A) and SlyA (B) are indicated by an arrow. A higher molecular weight protein (c) that reacted with the polyclonal antisera was used as loading control.
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Figure 2  Quade et al. 2012
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