Published in final edited form as:

Nat Struct Mol Biol. 2009 November ; 16(11): 1192–1194. doi:10.1038/nsmb.1681.

Regulation of a muralytic enzyme by dynamic membrane topology

Qingan Sun¹, Gabriel F. Kutyl¹, Arulandu Arockiasamy², Min Xu³, Ry Young¹, and James C. Sacchettini^{1,†}

¹Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station, Texas 77843-2128

²International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, 110 067 New Delhi, India

³Department of Microbiology, Immunology, & Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California 90095

Abstract

R²¹, the lysozyme of coliphage 21, has a N-terminal Signal-Anchor-Release (SAR) domain that directs its secretion in a membrane-tethered, inactive form and then its release and activation in the periplasm. Both genetic and crystallographic studies reveal that the SAR domain, once extracted from the bilayer, refolds into the body of the enzyme and effects muralytic activation by repositioning one residue of the canonical lysozyme catalytic triad.

Lyz^{P1} and R²¹, the lysozymes of coliphages P1 and 21, respectively, were the first endolysins shown to have N-terminal SAR (Signal Anchor Release) domains (Fig. 1a)¹. In contrast to canonical phage lysozymes, like T4 E, which accumulate fully folded and enzymatically active in the cytoplasm, SAR endolysins are first secreted as enzymatically inactive enzymes anchored to the membrane by the N-terminal SAR domain². This functional regulation is essential to avoid premature lysis of the infected host. The SAR domain behaves as a metastable transmembrane domain in the context of the energized membrane; it spontaneously escapes the bilayer at a low rate, so cells expressing a SAR endolysin will eventually undergo lysis. In a phage infection cycle, however, the SAR endolysins are activated synchronously when the membrane is depolarized by the triggering of the phage-encoded holin, a small membrane protein that opens holes in the bilayer^{3,4}. In any case, the escape of the SAR domain from the bilayer allows the enzyme to refold and become muralytically active. In Lyz^{P1}, activation is achieved when a free thiol from the newly-extracted SAR domain causes a disulfide bond isomerization, releasing a catalytic cysteine². In addition, the entire catalytic domain undergoes a radical conformational reorganization, unwinding 3 α -helices to form 3 β -strands in the active site and forming the catalytic cleft characteristic of lysozymes (Supplementary Fig. 1 online). The extracted SAR

[†]Corresponding author phone: 979-862-7636, fax: 979-862-7638, sacchett@tamu.edu.

Accession codes. Protein Data Bank: Atomic coordinates and structure factors for ^aR²¹ and ⁱR²¹ have been deposited with accession codes 3HDE and 3HDF, respectively.

AUTHOR CONTRIBUTIONS

Q.S. determined the structure of ⁱR²¹ and refined the structure of ^aR²¹ initially crystallized by A.A.; Q.S. also performed the in vitro assays. G.K. did the molecular biology, genetics, physiology and bioinformatics. M.X. provided key constructs. R. Y. and J. C. S. provided supervision. The writing of the manuscript was a collaborative effort by Q.S., G.K., J.C.S. and R.Y.

domain itself contributes only the liberating thiol, remains largely helical, and makes no intimate contacts with the body of the enzyme².

This elegant regulation is not general for SAR endolysins, however. While fifty-eight phage genomes encode endolysins with the Glu-8aa-Asp/Cys-5aa-Thr catalytic triad (Fig 1a) that characterizes the canonical T4 E lysozyme and other glycosylases, and most of these (43/58) have N-terminal SAR domains, only nine have a Cys residue within the SAR domain and a catalytic cysteine, as observed in Lyz^{P1} (Supplementary Table 1 online). This suggests that the remaining 34 SAR endolysins, including R²¹, must be regulated differently.

Previously, we demonstrated that the SAR domain of Lyz^{P1} is not essential for either the structure or the enzymatic activity, except for the disulfide linkage; even the position of the activating Cys within the SAR domain is very flexible². To determine whether this was also true for R²¹, we replaced its N-terminal 26 residues with the cleavable secretory signal sequence PelB_{ss} (Fig. 1a)⁵. When the *pelB_{ss}ΦR²¹₂₇₋₁₆₅* chimera was expressed in *Escherichia coli*, we observed no lysis even though large amounts of the processed R²¹₂₇₋₁₆₅ protein accumulated in the periplasm (Figs. 1b and c). Moreover, an assay of the purified protein did not detect lysozyme activity (Supplementary Fig. 2). The lack of *in vivo* and *in vitro* activity of the truncated R²¹ indicated that the SAR domain of R²¹ is necessary for its enzymatic function. Further, when Gly14 and Gly15 of the SAR domain were replaced with Leu residues, the R²¹ SAR domain lost its ability to escape the membrane and lysis was blocked unless CHCl₃ was added to disrupt the bilayer (Figs. 1b, c and d). Appending an artificial TMD to the N-terminus of full-length R²¹ resulted in a chimera that was membrane-tethered and, although not explicitly lytic, had sufficient enzymatic activity to convert all the induced cells to spherical morphology (Figs. 1b, c and d). Apparently, in this chimera, the location of the R²¹ SAR sequence distal to the TMD prevented its recognition by the *sec* system and caused it to be secreted into the periplasm with the rest of the polypeptide. Moreover, replacing the SAR domain of R²¹ with that of Lyz^{P1} resulted in a chimera that, although retaining the ability to be released from the membrane, was inactive (Figs. 1b and c). In contrast, Lyz^{P1} is still functional when its SAR domain is replaced by that of R²¹². Taken together, these results demonstrate that, besides controlling the topology of protein, the SAR domain in R²¹ plays a specific and more integral role in the catalytic activity of enzyme, compared to that in Lyz^{P1}.

To explore the structural basis for this novel regulation, we determined the crystal structures of the active, full-length enzyme, ^aR²¹, and the inactive enzyme, ⁱR²¹, which is missing the entire SAR domain, both to high resolution (Fig. 2a; Supplementary Table 2). ^aR²¹ shares the characteristic structure of the canonical T4 lysozyme, with the 42 residues distal to the SAR sequence forming a relatively independent catalytic domain, containing a catalytic triad (Glu35, Asp44 and Thr50; Figs. 1a and 2a, Supplementary Figs. 1 and 3a) connected by a long α-helix (Lys68 to Tyr89) to the cluster of C-terminal α-helices (helix α4 to α8). Despite marginal sequence identities (33.9% of R²¹ with Lyz^{P1}, 30.3% with P22 gp19, 18.8% with T4 E), the geometry of the catalytic triad in ^aR²¹ is nearly identical to those in other three known structures in the T4 E family (Supplementary Fig. 3b)^{2,6,7}.

In ^aR²¹, the SAR domain is folded into two anti-parallel α-helices, α1 (residues Pro3 to Gly15) and α2 (residues Ala17 to Thr26), which pack against the C-terminal helical bundle (α4 – α8) at an angle of about 45° (Fig. 2a; Supplementary Figs. 3a and 4). In the N-terminal domains of both T4 E and Lyz^{P1}, an α-helix (Ile3 - Glu11 in E; Asn31 – Glu42 in Lyz^{P1}; Fig. 1a) that interacts extensively with the C-terminal lobe terminates with the essential glutamate of the catalytic triad (Supplementary Fig. 1). In Lyz^{P1}, the SAR α-helix packs laterally to that essential α-helix. However, in ^aR²¹, the SAR helix α2 seems to play the same structural role of the first two turns of the essential helix in Lyz^{P1}. The critical Glu

of $^aR^{21}$ (Glu35) is located not on the helix itself, but on a loop directly downstream of the SAR helix. This loop is stabilized by a network of hydrogen bonds which includes residues of the SAR domain (Supplementary Fig. 3c). The catalytic Glu35 forms a salt bridge through its O ϵ with the side chain of Arg152 in helix α 8 of the C-terminal helical bundle, a feature well-known for T4 E 8 and also shared by the P22 and P1 enzymes (Supplementary Fig. 3d). Sequence alignment of R^{21} -like endolysins indicates that this Glu-Arg salt bridge is conserved throughout this family (Supplementary Fig. 5).

The structure of the inactive form, $^iR^{21}$, revealed several important features. The catalytic Glu35 can be only built to C β in $^iR^{21}$, presumably due to the flexibility of this region. Whereas in Lyz^{P1} , the active and inactive forms have radically different structures, the overall fold of $^iR^{21}$ is nearly identical to that of $^aR^{21}$, with every major secondary structural element preserved, except the changes to the active site (Fig. 2a). Like Lyz^{P1} , R^{21} has two disulfide bonds that provide structural stability, but unlike Lyz^{P1} , there is no difference in the disulfide bonding pattern between the active and inactive forms. In the catalytic domain, the loop region between Ser38 and Thr67, which includes two of the residues of the catalytic triad, Asp44 and Thr50, are superimposable (rmsd $\sim$ 0.4 Å) in $^aR^{21}$ and $^iR^{21}$ (Fig. 2b). However, in $^iR^{21}$, the absence of the two α -helices of the SAR domain and the adjacent H-bond network has a dramatic effect on the position of Glu35, which is displaced about 10 Å and replaced by Lys147. Given the indispensable role of the catalytic Glu demonstrated in T4 E 9 , the displacement and disorder of Glu35 account for the lack of enzymatic activity in $^iR^{21}$.

More significant differences were observed in the C-terminal domain (Glu96-Gln164; backbone rmsd $\sim$ 4.0 Å), including the tilt, rotation and unwinding one-turn of helix α 8 which contacts the SAR domain in $^aR^{21}$. Arg152 is turned towards the inner surface of the helix α 4- α 8 bundle in $^iR^{21}$ and loses the capability to form the salt bridge with Glu35 (Fig. 2c). Also helix α 7 rotates and gains one more turn at its end, and the kinked α 5-turn- α 6 is straightened into one α -helix in $^iR^{21}$ (Fig. 2a). Inspection of the predicted electrostatic surfaces reveals that the interface between the SAR domain and the body of the enzyme is dominated by hydrophobic contacts in $^aR^{21}$ (Supplementary Fig. 4). In $^iR^{21}$, the same surface is anionic and solvent-exposed, mainly due to rotation of helix α 8 (Fig. 2d).

The differences in the structures of $^iR^{21}$ and $^aR^{21}$ suggest a model for the activation event. The SAR domain, upon release from the membrane where it is presumably entirely helical, "jack-knives" into an α -helix-turn- α -helix structure to minimize the exposed hydrophobic surface (Fig. 2a). It packs against the non-polar face of helix α 4, which leads to dislodgement of helix α 8. Incorporation of the SAR domain into the R^{21} hydrophobic core leads to remodeling of the C-terminal domain and brings the critical Glu35 to its active position. In this perspective, the newly released SAR domain activates R^{21} through the spatially adjacent C-terminal helical bundle, rather than the sequence-close N-terminal domain.

Once synthesized, SAR endolysins require both strict post-secretory negative regulation and the means to become activated in a timely manner, since they are not sequestered from their substrate by the membrane. Inactive Lyz^{P1} has two levels of negative control: covalent inactivation of its active site Cys and a N-terminal catalytic domain with radical conformational disability, while $^iR^{21}$ appears to lack only the correct placement of its catalytic Glu. On the other hand, the Cys substitution for Asp in the catalytic triad of Lyz^{P1} potentially reduces enzyme activity in the T4 E context¹⁰ and also may require a host enzyme, like DsbA for activation¹. In contrast, R^{21} seems to be more poised for muralytic function, with its canonical catalytic triad and the minimally dysfunctional conformation of the inactive enzyme. The particular evolutionary path taken by these two SAR endolysins

would reflect the selective pressures exerted on the lysis process. However, our survey showed that the R²¹ strategy is more representative among the SAR endolysins, which includes those from phages encoding potent human toxins, like the cytolethal distending toxin¹¹, CDT, and the Shiga toxin¹²; moreover, in the latter case, the acute dispersal of the toxin in the mammalian gut is dependent on the muralytic action of the SAR endolysin^{13,14}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge the contribution of Erik McKee and Elizabeth Caronna in structure determination of ³R²¹. The work of Q.S., A.A. and J.C.S. was supported by funds to J.C.S. from the Robert A. Welch Foundation A-0015 and US National Institutes of Health grant PO1AI060342. The work of GK, MX and RY was supported by PHS grant NIGMS27099 and by the Program for Membrane Structure and Function, a Program of Excellence grant from the Office of the Vice President for Research at Texas A&M University. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under contract No. DE-AC02-06CH11357.

References

1. Xu M, Struck DK, Deaton J, Wang IN, Young R. Proc. Natl. Acad. Sci. USA. 2004; 101:6415–6420. [PubMed: 15090650]
2. Xu M, et al. Science. 2005; 307:113–117. [PubMed: 15637279]
3. Park T, Struck DK, Deaton JF, Young R. Proc. Natl. Acad. Sci. USA. 2006; 103:19713–19718. [PubMed: 17172454]
4. Park T, Struck DK, Dankenbring CA, Young R. J Bacteriol. 2007; 189:9135–9139. [PubMed: 17827300]
5. Lei SP, Lin HC, Wang SS, Callaway J, Wilcox G. J. Bacteriol. 1987; 169:4379–4383. [PubMed: 3040692]
6. Mooers BH, Matthews BW. Acta Crystallogr. Sect D: Biol. Crystallogr. 2006; 62:165–176. [PubMed: 16421448]
7. Bell JA, et al. Proteins. 1991; 10:10–21. [PubMed: 2062826]
8. Strynadka NC, James MN. EXS. 1996; 75:185–222. [PubMed: 8765301]
9. Rennell D, Bouvier SE, Hardy LW, Poteete AR. J. Mol. Biol. 1991; 222:67–88. [PubMed: 1942069]
10. Hardy LW, Poteete AR. Biochemistry. 1991; 30:9457–9463. [PubMed: 1892846]
11. Asakura M, et al. Proc. Natl. Acad. Sci. USA. 2007; 104:14483–14488. [PubMed: 17726095]
12. Plunkett G III, Rose DJ, Durfee TJ, Blattner FR. J. Bacteriol. 1999; 181:1767–1778. [PubMed: 10074068]
13. O'Brien AD, Marques LR, Kerry CF, Newland JW, Holmes RK. Microb. Pathog. 1989; 6:381–390. [PubMed: 2671581]
14. Ochoa TJ, Chen J, Walker CM, Gonzales E, Cleary TG. Antimicrob. Agents Chemother. 2007; 51:2837–2841. [PubMed: 17526759]

Figure 1. The R²¹ N-terminal domain and its physiological function

(a) N-terminal sequences of R²¹, Lyz^{P1} and T4 E. The N-terminal domains of R²¹, Lyz^{P1} and T4 E are shown aligned by the E-8aa-(D/C)-5aa-T catalytic triad (blue and asterisks). SAR domains are boxed in orange. Leu substitutions made in R²¹ are shown above Gly14 and Gly15. The PelB signal sequence (PelB_{ss}) and the artificial TMD (TMD_{art}; yellow) are shown with arrows indicating points of fusion in the chimeric constructs. Residues involved in α -helices that position the catalytic Glu are highlighted by a light blue box in Lyz^{P1} and T4 E. **(b) Lysis profiles.** (○) pZE-luc, (●) pZE-R²¹_{G14,15L}, (□) pZE-luc + 1mM CHCl₃ at 80 minutes after induction, (■) pZE-R²¹_{G14,15L} + 1mM CHCl₃ at 80 minutes after induction, (◇) pZE-R²¹, (◆) Lyz^{P1}₁₋₂₆ΦR²¹₂₇₋₁₆₅, (△) pZE-pelB_{ss}ΦR²¹₂₇₋₁₆₅, (▲) pZE-TMD_{art}ΦR²¹. **(c) Localization and processing of R²¹ and derivatives** after expression of the indicated chimeras. For the top two panels, lane 1 = total (T), lane 2 = periplasm (P), and lane 3 = spheroplast (S) fraction. For the bottom two panels, lanes 1, 2, and 3 represent the total (T), soluble (S), and membrane (M) fractions, respectively. **(d) Morphologies of cells** expressing the indicated pZE-R²¹ plasmids at 100 minutes after induction. In each panel, the scale bar represents 5 μ m.

Figure 2.

Structural basis of R²¹ activation. (a) Topological and conformational dynamics of R²¹ activation. Crystal structures of ⁱR²¹ (left) and ^aR²¹ (right) are represented in cartoon format: α-helix in cyan, β-strand in magenta, coil in salmon; the SAR domain is shown in orange, which is depicted as a membrane-spanning helix in ⁱR²¹. The catalytic triads are represented in stick-and-ball, disulfide linkages in yellow stick. (b) Alignment of the catalytic loop regions of ⁱR²¹ (green) and ^aR²¹ (salmon). Except for Glu35, which shows a 10 Å Cα displacement (dashed line), most of the catalytic loop (Ser38 to Thr67) of ^aR²¹ can be superimposed on the same region of ⁱR²¹ (RMS = 0.4 Å for 209 atoms). (c) Alignment of the C-terminal domains of ⁱR²¹ and ^aR²¹, colored as in (b). Beginning at Glu96, the backbone RMS between the two structures is 4.0 Å. Helix α8 in ⁱR²¹ tilts 30°, turns and unwinds compared to ^aR²¹, so turning Arg152 away from Glu35. (d) Polarity switching at the interface for the SAR domain. The imaginary electrostatic surface (positive = blue; negative = red) contacting the helices of the extracted SAR domain is shown for ^aR²¹ (right). The corresponding surface is shown for ⁱR²¹ at left, with the SAR helices superimposed as an orange backbone ribbon trace.