Biochemical and Biophysical Research Communications 441 (2013) 297–300

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Proposed docking interface between peptidoglycan and the target recognition domain of zoocin A

Yinghua Chen [a], Robin S. Simmonds [b], Russell Timkovich [a,*]

[a] Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487, United States
[b] Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand

ARTICLE INFO

*Article history:*
Received 3 September 2013
Available online 25 September 2013

*Keywords:*
Nuclear magnetic resonance
Exoenzyme
Cell wall specificity
Peptidoglycan
*Streptococcus*

ABSTRACT

A docking model is proposed for the target recognition domain of the lytic exoenzyme zoocin A with the peptidoglycan on the outer cell surface of sensitive bacterial strains. Solubilized fragments from such peptidoglycans perturb specific backbone and side chain amide resonances in the recombinant form of the domain designated rTRD as detected in two-dimensional $^{1}H$–$^{15}N$ correlation NMR spectra. The affected residues comprise a shallow surface cleft on the protein surface near W115, N53, N117, and Q105 among others, which interacts with the peptide portion of the peptidoglycan. Calculations with AutoDock Vina provide models of the docking interface. There is approximate homology between the rTDR-peptidoglycan docking site and the antigen binding site of Fab antibodies with the immunoglobin fold. EDTA was also found to bind to rTRD, but at a site distinct from the proposed peptidoglycan docking site.



## 1. Introduction

*Streptococcus equi* subspecies *zooepidemicus* 4881 secretes an antibacterial exoenzyme zoocin A that hydrolyzes the peptidoglycan outer cell wall of sensitive strains, while not affecting the wall of resistant strains including 4881 [1]. Zoocin A is composed of two domains, designated the N-terminal catalytic (CAT) and C-terminal target recognition (TRD) domains, separated by an unstructured Thr/Pro-rich linker peptide. The domains have been cloned separately and expressed in *Escherichia coli* as rCAT and rTRD [2]. The CAT domain is a zinc-containing endopeptidase similar to the catalytic domain of lysostaphin and the single domain of the *Staphylococcus aureus* autolysin LytM [3]. The TRD domain showed no sequence homology to any known protein or domain in publically accessible sequence databases. A recent NMR-based solution structure for rTRD also showed it possessed a unique protein fold that could not be classified into any known folding family [4]. However, it did roughly resemble the immunoglobin fold of Fab antibodies, which is characterized by two crisscrossing spans of beta sheets.

Fig. 1 shows the chemical structure of the repeating unit of peptidoglycan from a typical sensitive bacterial strain. The carbohydrate backbone polymer consists of alternating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). The carboxylate of the lactic acid moiety of NAM makes a peptide bond to a tetrapeptide containing D-isomers and an isopeptide bond as shown. A variable number of L-alanyl residues following the terminal D-alanine form what is called the cross bridge because they are bonded to the lysyl side chain of a tetrapeptide originating off of another NAM residue by an omega-*N*-peptide bond. This second tetrapeptide in general is from a distinct carbohydrate strand and it in turn has a cross bridge to still another strand and so forth, leading to a complicated interlocking three dimensional insoluble polymer coating for the cell, its exoskeleton. This article reports experiments and calculations that lead to a proposed model for the docking of TRD to this exoskeleton.

## 2. Materials and methods

$^{15}N$-enriched rTRD was prepared as described previously [4]. Solubilized peptidoglycan fragments were prepared also as described previously [5]. This preparation involves digesting purified peptidoglycan polymer from a sensitive strain with a combination of two enzymes: zoocin A to hydrolyze peptide bonds and mutanolysin, a glycosidase, to hydrolyze carbohydrate bonds. In the present study the enzymatic digestions were performed in a volatile buffer (0.1 M ammonium bicarbonate) for 24 h. Undigested solid polymer was removed by centrifugation and the supernatant was lyophilized then re-dissolved in just water to form working stock solutions. The starting polymer was obtained from growing cells and is heterogeneous because of incomplete formation of the tetrapeptides, variable length of cross bridges, and

* Corresponding author. Address: Department of Chemistry, PO Box 870336, University of Alabama, Tuscaloosa, AL 35487-0336, United States.
*E-mail address:* rtimkovi@bama.ua.edu (R. Timkovich).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.087

incompletely terminated cross bridges. Add to this the complicated interlocking three dimensional network of the starting polymer, and even exhaustive digestions produce a highly heterogeneous mixture. The solubilized fraction was examined by LC–MS on an in-house Bruker HCTultra PTM Discovery System with electrospray interface using a capillary column (Agilent ZORBAX SB-C18, 150 × 0.5 mm, 5 mm) eluted with an acetonitrile–water gradient at 10 μL per minute. Masses detected ranged from 500 to 1924 Daltons. Some of the components were identified by their mass as dimers, trimers, etc. of the core displayed in Fig. 1, but incomplete resolution precluded assignment of all components. Peptidoglycan was quantified in the soluble fraction by acid hydrolysis of portions in 6 M HCl at 110 °C for 24 h, followed by quantification of the amount of alanine. From Fig. 1 it can be seen that the core repeating unit of the peptidoglycan contains roughly four equivalents of alanine. One unit of peptidoglycan was therefore defined as that amount of solubilized fraction that would contain 4 mol of alanine. Molar ratios of peptidoglycan to rTRD are the ratio of units to mols of rTRD. This is a very rough approximation because it is unlikely that all the fragments interact with TRD with similar affinity, but, it does establish some scale for the relative concentrations.

Two dimensional $^{1}H–^{15}N$ correlation spectra (heteronuclear single quantum correlation) were taken as described previously and interpreted with assignments made previously [4]. These spectra contain the fingerprint region of NH functional groups including the main chain amide backbones with the nitrogen shift along one axis and the shift of the attached proton along the other. The chemical shifts are very sensitive to perturbations on the protein such as would accompany a docking event. Docking simulations were performed with the software application AutoDock Vina [6]. Calculations employed its exhaustiveness parameter, a measure of how long it searches the potential docking region for modes of docking, with values from 8 to 16.

Coordinate files for models of peptidoglycan were constructed using standard library residues and parameters from CNSolve [7]. Carbohydrate residues were constrained to the low energy chair conformation. The coordinate file for EDTA was taken from PDB accession entry 1NNF for the ferric iron binding protein from *Haemophilus influenza* [8].

## 3. Results and discussion

The unfractionated mixture of peptidoglycan fragments was added stepwise to samples of rTRD and $^{1}H–^{15}N$ spectra were recorded. At low molar ratios of peptidoglycan to rTRD of 0.04–0.32, progressive effects were observed for only select resonances. At higher molar ratios of 0.64–2.5, all observable peaks diminished in intensity. Above a molar ratio of 3, no peaks could be observed, even though the mixture was still completely soluble. This spectral bleaching effect may be due to rTRD aggregating with the larger peptidoglycan fragments present at lower concentrations in the heterogeneous digestion mixture which would then decrease the relaxation times of resonances potentially rendering them unobservable by NMR.

The residues most affected at the lowest levels of peptidoglycan were W115 HE1, N53 HD21 and 22, N117 HD21 and 22, Q105 HN, and M95 HN. The perturbations are shown in Fig. 2. In some of the cases it is obvious that the resonance has just shifted, while in others, the effects were so large that the new locations cannot be determined, either because they now overlap with other peaks or have disappeared by some mechanism such as a major decrease in relaxation time, or chemical exchange averaging. These perturbation sites are localized into a small cleft or depression on the surface of rTRD.

Because of the size of the physiological peptidoglycan on the outer cell wall of zoocin sensitive cells, this article will refer to docking rather than ligand or substrate binding. Docking sites were computed using a model consisting of the four carbohydrate residues from Fig. 1 in the chair conformation and using the entire surface of rTRD as the search site. Multiple docking sites with low binding energies were computed over the entire surface. Next a fuller peptidoglycan model such as depicted in Fig. 1 was used with four carbohydrate units, a tetrapeptide attached to the central NAM monomer, a di-L-Ala cross bridge, and another tetrapeptide without carbohydrate attached to the cross bridge via an omega-*N*-peptide bond. Docking sites were calculated and again there were multiple sites distributed over the molecular surface interacting mainly with the carbohydrate residues. The polar carbohydrate residues apparently had an affinity in calculation for multiple surfaces areas on rTRD. The carbohydrate residues can be ruled out as determining the docking site, because both sensitive and resistant strains have exactly the same carbohydrate composition. The specificity resides in the peptide portion whereby the sensitive strains have mostly two alanyl cross bridge residues while the resistant strains have more alanyl residues [9]. So the model was further minimized to two tretrapeptides crosslinked by a di-alanyl cross bridge. Again the entire surface of rTRD was used as the search site. This time docking sites were calculated that consistently placed a portion of the peptide into the depression on the surface of rTRD that contained the residues most affected in the experimental $^{1}H–^{15}N$ spectra. An illustration is given in Fig. 3.

It is unlikely that there is much local conformational flexibility in the interlocked three dimensional peptidoglycan of the cell wall, but it is also possible that there is considerable conformational heterogeneity over the entire surface and rTRD may prefer to dock with certain conformations. The standard distribution of AutoDock Vina allows for free rotation of up to 32 bonds in the ligand. The peptide model had a total of 40 conformationally meaningful freely rotatable single bonds, excluding the CαCβ bond of alanyl residues. Multiple simulations were computed wherein the set of rotatable bonds was varied. In all cases the main chain Ramachandran phi and psi angles for all residues were allowed to rotate and different combinations of side chain single bonds were made rotatable. To save calculation time, the search site was restricted to a hemisphere of rTRD containing the proposed docking site first found

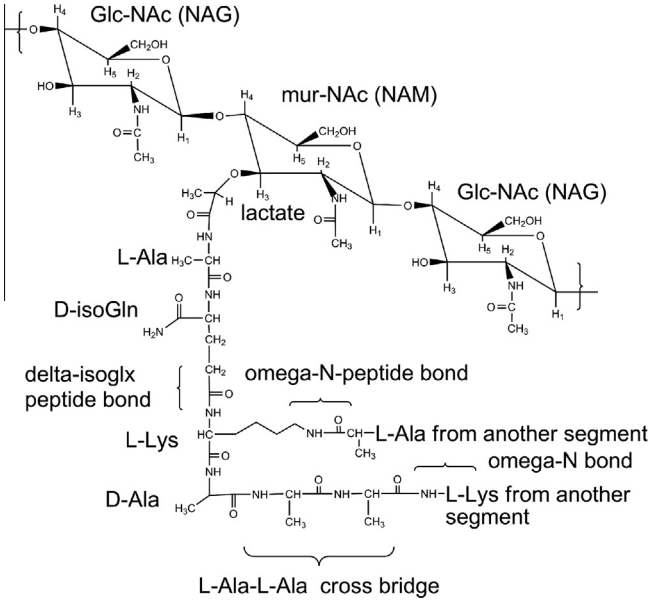


**Fig. 1.** Typical chemical structure of the peptidoglycan of zoocin-sensitive bacteria.

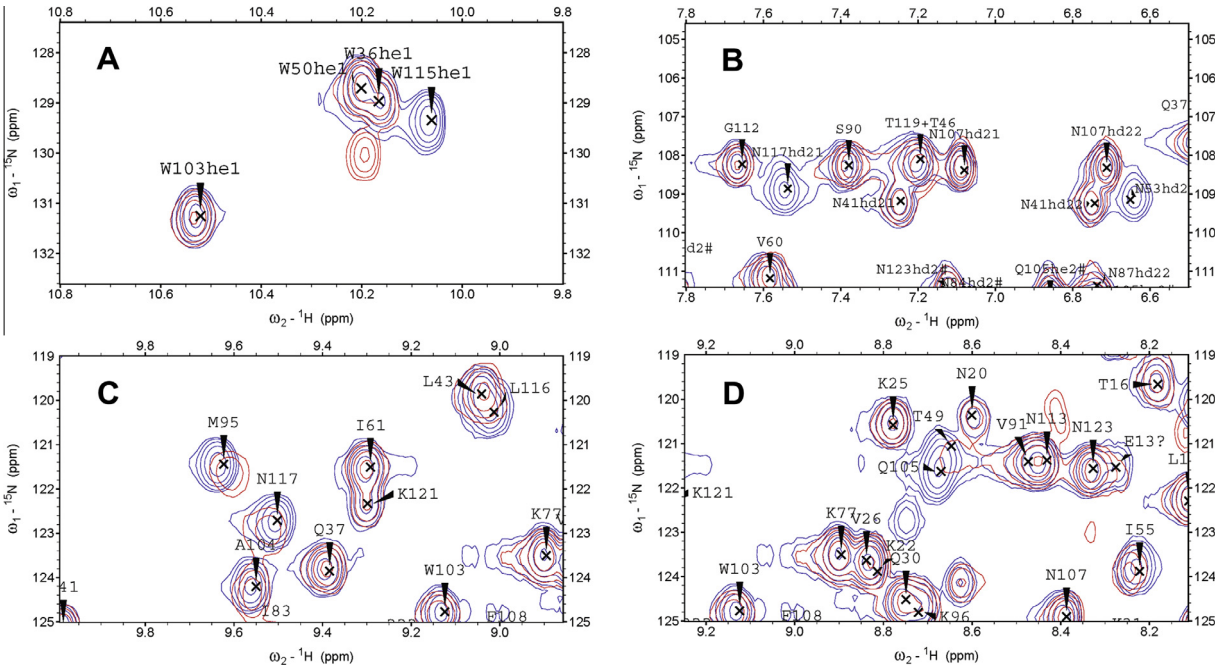


**Fig. 2.** Select regions of the $^{1}H–^{15}N$ correlation spectra of rTRD in the absence (blue contours) and presence (red contours) of solubilized peptidoglycan fragments at a mol ratio of 0.32 peptidoglycan to rTRD. The panels illustrate some of the most drastically perturbed resonances as follows: A, W115HE1; B, N117HD21 and N53HD2; C, M95 and N117 HN; D, Q105 HN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

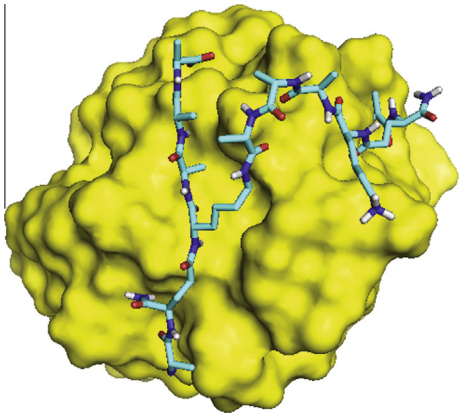

**Fig. 3.** A cartoon showing the molecular surface of rTRD with the unstructured 19 residue amino terminal residues removed for clarity, binding in a surface cleft a model fragment of the peptidoglycan from a sensitive strain consisting of two tetrapeptides and a di-alanyl cross bridge as given in Fig. 1.

in the full surface search. The calculations did not reveal a preference and most docking models were close to extended conformations. Coordinates for a representative set of models are provided in Supplementary Data Coordinates for Models.

The docking simulations by themselves constitute only a marginal case for the identification of the active interface on rTRD. The binding energy for a good docking model in Vina is in the range of −13 to −16 kcal, while the present results ranged from −3.5 to −6.8 kcal. For a family of docking models produced in a single simulation calculation, Vina calculates the root mean squared displacement (rmsd) of all heavy atoms between the model with the most negative binding energy (highest affinity) and other members of the family. The smaller the values, the more similar are the models, and the greater is confidence that a unique binding mode has been found. For a good result such as the test case distributed with Vina, the rmsd's ranged from 1.76 to 3.37 Å. In the present case they ranged from 1.82 to 14.2 Å. This dispersion is graphically evident in the Supporting Information coordinate files. A major source of this is "strand confusion," whereby the two Ala-D-isoGln-Lys-D-Ala segments on either side of the cross bridge are interchanged in the docking model. The key is that despite the dispersion in the models, they localized into the same surface area implicated by experiment.

In spite of the limitations of the calculations, there were some intriguing and suggestive results. Model 1 of the deposited coordinates showed strong hydrogen bonds between the peptidoglycan and rTRD residues including: N53 side chain amide HD22 and the carbonyl of a peptidoglycan Lys carbonyl O, N53 side chain amide carbonyl OD1 and the HN of the same Lys, Q105 side chain amide HE22 to the carbonyl of a peptidoglycan Ala, and K96 HN to the carbonyl OE1 of the D-isogln residue. Hydrogen bonds involving the side chain amides of N53 and Q105 appeared in other, but not all, models. These proton resonances were among those most strongly perturbed in the experimental spectra.

As noted previously, there is some homology between the folding pattern of TRD and the immunoglobin fold of the $V_H$ and $V_L$ domains of the Fab fragment of an IgG immunoglobin [4]. This homology extends to the active surface interface of each protein. Supplementary Data presents a cartoon, Fig. S1, comparing the proposed peptidoglycan docking site on rTRD with the consensus antigen binding site on $V_H$ domains in the region referred to as the hypervariable loop or the complimentary determining region. While not an exact match, there is homology in that the rTRD site lies in a groove formed by the antiparallel beta sheet C and the antigen binding site lies at the hypervariable loop formed an the end of the homolog $V_H$ sheet C. Fig. S2 presents topology diagrams to emphasize this correspondence. The proposed docking site on rTRD falls in line with the hypothesis that TRD is a very crude, primitive form of an antibody.

By serendipity, it was discovered that EDTA also binds to a specific site on rTRD. Supplementary Data contains two annotated and superimposed $^{1}H–^{15}N$ correlation spectra of rTRD without and with added EDTA (Fig. S3). Select resonances were perturbed, given

in Table S1, and these map the proposed binding site. It should be noted that the changes were not as drastic as observed upon peptidoglycan addition. Since the perturbations arose upon simple addition of EDTA, there was the obvious possibility that the EDTA was chelating and removing a metal ion associated with the protein, perhaps in some structural role. This hypothesis was proven false because dialysis which removed the EDTA caused the resonances to revert back to their original positions. Fig. S1 shows the EDTA binding site on TRD in relation to the peptidoglycan docking site, and they are distinct and almost diametrically opposite.

Vina calculations with EDTA as a ligand revealed weak binding modes with energies around −4 kcal and superimposition rmsd's as large as 23 Å. This latter result was due to the fact that two discrete binding sites were calculated. One was in the same grove postulated as the peptidoglycan docking site. It must be stressed that there was no experimental evidence suggesting such a binding site. It may be that the hydrophilic grove just is an attractive calculation site for the polar groups in EDTA. The second site corresponded to the experimentally implicated binding site. Table S1 contrasts observed resonances perturbed by EDTA binding with the nearest neighbors to the Vina calculated site. Supplementary Data Coordinates for Models provides coordinates for models of the bound EDTA.

## Acknowlegements

The authors are grateful to Dr. Shaw Gargis and Prof. Gary Sloan for their instruction and assistance in preparing the solubilized peptidoglycan fragments and enormously helpful discussions and guidance.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.087.

## References

[1] R.S. Simmonds, J. Naidoo, C.L. Jones, J.R. Tagg, The stretococcal bacteriocin-like inhibitory substance, zoocin A, reduces the proportion of *Streptococcus mutans* in an artificial plaque, Microb. Ecol. Health Dis. 8 (1995) 281–292.
[2] R.S. Simmonds, W.J. Simpson, J.R. Tagg, Cloning and sequence analysis of *zooA*, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory substance having a domain structure similar to that of lysostaphin, Gene 189 (1997) 255–261.
[3] Y. Chen, R.S. Simmonds, G.L. Sloan, R. Timkovich, The metal binding site of zoocin A, J. Biol. Inorg. Chem. 13 (2008) 855–860.
[4] Y. Chen, R.S. Simmonds, J.K. Young, R. Timkovich, Solution structure of the recombinant target recognition domain of zoocin A, Proteins 81 (2013) 722–727.
[5] S.R. Gargis, H.E. Heath, L.S. Heath, P.A. LeBlanc, R.S. Simmonds, B.A. Abbott, R. Timkovich, G.L. Sloan, Use of 4-sulfophenyl isothiocyanate labeling and mass spectrometry to determine the site of action of the streptococcolytic peptidoglycan hydrolase zoocin A, Appl. Environ. Microbiol. 75 (2009) 72–77.
[6] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J. Comput. Chem. 31 (2010) 455–461.
[7] A.T. Brunger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, R.J. Read, L.M. Rice, T. Simonson, G.L. Warren, Crystallography & NMR system: a new software suite for macromolecular structure determination, Acta Cryst. D54 (1998) 905–921.
[8] S.R. Shouldice, D.R. Dougan, R.J. Skene, L.W. Tari, D.E. McRee, R.-H. Yu, A.B. Schryvers, High resolution structure of an alternate form of the ferric ion binding protein from *Haemophilus influenzae*, J. Biol. Chem. 278 (2003) 11513–11519.
[9] S.R. Gargis, A.S. Gargis, H.E. Heath, L.S. Heath, P.A. LeBlanc, M.M. Senn, B. Berger-Bachi, R.S. Simmonds, G.L. Sloan, Zif, the zoocin a immunity factor, is a femabx-like immunity protein with a novel mode of action, Appl. Environ. Microbiol. 75 (2009) 6205–6210.