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Structural basis of resistance of *Helicobacter pylori* DnaK to antimicrobial peptide pyrrhocoricin

Musammat F. Nahar, and Anna Roujeinikova

***Abstract*—**Bacterial molecular chaperone DnaK plays an essential role in protein folding, stress response and transmembrane targeting of proteins. DnaKs from many bacterial species, including *Escherichia coli*, *Salmonella typhimurium* and *Haemophilus infleunzae* are the molecular targets for the insect-derived antimicrobial peptide pyrrhocoricin. Pyrrhocoricin-like peptides bind in the substrate recognition tunnel. Despite the high degree of cross-species sequence conservation in the substrate-binding tunnel, some bacteria are not sensitive to pyrrhocoricin. This work addresses the molecular mechanism of resistance of *Helicobacter pylori* DnaK to pyrrhocoricin. Homology modelling, structural and sequence analysis identify a single aminoacid substitution at the interface between the lid and the β-sandwich subdomains of the DnaK substrate-binding domain as the major determinant for its resistance.

Keywords*—Helicobacter pylori*, molecular chaperone DnaK, pyrrhocoricin, structural biology.

I. Introduction

Helicobacter pylori is a gram-negative bacterium that colonises the stomachs of roughly 50% of the world's population[1] and is associated with numerous severe gastroduodenal diseases including gastric and duodenal ulcers, mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma[2, 3]. Eradication of *H. pylori* has been shown to reduce the recurrence of gastric cancer in patients who received endoscopic resection of early gastric cancer and the recurrence of both gastric and duodenal ulcers in patients with peptic ulcer disease[4, 5]. At present, there is no specific single drug that could effectively cure the *H. pylori* infection; current treatments involve the use of a combination of a proton pump inhibitor with two broad-spectrum antibiotics (mainly clarithromycin and either amoxicillin or metronidazole)[6]. An increasing prevalence of resistance to the antibiotic components of such regimens[7] requires development of drugs that specifically interact with *H. pylori*.

The discovery that bacterial molecular chaperones DnaKs are the molecular targets for the insect-derived antimicrobial peptide pyrrhocoricin (VDKGSYLPRPTPPRPIYNRN)[8, 9] attracted interest to these enzymes as new targets for drug design. As a drug target, DnaK has not yet experienced selective pressure in the clinical setting. Blocking DnaK activity inhibits the essential molecular chaperone function implicated in protein folding, stress response and transmembrane targeting of proteins (for the review on DnaK cellular functions, see [10]), thereby killing the bacteria. Non-toxicity to mammalian cells and good serum stability of pyrrhocoricin-derived peptides make them promising drug candidates in treating emerging/re-emerging antimicrobial-resistant bacterial pathogens[9]. This highlights the importance of detailed structural characterization of inhibitor-protein interactions with a view to facilitating rational drug design.

We have recently established that pyrrhocoricins target the substrate-binding tunnel of DnaK[11]. This site shows very little structural variation across different bacterial species. Nevertheless, both pyrrhocoricin-sensitive and pyrrhocoricin-resistant species have been identified. Native pyrrhocoricin kills a broad range of bacterial species, including *E. coli*, *S. typhimurium*, *H. infleunzae* and *Agrobacterium tumefaciens*. Species that are not sensitive to this peptide include *Helicobacter pylori*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Haemophilus ducreyi*[12]. This work addresses the molecular mechanism of resistance of *H. pylori* DnaK to pyrrhocoricin. The high level of sequence identity (approximately 60%) between *H. pylori* and *E. coli* DnaK substrate binding domains allowed us to build an accurate homology model for the *H. pylori* protein, based on the known 3D coordinates of its *E. coli* counterpart[11], and pinpoint the single aminoacid substitution that confers resistance.

II. Materials and Methods

The homology model of *H. pylori* DnaK SBD was constructed using MODELLER (9v7)[13, 14] based on the coordinates of the 2.1-Å resolution crystallographic model of the complex of *E. coli* DnaK SBD with pyrrhocoricin (PDB RSCB 3DPO)[11]. The spatial restraints, including distance restraints and torsion angle restraints, were derived from the sequence alignment and used in the automated 3D-model construction of the protein. The model was further optimized with the internal optimizer of MODELLER. Model quality was assessed using the Prosa2003 [15, 16], ProQ[17] and Verify3D[18] quality scores.

M. N. and A. R. (the corresponding author) are with the Department of Microbiology, Building 76, Monash University, Clayton Campus, Victoria 3800, Australia (phone: +61 399029194; fax +61 399029222; e-mail: Anna.Roujeinikova@med.monash.edu.au).

Multiple sequence alignment was performed with ClustalW2 using the default parameters. Structural superimpositions were carried out using the Swiss PDB viewer[19]. Figures were prepared using PYMOL[20].

III. Results and Discussion

DnaKs from different bacteria have a strictly conserved structural feature proven to be indispensible for their function: a 'latch' between the lid and the β-sandwich subdomains formed by residues Asp431, Arg467, Asp540, His544 and Lys548 (*E. coli* numbering) [21] (Fig. 1).

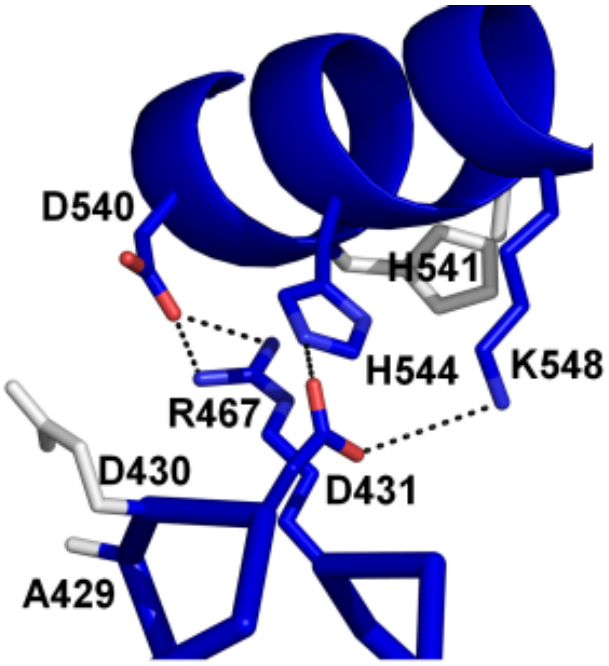

Fig. 1 The functionally important network of hydrogen bonds and ion pairs (called the 'latch') at the interface between the lid and the β-sandwich subdomains [21].

Analysis of the crystal structure of the *E. coli* DnaK substrate-binding domain (SBD) in complex with a pyrrhocoricin-derived peptide inhibitor[11] reveals that this peptide acts as a site-specific, dual mode (competitive and allosteric) inhibitor. The competitive inhibition mode is demonstrated by the fact that it occupies the conventional substrate-binding tunnel of SBD (Fig. 2), mimicking the natural substrate. The detailed comparative analysis of the protein-ligand contacts in the peptide inhibitor complex and in the complex with the non-inhibiting peptide suggests that the allosteric effect of pyrrhocoricin is due to the introduction of a bulky aliphatic cyclohexylalanine (Z) side chain at the position of Leu3 in the non-inhibiting peptide, which causes disruption of the 'latch' between the β-sandwich and the lid subdomains (Fig. 3). Disruption of the 'latch' by other means (e.g. site-directed mutagenesis) renders DnaK inactive. The pyrrhocoricin binding is therefore likely to affect the refolding DnaK activity in a similar way. The structural basis behind this phenomenon is as follows: introduction of a bulky aliphatic side chain of cyclohexylalanine (Cha) at the position of Leu in the non-inhibiting peptide induces a conformational rearrangement in the protein that creates a more hydrophobic environment for this large apolar group at a cost of breaking several hydrogen bonds[11] (Fig. 3).

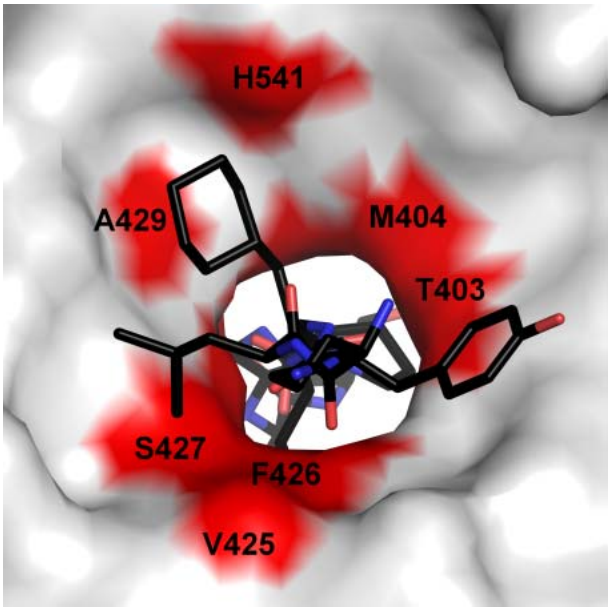

Fig. 2 The pyrrhocoricin binding site of *E. coli* DnaK. The peptide inhibitor is drawn in a stick representation with carbons in black. The molecular surface of DnaK is shown, and the positions of the residues that form the inhibitor binding site are highlighted in red.

In order to gain an understanding of why pyrrhocoricin inhibits *E. coli* DnaK but not the *H. pylori* chaperone, we created a 3D homology model of the complex of *H. pylori* DnaK with pyrrhocoricin-like peptide using the coordinates of the crystal structure of the complex of *E. coli* DnaK SBD with a pyrrhocoricin-derived peptide[11]. The peptide was excluded from the initial calculations for the protein moiety. To model the peptide binding in the substrate-binding tunnel of *H. pylori* DnaK, the 3D atomic model of the latter was superimposed with the crystal structure of the *E. coli* DnaK SBD complex with the inhibitor peptide. The conformations of the side chains in the *H. pylori* protein model around the binding cavity were manually adjusted to remove clashes with the peptide atoms using the manipulation tools implemented in Coot[22]. This model was further optimised by structure idealization through iterative manual model re-building and simulated annealing calculations using an approach similar to that previously employed by Roujeinikova for modelling of the peptidoglycan binding by the motor protein B[23]. The detailed comparative analysis of the generated model and the crystal structure of the inhibitor complex of *E. coli* DnaK SBD shows that the residues forming the substrate binding tunnel are highly conserved between the two proteins. In contrast, the 'latch' at the interface between the lid and the β-sandwich subdomains shows different residue composition.

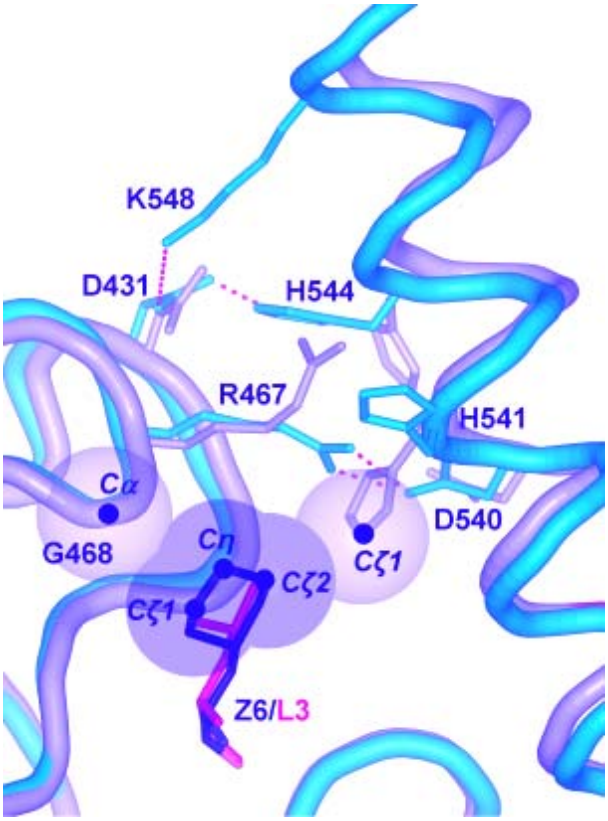

Fig. 3 Superposition of the pyrrhocoricin (carbons in black) and the NR peptide bound to SBD in their respective complexes, showing the broken latch and the additional hydrophobic contacts stabilizing cyclohexylalanine (Z).

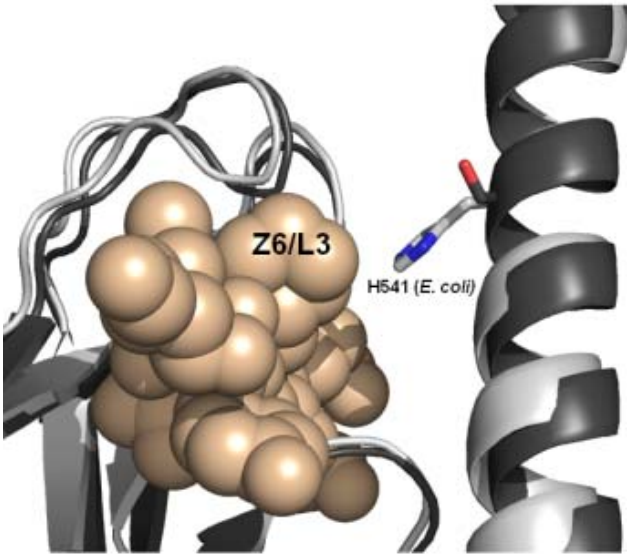

Fig. 4 Superimposition of the crystal structure of the *E. coli* DnaK complex with a pyrrhocoricin-derived peptide with the homology model of *H. pylori* DnaK. The inhibitor peptide is drawn in all-atom representation. The side chains of His541 (*E. coli* DnaK) and Ser535 (*H. pylori* DnaK) are shown.

E. coli	KMVRDAEANAEADRKFEELVQTRNQGDHLLHSTRK 548	
Klebsiella pneumoniae	KMVREAEANAESDRKFEELVQTRNQGDHLLHSTRK 548	pyrrhocoricin-sensitive
Salmonella typhimurium	KMVRDAEANAESDRKFEELVQTRNQGDHLLHSTRK 548	
Micrococcus luteus	RMVKDAEAHADEDRKRREAADRRNQARQSAYSVDK 525	
H. pylori	KMVKDAELHKEEDAKKKEVIEARNHADSLAHQTQK 542	
Haemophilus influenzae	QMVRDAEANADADRKFEEVVQARNQADGIAHATRK 547	pyrrhocoricin-resistant
Pseudomonas aeruginosa	QMVRDAEANAEEDRKFEELAAARNQGDALVHATRK 548	
Staphylococcus aureus	RMVKDAEVNAEADKKRREEVDLRNEADSLVFQVEK 518	

Fig. 5 Sequence comparison of the region surrounding the residue His541 in E. coli DnaK with other known DnaKs. Highlighted are the residues at positions equivalent to His541 in the *E. coli* protein.

The structure superimposition reveals that the Ser535 residue of *H. pylori* protein occupies a position that is structurally equivalent to position 541 (histidine) in the *E. coli* protein (Fig. 4). Our previous analysis suggests that the interactions between His541 and the inhibitor drive the conformational change that breaks the 'latch' and renders the chaperone inactive. The side chain of His541 shields the large apolar side chain of cyclohexylalanine from the solvent in the inhibitor complex of *E. coli* DnaK – the function that the smaller side chain of serine would not be able to perform. Sequence alignment of DnaKs from pyrrhocoricin-sensitive and pyrrhocoricin-resistant bacteria reveals that the former have a large side chain (histidine or glutamine) at the position equivalent to 541 of *E. coli* DnaK, whereas the latter have a small side chain (glycine, alanine or serine) at this position (Fig. 5). Our analysis therefore suggests that the presence of a serine residue at position 535 of *H. pylori* DnaK is the major determinant for its resistance to pyrrhocoricin. Our efforts are currently being directed towards testing this hypothesis using site-directed mutagenesis.

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