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A Comprehensive Study of the Interaction between Peptidoglycan Fragments and the Extracellular Domain of *Mycobacterium tuberculosis* Ser/Thr Kinase PknB

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Abstract

The *Mycobacterium tuberculosis* Ser/Thr kinase PknB is implicated in the regulation of bacterial cell growth and cell division. The intracellular kinase function of PknB is thought to be triggered by peptidoglycan (PGN) fragments that are recognized by the extracytoplasmic domain of PknB. The PGN in the cell wall of *M. tuberculosis* has several unusual modifications, including the presence of *N*-glycolyl groups (in addition to *N*-acetyl groups) in the muramic acid residues and amidation of D-Glu in the peptide chains. Using synthetic PGN fragments incorporating these diverse PGN structures, we analyzed their binding characters through biolayer interferometry (BLI), NMR spectroscopy, and native mass spectrometry (nMS) techniques. The results of BLI showed that mucopeptides containing 1,6-anhydro-MurNAc and longer glycan chains exhibited higher binding potency and that the fourth amino acid of the peptide stem, D-Ala, was crucial for

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Conflict of Interest

The authors declare no conflict of interest.

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protein recognition. Saturation transfer difference (STD) NMR spectroscopy indicated the major involvement of the stem peptide region in the PASTA-PGN fragment binding. nMS suggested that the binding stoichiometry was 1:1. The data provide the first molecular basis for the specific interaction of PGN with PknB and firmly establish PGNs as the effective ligands of PknB.

Keywords

biolayer interferometry; biomolecular interactions; proteins; peptidoglycans; NMR spectroscopy

Peptidoglycan (PGN) is an important component of the cell wall of all bacteria, as it provides both shape and structural integrity to the cell.^[1] PGN mostly consists of long glycan chains with repeating $\beta(1\rightarrow4)$ disaccharide units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are linked through peptide bridges to form a mesh-like network. The stem peptide crosslinks with other fragments by the third amino acid that is usually a diaminocarboxylic acid, such as L-Lys (in most Gram-positive bacteria) or *meso*-diaminopimelic acid (*meso*-DAP, in Gram-negative bacteria and some Gram-positive bacteria, e.g., *Bacillus subtilis* and *Mycobacteria*). PGN has a conserved structure in many bacteria, but modifications are observed in some cases.^[2] The PGN layer of *M. tuberculosis* has modifications in both the peptide stem and the glycan backbone. In the peptide, the carboxylic acids of D-Glu and *meso*-DAP are extensively amidated. In the glycan chain, *N*-glycolylmuramic acid (MurNGlyc) is also generated through the catalysis of an enzyme, *N*-acetylmuramic acid hydroxylase (NamH),^[3] in addition to the conventional MurNAc. The 1,6-anhydroglycan structures have also been observed by means of LC-MS analysis of *M. tuberculosis* muropeptides.^[4] In addition to the typical 3--4 crosslinks of the peptide stem (linking the carbonyl group of D-Ala to the side-chain amine group of *meso*-DAP of an acceptor stem), *M. tuberculosis* has many 3--3 crosslinks (linking the carbonyl group of *meso*-DAP to the other *meso*-DAP in an acceptor stem).^[5]

Reversible protein phosphorylation plays an important role in signal transduction and bioactivity regulation in organisms. The most widely distributed eukaryotic-like protein kinases in bacteria are Ser/Thr protein kinases (STPKs).^[6] *M. tuberculosis* possesses genes encoding 11 STPKs, namely, PknA--PknL, excluding PknC. The gene that encodes PknB is located in an operon containing five genes, two of which are involved in PGN synthesis,^[7] and PknB is implicated as a regulator of cell growth and division. PknB is a receptor like transmembrane protein comprising an intracellular catalytic kinase domain, a juxtamembrane linker, a short transmembrane segment, and the extracellular domain.^[8] So far, a number of probable substrates of PknB have been identified. For example, Wag31 (DivIVA) is involved in cell division;^[9] two β -ketoacyl AcpM synthases, KasA and KasB, are related to mycolic acid biosynthesis;^[10] and PBPA and GlmU are two essential enzymes for peptidoglycan biosynthesis.^[11] However, the exploration of the extracellular stimuli of PknB and the understanding of their interaction remain in the nascent stage.

The extracellular domain of PknB (ED-PknB) is made up of four penicillin-binding-associated and serine/threonine kinase-associated (PASTA) domains, which exhibit a linear organization.^[12] The PASTA domain is a conserved region in the membrane-associated

Ser/Thr kinases that are involved in cell-wall synthesis.^[13] This domain contains three β strands and an α helix, with a loop region of variable length between the first and second β strands.^[14] The PASTA repeat was initially identified in the penicillin-binding protein PBP2X of *Streptococcus pneumoniae*.^[15] The β -lactam ring of cefuroxime, a cell wall synthesis inhibitor that binds to the PASTA domain, is similar to the un-crosslinked PGN,^[16] which led to the prediction that the PASTA domain could be a PGN binding region. Later, PrkC, a three PASTA domain containing STPK from *Bacillus subtilis*, was revealed to bind to mucopeptides, either from purified PGN or the supernatants of the bacterial cultures.^[13] The extracellular domain serves as the receptor for PGN fragments during spore germination. The physical interaction of PrkC PASTA domain with mucopeptides was proved recently by NMR spectroscopy techniques and protein mutagenesis.^[17]

The presence of four PASTA domains in the ED-PknB suggests that this region binds to PGN fragments. The Husson and Boons groups provided the first evidence for the recognition of PknB PASTA domains with synthetic PGN fragments by the surface plasmon resonance (SPR) method.^[18] In this study, the ED-PknB was suggested to bind strongly to the *meso*-DAP-containing PGNs, and ED-PknB was found to be required for localizing PknB at the mid-cell and the cell poles, at which active PGN turnover occurred. The binding assay was done with conventional monosaccharide-containing PGN fragments that exist in most bacteria, without the unusual modifications in *M. tuberculosis* PGNs.

We successfully synthesized a series of *Mycobacterium* PGN structures, as shown in Scheme 1<schrl>, including disaccharide mucopeptides, 1,6-anhydromuramyl-containing mucopeptides, and glycolyl-modified mucopeptides.^[19] Using a combination of biolayer interferometry (BLI), saturation transfer difference (STD)-NMR spectroscopy, and native mass spectrometry (nMS) techniques, we provide a more complete picture of the binding of PGN fragments to the ED-PknB with these diverse variations.

BLI is a label-free optical technology that is used to measure biomolecular interactions, for example, protein--liposome,^[20] protein--protein,^[21] and protein--nucleic acid interactions.^[22] BLI analyzes the interference pattern of white light reflected from two interfaces: A layer of immobilized protein on the biosensor tip and an internal reference layer. We used this method to analyze the interactions between ED-PknB and synthetic PGN fragments characteristic of *M. tuberculosis*, especially for PGN disaccharide structures **1--5** (Scheme 1) with or without modifications.

ED-PknB was prepared according to a reported method,^[18] and this was followed by biotinylation for BLI analysis. The detailed kinetic assay procedure and sensorgrams for other compounds are provided in Figure S1 in the Supporting Information. The binding affinity of synthetic PGN fragments to ED-PknB is shown in Table 1.

As shown in Table 1, glycolylation modification of muramic acid resulted in reduced binding, regardless of other structural differences (i.e., **1** vs. **2** and **4** vs. **5**), which implied that the MurNGlyc moiety might contribute more to stabilization of the cell wall than the activation of bacterial growth. By comparing the compounds containing the *N*-acetylmuramyl group (i.e., **6** vs. **4**), it was inferred that elongation of the glycan chain with

one *N*-acetyl glucosamine enhanced the binding: **4** was threefold more potent than **6**. It was also found that structures containing the 1,6-anhydromuramyl group showed stronger interaction with ED-PknB than their nonanhydro analogues: **4** and **5** showed higher affinities than **1** and **2**, respectively. Furthermore, tripeptide-containing structures displayed much weaker binding abilities than their corresponding tetrapeptide containing ones (i.e., **3** vs. **4**), as shown in a previous report.^[18] These results indicate that D-Ala, the fourth amino acid in the peptide stem, is quite important for the recognition between PGN fragments and the ED-PknB.

STD NMR spectroscopy has emerged as a fast and versatile tool to investigate biomolecular interactions in solution. This powerful technique not only demonstrates the existence of physical binding, but it permits determining which part of the ligand molecule is closer to the receptor, which thus defines the ligand binding epitope.^[23]

Therefore, on the basis of the results of the BLI assay, STD NMR spectroscopy was used to investigate, at atomic resolution, the interaction between the best ligand, **4**, and ED-PknB. As shown in Figure 1, several changes in the shape and the relative intensities of the individual signals in the STD NMR spectrum were observed, which clearly demonstrated that there was direct interaction between the PASTA domain and the synthetic mucopeptide. Comparison of the STD relative enhancements allowed us to map the interacting epitope qualitatively and suggested that the peptide stem, in particular the aliphatic side-chain moieties, were the most involved in the binding process. Additionally, the data also indicated that the saccharidic backbone contributed to the interaction with the protein, but only to a minor extent.

Similar results were obtained from STD NMR spectroscopy analysis of **1** in the presence of ED-PknB (Figure 1 B). Although in this case the interaction was weaker, as suggested by the lower signal-to-noise ratio, the most prominent signals belonged once again to L-Ala, *meso*-DAP, and D-Ala, which suggested that the amino acid portion was in closer contact with the protein. The clear interaction of D-Ala with PknB would be a basis for the enhancement in the binding. The STD NMR spectroscopy analyses therefore confirmed the kinetic results in a direct way. The study of the interaction between ED-PknB and other PGN derivatives, such as the immunoactive peptide heptanoyl- γ -D-Glu-L-*meso*-DAP-D-Ala (FK565)^[24] and the Lys-type PGN fragment MurNAc-L-Ala-D-isoGln-L-Lys,^[25] was also performed. All the NMR spectroscopy results highlighted the major contribution of the peptide stem for recognition and binding, as displayed in Figure S2.

From the results obtained by STD NMR spectroscopy and BLI, 1,6-anhydro-MurNAc-containing fragment **4** showed a more apparent interaction than regular MurNAc-containing fragment **1**. Presumably because 1,6-anhydro-MurNAc is an important motif during the biosynthesis of the bacterial cell wall, PknB might recognize certain stages of the structure of PGN.

To detect proteins or protein--ligand complexes in their native or near-native states in solution, nMS has become a powerful technique for structural biology.^[26] It provides information about the composition, stoichiometry, and structural properties of protein

complexes.^[27] The highest affinity ligand from the kinetic study, compound **4**, was chosen to conduct the nMS analysis with ED-PknB, as shown in Figure 2. The molecular weights of the protein and ligand **4** are 28[^]569 and 920.9240 Da, respectively. The signals belonging to the protein in the free state are represented by the ion series with blue dots. The observation of the ion series with red dots indicates a complex with a molecular weight of 29[^]490 Da; this suggests that the binding molar ratio of ED-PknB/**4** was 1:1.

In conclusion, our results illustrate that the synthetic *Mycobacterium* PGN fragments are direct ligands of the PASTA domains of PknB. The affinity of the interaction was increased for the structures characterized by a tetrapeptide versus tripeptide stem peptide, disaccharide versus monosaccharide glycan chain, *N*-acetyl- versus *N*-glycolylmuramic acid, and 1,6-anhydro-MurNAc versus MurNAc. 1,6-Anhydro-MurNAc is an important motif during the biosynthesis of the bacterial cell wall and during PGN remodeling by lytic transglycosylases; the higher affinity for this moiety suggested that PknB might recognize and modulate certain stages of cell-wall synthesis or turnover. The stem peptide part was largely involved in molecular recognition and binding to ED-PknB, as revealed by STD NMR spectroscopy analysis. The 1:1 binding stoichiometry supports the previously proposed molecular model for PknB activation, that is, the binding of PGN fragments to ED-PknB could facilitate the accumulation of PknB at the cell poles; this would lead to local high concentrations of kinase domain, which could then dimerize easily.^[18]

Our work provides insight into the molecular basis of binding of PGN fragments to the PASTA domains of PknB, including the role of specific constituents and modifications of the PGN fragments that are critical for binding. Genes coding PknB-like protein kinases with extracytoplasmic PASTA domains are also present in many other Gram-positive bacteria.^[28] Our results thus contribute to understanding the biology of these similar proteins.

Experimental Section

Experimental procedures and additional data including detailed BLI analysis of the interaction of ED-PknB and synthetic PGN fragments and the NMR spectroscopy study of the peptide stem (FK565) and Lys-type PGN can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- 1). Höltje J-V, Microbiol. Mol. Biol. Rev. 1998, 62, 181—203. [PubMed: 9529891]
- 2). Sukhithasri V, Nisha N, Biswas L, Anil Kumar V, Biswas R, Microbiol. Res. 2013, 168, 396—406. [PubMed: 23578963]
- 3). Raymond JB, Mahapatra S, Crick DC, Pavelka MS, Jr., J. Biol. Chem. 2005, 280, 326—333. [PubMed: 15522883]
- 4). Mahapatra S, Crick DC, McNeil MR, Brennan PJ, J. Bacteriol. 2008, 190, 655—661. [PubMed: 18024514]
- 5). Lavollay M, Arthu M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L, Mainardi JL, J. Bacteriol. 2008, 190, 4360—4366. [PubMed: 18408028]
- 6). Leonard CJ, Aravind L, Koonin EV, Genome Res. 1998, 8, 1038—1047. [PubMed: 9799791]
- 7). Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry III CE, Tekai F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream M-A, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG, Nature 1998, 393, 537—544. [PubMed: 9634230]
- 8). Chakraborti PK, Matange N, Nandicoori VK, Singh Y, Tyagi JS, Visweswariah SS, Tuberculosis (Oxford, U.K.) 2011, 91, 432—440.
- 9). Kang CM, Nyayapathy S, Lee JY, Suh JW, Husson RN, Microbiology 2008, 154, 725—735. [PubMed: 18310019]
- 10). Molle V, Brown AK, Besra GS, Cozzzone AJ, Kremer LJ, Biol. Chem. 2006, 281, 30094—30103.
- 11). a) Dasgupta A, Datta P, Kundu M, Basu J, Microbiology 2006, 152, 493—504; [PubMed: 16436437] b) Parikh A, Verma SK, Khan S, Prakash B, Nandicoori VKJ, Mol. Biol. 2009, 386, 451—464.
- 12). Barthe P, Mukamolova GV, Roumestand C, Cohen-Gonsaud M, Structure 2010, 18, 606—615. [PubMed: 20462494]
- 13). Shah IM, Laaberki MH, Popham DL, Dworkin J, Cell 2008, 135, 486—496. [PubMed: 18984160]
- 14). Yeats C, Finn RD, Bateman A, Trends Biochem. Sci. 2002, 27, 438—440. [PubMed: 12217513]
- 15). Pares S, Mouz N, Petillot Y, Hakenbeck R, Dideberg O, Nat. Struct. Biol. 1996, 3, 284—289. [PubMed: 8605631]
- 16). Gordon E, Mouz N, Duee E, Dideberg O, J. Mol. Biol. 2000, 299, 477—485. [PubMed: 10860753]
- 17). Squeglia F, Marchetti R, Ruggiero A, Lanzetta R, Marasco D, Dworkin J, Retoukhov M, Molinaro A, Berisio R, Silipo A, J. Am. Chem. Soc. 2011, 133, 20676—20679. [PubMed: 22111897]
- 18). Mir M, Asong J, Li X, Cardot J, Boons GJ, Husson RN, PLoS Pathog. 2011, 7, e1002182. [PubMed: 21829358]
- 19). Wang Q, Matsuo Y, Pradipta AR, Inohara N, Fujimoto Y, Fukase K, Org. Biomol. Chem. 2016, 14, 1013—1023. [PubMed: 26631868]
- 20). Wallner J, Lhota G, Jeschek D, Mader A, Vorauehr-Uhl K, J. Pharm. Biomed. Anal. 2013, 72, 150—154. [PubMed: 23146240]
- 21). Shah NB, Duncan TM, J. Visualized Exp. 2014, 84, 51383.
- 22). Sultana A, Lee JE, Curr. Protoc. Protein Sci. 2015, 79, 19.25.1—19.25.26.
- 23). a) Marchetti R, Perez S, Arda A, Imbert A, Barbero JJ, Silipo A, ChemistryOpen 2016, 5, 274—296; [PubMed: 27547635] b) Berteau O, Sandström C, Bielicki J, Anson DS, Kenne L, J. Am. Chem. Soc. 2003, 125, 15296—15297; [PubMed: 14664566] c) Viegas A, Manso J, Corvo MC, Marques MMB, Cabrita EJ, J. Med. Chem. 2011, 54, 8555—8562; [PubMed: 22091869] d) Angulo J, Nieto PM, Eur. Biophys. J. 2011, 40, 1357—1369; [PubMed: 21947507] e) Tsvetkov YE, Burg-Roderfeld M, Loers G, Ardá A, Sukhova EV, Khatuntseva EA, Grachev AA, Chizhov AO, Siebert HC, Schachner M, Jiménez-Barbero J, Nifantiev NE, J. Am. Chem. Soc. 2012, 134, 426—435. [PubMed: 22087768]

- 24). Mine Y, Yokota Y, Wakai Y, Fukada S, Nishida M, Goto S, Kuwahara S, J. Antibiot. 1983, 36, 1045—1050. [PubMed: 6415029]
- 25). Inamura S, Fujimoto Y, Kawasaki A, Shiokawa Z, Woelk E, Heine H, Lindner B, Inohara N, Kusumoto S, Fukase K, Org. Biomol. Chem. 2006, 4, 232—242. [PubMed: 16391765]
- 26). Zhang H, Cui W, Gross ML, Blankenship RE, FEBS Lett. 2013, 587, 1012—1020. [PubMed: 23337874]
- 27). a) Duijn E. van, J. Am. Soc. Mass Spectrom. 2010, 21, 971—978; [PubMed: 20116282] b) Ishii K, Noda M, Yagi H, Thammaporn R, Seetaha S, Satoh T, Kato K, Uchiyama S, Sci. Rep. 2015, 5, 18167. [PubMed: 26657688]
- 28). Boitel B, Ortiz-Lombardia M, Duran R, Pompeo F, Cole ST, Cervenansky C, Alzari PM, Mol. Microbiol. 2003, 49, 1493—1508. [PubMed: 12950916]

A) ^1H NMR spectrum of **4** (bottom), and 1D STD NMR spectrum of **4** in the presence of ED-PknB (1:50 ratio; top). B) ^1H NMR spectrum of **1** (bottom), and 1D STD NMR spectrum of **1** in the presence of ED-PknB (1:50 ratio; top).

Figure 2.

Native mass spectra of ED-PknB in the presence or absence of **4**. Ion series of the free ED-PknB (●; ($28\,571.32 \pm 0.04$) Da) and the 1:1 complex of PknB/4 (●; ($29\,490.88 \pm 0.00$) Da).

Scheme 1.

Synthetic PGN fragments from *Mycobacterium tuberculosis*.

Table 1

Binding affinity (K_D) values of synthetic PGN fragments for the ED-PknB determined by BLI analysis.

Compound	K_D [μM]
DS-4P _{A-iQ-DAP-A} (<i>N</i> -Ac) 1	38.4
DS-4P _{A-iQ-DAP-A} (<i>N</i> -Glyc) 2	555
DS(anh)-3P _{A-iQ-DAP} (<i>N</i> -Ac) 3	730
DS(anh)-4P _{A-iQ-DAP-A} (<i>N</i> -Ac) 4	12.0
DS(anh)-4P _{A-iQ-DAP-A} (<i>N</i> -Glyc) 5	185
MS(anh)-4P _{A-iQ-DAP-A} (<i>N</i> -Ac) 6	35.8