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Chemical Basis of Peptidoglycan Discrimination by PrkC, a Key Kinase Involved in Bacterial Resuscitation from Dormancy

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Supporting Information

ABSTRACT: Bacterial Ser/Thr kinases modulate a wide number of cellular processes. In *Bacillus subtilis*, the Ser/Thr kinase PrkC has been shown to induce germination of bacterial spores in response to DAP-type but not Lys-type cell wall muropeptides. Muropeptides are a clear molecular signal that growing conditions are promising, since they are produced during cell wall peptidoglycan remodeling associated with cell growth and division of neighboring bacteria. However, whether muropeptides are able to bind the protein physically and how the extracellular region is able to distinguish the two types of muropeptides remains unclear. Here we tackled the important question of how the extracellular region of PrkC (EC-PrkC) senses muropeptides. By coupling NMR techniques and protein mutagenesis, we exploited the structural requirements necessary for recognition and binding and proved that muropeptides physically bind to EC-PrkC through DAP-moiety-mediated interactions with an arginine residue, Arg500, belonging to the protein C-terminal PASTA domain. Notably, mutation of this arginine completely suppresses muropeptide binding. Our data provide the first molecular clues into the mechanism of sensing of muropeptides by PrkC.

During growth, bacteria turn over their cell wall material through the actions of peptidoglycan hydrolases and amidases.[1] Peptidoglycan (PGN) is an essential bacterial cell wall polymer formed by glycan chains of $\beta(1-4)$-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) cross-linked by short peptide stems. Depending on the amino acid located at the third position of the peptide stem, PGN is classified as either Lys-type or *meso*-diaminopimelic acid (DAP)-type. Release of PGN fragments (muropeptides) in the bacterial milieu is also associated with key peptidoglycan hydrolases in bacterial revival from dormancy, a metabolically inactive state that allows them to survive adverse physicochemical conditions or in case of nutrient starvation.[2−4] Consistently, PGN-derived muropeptides induce resuscitation of *Bacillus subtilis* (germination of spores).[5] Muropeptide-driven exit from dormancy requires a member of the serine/threonine kinase (STPK) family, denoted as PrkC.[5] Proteins of this family are expressed in many prokaryotes, including a broad range of pathogens, and modulate a wide number of cellular processes, such as biofilm formation,[6] cell wall biosynthesis and cell division,[7] sporulation,[6,8] and stress response.[9]

PrkC is a membrane protein that comprises an intracellular kinase domain joined by a transmembrane segment to a large extracellular region.[5] Notably, *B. subtilis* spores germinate in response to DAP-type muropeptides, which constitute the *B. subtilis* cell wall, but not in response to L-Lys-type muropeptides.[5] This finding suggests that PrkC extracellular domains exhibit specificity of muropeptide sensing.[5] However, whether muropeptides are able to bind the protein physically and how the extracellular region is able to distinguish the two types of muropeptides is hitherto unknown.

In a previous study, we determined the crystal structure of the extracellular region of a close homologue of PrkC from *Staphylococcus aureus*.[10] This structure shows that the extracellular part of PrkC is formed by three penicillin-binding-associated and Ser/Thr kinase-associated (PASTA) domains and an unpredicted immunoglobulin (IG)-like domain.[10] However, the absence of significant sequence conservation of surface residues did not allow for identification of a binding pocket. In the present work, we have addressed a gap in our initial study, as we address the important question of how the extracellular region of PrkC (EC-PrkC) senses muropeptides. To this end, we isolated and identified by 2D NMR spectroscopy those muropeptides responsible for bacterial revival [$GlcNAcMurNAcAla_2GluDAP$ (**1**) and $GlcNAc_2MurNAc_2Ala_4Glu_2DAP_2$ (**2**); Figure S1 in the Supporting Information (SI)] from *B. subtilis* peptidoglycan (see the SI for a complete NMR discussion and Tables S1 and S2).[5] Thus, here we report saturation transfer difference (STD) NMR, biophysical, and biochemical experiments that reveal the first full picture of the binding of muropeptides to EC-PrkC and the chemical basis for the discrimination of PGN types.

STD NMR spectroscopy is among the most efficient and established methods for obtaining structural details of substrate−protein complexes by epitope mapping.[11−16] ^{1}H NMR and STD NMR analysis of **1** in the presence of EC-PrkC

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clearly revealed that **1** binds EC-PrkC (Figure 1). To determine the binding epitope of **1**, relative STD effects were calculated

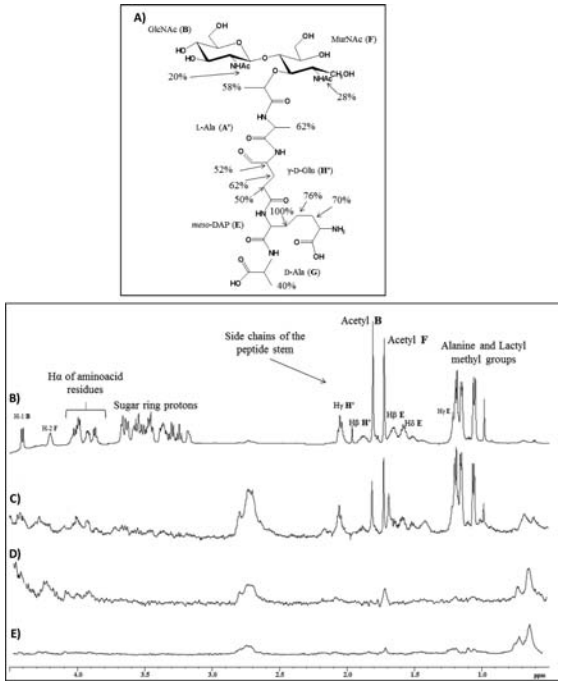

Figure 1. (A) Chemical structure and epitope binding of **1** to EC-PrkC; the percentages refer to the relative STD effects. (B) ^{1}H NMR spectrum of **1**. (C–E) 1D STD NMR spectra of **1** in the presence of (C) EC-PrkC (1:50 ratio) and the (D) R500A and (E) R500E mutants (1:100 ratio). Signals at about 2.9 and 0.5 ppm in (C–E) are residual protein resonances.

from the STD amplification factors. STD NMR signals were observed for side chains of peptide stems (1–2 ppm), indicating that this region makes the closest contacts with the EC-PrkC binding site (Figure 1B,C). In particular, the strongest signals involve the DAP residue (H_β protons at 1.65 ppm; Figure 1C). Significant STD NMR signals were also observed for other side-chain protons of the peptide stem, whereas low-intensity STD signals were recorded for the carbohydrate moieties (Table 1 and Figure 1).

Table 1. Relative STD Effects of 1 Bound to EC-PrkC

	STD (%)
H_β *meso*-DAP (E)	100
H_γ *meso*-DAP (E)	76
H_δ *meso*-DAP (E)	70
H_α *i*-Glu (H′)	52
H_β *i*-Glu (H/H′)	62
H_γ *i*-Glu (H/H′)	50
H_β Ala (G)	40

STD NMR spectra of muropeptide **2** that revealed its binding epitope to EC-PrkC (Figure 2) confirmed a major involvement of its peptide stem upon binding (Figure 2 and Table 2). Because of signal overlap in the region of the spectrum between 3 and 5.2 ppm (Figure S2), we also carried out STD–total correlation spectroscopy (TOCSY) experiments (Figure 2C). The spectra showed the highest STD contribution for proton signals of DAP (H_β, I_{STD} = 100%;

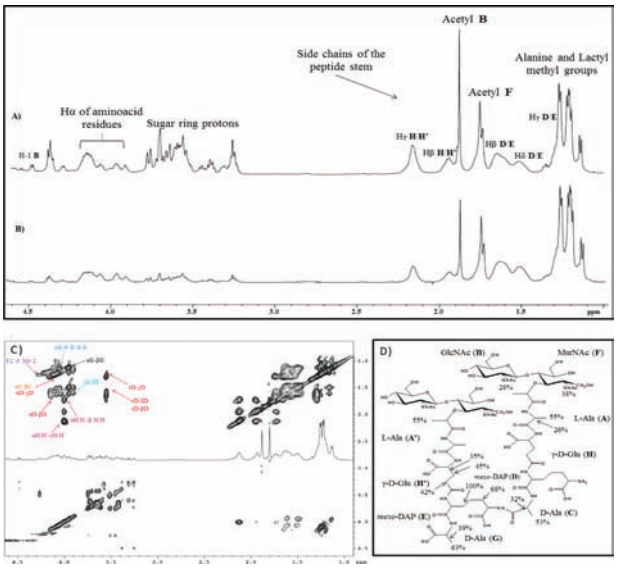

Figure 2. (A) ^{1}H NMR spectrum of **2**. (B) 1D STD NMR spectrum and (C) 2D STD TOCSY spectrum of **2** in the presence of EC-PrkC. (D) Chemical structure and epitope binding of **2** to EC-PrkC; the percentages refer to the relative STD effects.

Table 2. Relative STD Effects of 2 Bound to EC-PrkC

	STD (%)		STD (%)
H_β *meso*-DAP (E)	100	H_α i-Glu (H/H′)	35
H_δ *meso*-DAP (E)	68	H_β *i*-Glu (H/H′)	45
H_β *meso*-DAP (D)	68	H_γ *i*-Glu (H/H′)	42.5
H_γ *meso*-DAP (D)	55.2	H_α Ala (G)	38.8
H_δ *meso*-DAP (D)	43.8	H_2 MurNAc (F)	15.2
H_β Ala (G)	63.6	H_1 GlcNAc*ol* (B)	15.2
H_α Ala (G)	39	H_3 MurNAc (F)	15.1
H_α Ala (C)	32	H_6 MurNAc (F)	14.7
H_β Ala (C)	53.4	H_3 GlcNAc*ol* (B)	12.9
H_α Ala (A)	26	CH_3Ac GlcNAc*ol* (B)	27.8
H_β Ala (A)	55	CH_3Ac MurNAc (F)	38

Figure 2) and only a small involvement (<20%) of the sugar backbone (Figure 2). For both muropeptides, rotational Overhauser spectroscopy (ROESY) and transverse ROESY (tr-ROESY) spectra showed no significant conformational change upon protein binding (Figure S3).

The key involvement of the DAP residue in protein recognition agrees well with the previous finding that only muropeptides containing DAP in their peptide stem resuscitate *B. subtilis*, whereas L-Lys-type muropeptides do not.[5] In further support of the above-proposed model of binding, we studied the interaction of EC-PrkC with a DAP-containing tripeptide (L-Ala-γ-D-Glu-*m*-DAP) lacking the carbohydrate moiety and, as negative control, a lysine-containing tripeptide (L-Ala-γ-D-Glu-L-Lys) (see the SI for a complete discussion, Figures S4–S8 and Tables S3 and S4). The NMR binding data showed unambiguous albeit weak binding of EC-PrkC with the DAP-type peptide (Figure S4), whereas the same experiments carried out for the Lys-type peptide showed no binding (Figure S5).

Overall, these data confirm a key role of DAP in the interaction with EC-PrkC (Figure S6) and provide a rationale for the ability of EC-PrkC from *B. subtilis* to recognize DAP-type but not Lys-type PGN.[5] On the other hand, the weakness of the NMR signals observed for the DAP-type peptide (Figure S4) evidence the importance of the carbohydrate moiety in the

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overall recognition process, consistent with the finding that this peptide fails to induce germination.[5]

Prompted by the observed key role of DAP in protein recognition, we carried out a statistical survey in the Protein Data Bank (PDB) to identify the structural determinants responsible for DAP binding to proteins. Notably, in all structures of protein complexes with DAP, the carboxylate end of DAP forms a salt bridge with an arginine side chain (Figure S9). This finding, together with our STD data (Figures 1 and 2), suggests that muropeptide binding occurs mainly through the interaction of DAP with an arginine residue of EC-PrkC.

We derived a homology model of EC-PrkC from *B. subtilis* using the structure of EC-PrkC from *Staphylococcus aureus* as a template.[10] EC-PrkC contains two arginine residues: Arg614, which is in the IG-like domain and involved in a salt bridge with Glu604 (Figure 3), and Arg500, which is in the PASTA3

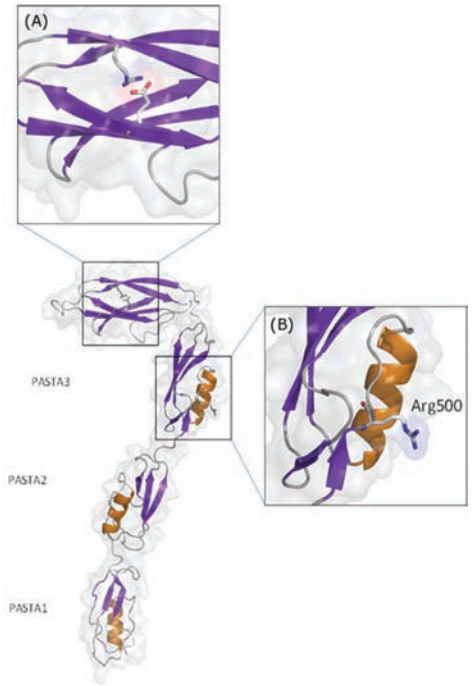

Figure 3. Structure of EC-PrkC from *B. subtilis* derived by homology modeling. The two insets show enlargements of (A) the salt bridge between Arg614 and Glu604 in the IG-like domain and (B) the location of Arg500 in PASTA3.

domain and solvent-exposed (Figure 3). These considerations led us to hypothesize that Arg500 is involved in muropeptide binding in a fashion similar to those observed in the PDB (Figure S9). To corroborate this hypothesis, we mutated Arg500 to alanine (R500A) and to glutamic acid (R500E), which we predicted to be more disruptive of the Arg−DAP interaction. STD experiments unequivocally showed that neither R500E nor R500A are able to bind muropeptides, as no STD signals were observed in either case (Figure 1D,E).

Circular dichroism (CD) spectroscopy excluded the possibility that the differences in the muropeptide binding of the two mutants were due to defects in protein folding induced by the mutations (Figure 4). The inability of the R500E and R500A mutants to bind muropeptides shows that Arg500 is the primary site of interaction with muropeptides. The combination of these results with those from the STD experiments on wild-type EC-PrkC (Figures 1 and 2) proves that PrkC senses

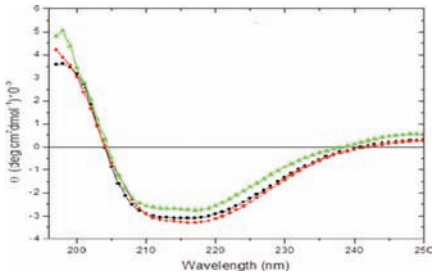

Figure 4. CD spectra of R500A (black) and R500E (red) mutants of EC-PrkC compared to the CD spectrum of wild-type EC-PrkC (green).

muropeptides through interactions of the negatively charged DAP side chain with the side chain of Arg500. Furthermore, the inability of the R500A mutant to bind muropeptides (Figure 1D,E) shows that the contribution of sugar moieties, although measurable (Figure S4), is not sufficient for binding in the absence of DAP−Arg interactions.

Using a set of biophysical techniques, we also analyzed the effect of muropeptide interactions on the protein oligomerization state (see the SI). To make sure we conducted these experiments in muropeptide saturating conditions, we measured the binding affinity of muropeptides to EC-PrkC by isothermal titration calorimetry (see the SI). Using the typical blend of muropeptides released during cell wall remodeling (muropeptides **2** and **1** in a 3:1 molar ratio), we observed a low-millimolar protein−ligand affinity, with K_d = 1.2 mM (K_a = 801.0 ± 33.3 M^{-1}, ΔH = −14.7 ± 0.4 kcal · mol^{-1}; see Figure S10 and the SI).

Similar to PknB kinase from *Mycobacterium tuberculosis* and to PrkC from *S. aureus*,[17,18] the kinase domain of PrkC from *B. subtilis* undergoes self-phosphorylation.[8] Activation of PrkC via self-phosphorylation typically occurs through dimerization, as demonstrated by in vivo studies.[6] However, consistent with our previous data,[10] EC-PrkC is not able to form dimers in solution (Figure 5 and Figure S11), and the presence of muropeptide

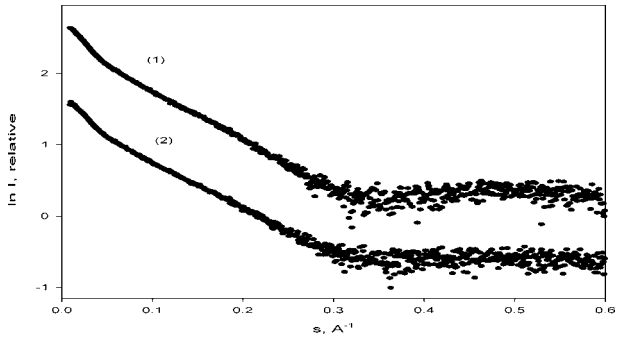

Figure 5. Small-angle X-ray scattering profiles of EC-PrkC (1) without and (2) with addition of **2**. The plot displays the logarithm of the scattering intensity as a function of momentum transfer $s = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and λ = 0.15 nm is the X-ray wavelength. R_g and D_{max} values (Table S3) identify protein monomers. Curves have been arbitrarily displaced along the logarithmic axis for better visualization.

under saturating conditions (Figure S10) does not affect the protein oligomerization state (Figures S11 and S12 and Table S5).

In summary, we have presented in this work two major results. We have clearly demonstrated that in solution EC-PrkC

is able to bind DAP-type muropeptides physically (Figures 1 and 2) and that DAP is the critical element in the binding to the protein. We have also shown that this recognition occurs through interactions of DAP with Arg500, as a mutation of this amino acid in EC-PrkC completely impaired muropeptide binding (Figure 1D,E). This finding agrees well with the key role played by arginine in the specific recognition of DAP-type muropeptides by peptidoglycan recognition proteins.[19] In this scenario, the key role of Arg500 in binding provides a clear explanation for the ability of PrkC from *B. subtilis* to discriminate between DAP- and Lys-type muropeptides in bacterial revival.[5] Using this mechanism, *B. subtilis* bacteria, which possess a DAP-type PGN, can cross-talk and trigger resuscitation by its own cell wall turnover.

Consistent with our results, very recent data have shown that PknB kinase from *M. tuberculosis* also possesses a strong preference for DAP-type muropeptides similar to the bacterial cell wall fragments, although the protein interaction site and reasons for specificity are yet to be identified.[20] Furthermore, the observed inability of EC-PrkC to form dimers in vitro points to a more complex protein dimerization mechanism, which may either involve the PrkC transmembrane portion or require a third molecule, available in vivo, in a manner similar to that observed for the fibroblast growth factor receptor (FGFR).[21] Finally, the definition of structural determinants of muropeptide-driven revival from dormancy is precious to the development of low-molecular-weight entities of therapeutic interest.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, supporting results, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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