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A microplate assay for the coupled transglycosylase–transpeptidase activity of the penicillin binding proteins; a vancomycin-neutralizing tripeptide combination prevents penicillin inhibition of peptidoglycan synthesis

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ABSTRACT

A microplate, scintillation proximity assay to measure the coupled transglycosylase–transpeptidase activity of the penicillin binding proteins in *Escherichia coli* (Eco) membranes was developed. Membranes were incubated with the two peptidoglycan sugar precursors UDP-N-acetyl muramylpentapeptide (UDP-MurNAc(pp)) and UDP-[^{3}H]N-acetylglucosamine in the presence of 40 μM vancomycin to allow *in situ* accumulation of lipid II. In a second step, vancomycin inhibition was relieved by addition of a tripeptide (Lys-D-ala-D-ala) or UDP-MurNAc(pp), resulting in conversion of lipid II to cross-linked peptidoglycan. Inhibitors of the transglycosylase or transpeptidase were added at step 2. Moenomycin, a transglycosylase inhibitor, had an IC_{50} of 8 nM. Vancomycin and nisin also inhibited the assay. Surprisingly, the transpeptidase inhibitors penicillin and ampicillin showed no inhibition. In a pathway assay of peptidoglycan synthesis, starting from the UDP linked sugar precursors, inhibition by penicillin was reversed by a 'neutral' combination of vancomycin plus tripeptide, suggesting an interaction thus far unreported.

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1. Introduction

Peptidoglycan is the major structural component of the bacterial cell wall. It is a polymer of a repeating disaccharide–peptide unit, where the pentapeptide chains attached to adjacent sugar molecules are cross-linked. The disaccharide unit is synthesized on a lipid precursor from the sugar precursors UDP-N-acetyl glucosamine (UDP-GlcNAc) and UDP-N-acetyl-muramylpentapeptide (UDP-MurNAc(pp)). The MraY enzyme condenses UDP-MurNAc(pp) with undecarprenyl pyrophosphate present in the membrane to form lipid I. Subsequently, MurG catalyzes transfer of GlcNAc to lipid I to form lipid II. The final stage of peptidoglycan synthesis occurs in the periplasm: polymerization of the disaccharide unit (GlcNAc-MurNAc(pp)) by the transglycosylase (TG) to form peptidoglycan and simultaneous crosslinking of the peptide chains by the transpeptidase (TP) to form crosslinked peptidoglycan (Fig. 1A). The lipid carrier is released and recycled by the action of the lipid pyrophosphorylase. All enzymes and the lipid carrier are present in the membrane. The coupled TG and TP activities are catalyzed by penicillin binding proteins (PBP), e.g. PBP1a, 1b of *Escherichia coli* [1,2].

The TG and TP, being membrane-associated, and on the periplasmic surface are accessible to drug molecules and are attractive drug targets, especially since peptidoglycan is absent in eukaryotes. The TP is the target of the β-lactams, one of the most successful antibiotic classes to date. This target is being currently revisited, by the discovery of novel β-lactams and PBP inhibitors [3,4] as well as β-lactamase inhibitors [5] and combinations of the two [6] that overcome resistance to previous generations of β-lactams. However, both enzymes, and, in particular, the TG, are difficult to assay in a format amenable to high throughput screening.

Measuring the peptidoglycan TG activity is challenging, because the substrate, lipid II, is difficult to synthesize [7–9] and the TG product, uncross-linked peptidoglycan, is not easily distinguished

Abbreviations: UDP-GlcNAc, UDP-N-acetyl glucosamine; UDP-MurNAc(pp), UDP-N-acetyl-muramylpentapeptide; TG, transglycosylase; TP, transpeptidase; V + T, vancomycin + neutralizing tripeptide; PBP, penicillin binding protein; UDP-MurNAc-(pp), UDP-N-acetyl-muramylpentapeptide; SPA, scintillation proximity assay; WGA, wheat germ agglutinin.

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from the substrate; paper chromatography or HPLC may have to be used. Binding assays to detect inhibitors of the TG that compete with moenomycin have been reported [10]. Lipid II can be accumulated *in situ* in membranes using detergent [11] or a PBP1b mutant [12] and a high throughput, continuous assay has been described [13].

Several assays have been described for the TP activity of the PBPs, but most are binding assays and are not truly reflective of the catalytic activity. The most commonly used is the binding of a β-lactam to the PBPs to screen for agents that compete with this binding [14,15]. Monitoring the enzyme activity of the TP is even more challenging than that of the TG, since its product, crosslinked peptidoglycan, is not easily distinguished from uncrosslinked peptidoglycan. An exception is the scintillation proximity assays (SPAs) reported from our research unit that can easily assay the transpeptidase, albeit in the environment of the membrane, either in isolation, [16] or coupled with enzymes in the pathway of peptidoglycan biosynthesis [12,17,18]. These showed that wheat germ agglutinin coated SPA (WGA-SPA) beads in the presence of a detergent, such as N-lauryl sarcosine (sarkosyl), can specifically capture and distinguish crosslinked peptidoglycan from uncrosslinked peptidoglycan or lipid II in membranes of Eco [16–19]. The other two species can be captured by the same beads in the absence of detergent. Here a simple, coupled TG–TP assay is described.

2. Materials and methods

2.1. Materials

Wheat germ agglutinin-coated SPA (WGA-SPA) beads were from Amersham International plc. U.K. UDP-[^{3}H]-N-acetyl glucosamine was from NEN Dupont, USA. Other chemicals were from Sigma Chemical Co. USA. Moenomycin was a gift from Hoechst, India.

2.2. Enzyme and substrates

UDP-N-acetyl muramyl pentapeptide (UDP-MurNAc(pp)) was purified from *Bacillus cereus* [17]. Membranes were prepared from *E. coli* AMA1004 [17].

2.3. Enzyme assays

All enzyme reactions were performed in 96 well flexible plates (Wallac) in a final volume of 25 μl. Reactions were stopped by the addition of 5 μl of 90 mM EDTA. For the SPAs to measure crosslinked peptidoglycan 170 μl of WGA-SPA beads (0.5 mg) in 50 mM HEPES ammonia pH 7.3 was added along with sarkosyl to a final concentration of 0.2% [18]. For measurement of lipid II the sarkosyl was left out of the WGA-SPA bead mixture [17–19].

For paper chromatography analysis, a second set of reactions was run in parallel to the SPA and analyzed by paper chromatography [17]. Peptidoglycan remains at the origin, whereas lipid II has an R_f of ~0.9 [17,20]. The chromatogram was cut into pieces and the radioactivity measured in a liquid scintillation counter.

2.4. Peptidoglycan pathway assay

The membrane steps of peptidoglycan synthesis, starting from the UDP-linked precursors, was performed similar to the method described [17]. *E. coli* membranes (4 μg protein) were incubated for 90 min at 37 °C with 15 μM UDP-MurNAc(pp), 2.5 μM UDP-[^{3}H]GlcNAc (0.2 μCi) in 50 mM HEPES ammonia pH 7.5, 10 mM $MgCl_2$, 8% DMSO. Reactions were carried out in triplicate, unless otherwise specified. The enzyme blank was a reaction without UDP-MurNAc(pp) and, for each type of capture condition, the cpm obtained in this reaction was subtracted from that of reactions containing both sugar precursors (complete or 100% reaction) as a measure of 'Activity'.

2.5. Transglycosylase–transpeptidase assay

The TG substrate, lipid II, was synthesized in *E. coli* membranes (4 μg) incubated for 120 min at 37 °C with 15 μM UDP-MurNAc(pp), 4.16 μM UDP-[^{3}H]GlcNAc (0.5 μCi), 40 μM vancomycin in 50 mM HEPES-ammonia pH 7.5, 10 mM $MgCl_2$, in 15 μl. Subsequently, in a second step, the TG–TP reaction was carried out by adding 10 μl of a solution containing UDP-GlcNAc (to 250 μM) to dilute out the radiolabel, DMSO (to 8%), Nα,Nε diacetyl Lysine-D-alanine-D-alanine (Lys-D-ala-D-ala) tripeptide (to 400 μM) to neutralize vancomycin and the reaction was incubated for 5 min at 37 °C. Inhibitors were added at this step. The reaction was stopped and analyzed by SPA or paper chromatography as described above; for experiments where products were analyzed in parallel by paper chromatography 1.2 μCi UDP-[^{3}H]GlcNAc was used. Reactions were carried out in triplicate, unless otherwise specified. For the enzyme blank, reactions were stopped and products measured at the end of step 1; an alternative blank used was a reaction containing no UDP-MurNAc(pp) at step 1.

3. Results

3.1. Assay principle

A coupled TG–TP assay was envisaged as a two step process with an inhibitor of the TG used in step 1 to allow accumulation of the TG substrate, lipid II, *in situ* in *E. coli* membranes (Fig. 1B and C). In a second step, the plan was to relieve inhibition of the TG to allow the coupled TG–TP to convert the lipid II to crosslinked peptidoglycan.

Experiments using moenomycin to inhibit the TG resulted in accumulation of lipid II, but the inhibitory effect of moenomycin could not be reversed by dilution. Vancomycin inhibits MraY, MurG, the TG and also the TP [21]. Experiments in our laboratory, suggested that the TG was more sensitive to inhibition by vancomycin than the MraY and MurG. If so, it was likely that vancomycin could be used at a concentration that inhibited the TG, but not MraY or MurG, so as to cause accumulation of lipid II *in situ* in *E. coli* membranes. The plan was to subsequently neutralize vancomycin by a peptide containing D-ala-D-ala or by the substrate UDP-MurNAc(pp) to allow catalysis by the TG and TP.

3.2. In situ synthesis of lipid II: titration of vancomycin concentration

The concentration of vancomycin was titrated in the peptidoglycan pathway assay with the aim of finding a condition where lipid II was formed, but not peptidoglycan (Table 1). This assay monitors the membrane steps of peptidoglycan synthesis starting from the UDP-linked sugar precursors [18]. One set of reactions was captured by WGA-SPA beads to monitor lipid II and a second set with WGA-SPA beads plus sarkosyl to monitor the quantity of cross-linked peptidoglycan synthesized [18].

At very low concentrations of vancomycin no inhibition of any of the enzymes occurred, resulting in synthesis of cross-linked peptidoglycan. However at 30–60 μM vancomycin the counts were highest in the SPA without detergent and minimal in that with detergent indicating a significant quantity of lipid II and a minimal amount of crosslinked peptidoglycan were formed (Table 1). This was confirmed by paper chromatography analysis (data not

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