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Short communication

Peptidoglycan glycosyltransferase-ligand binding assay based on tryptophan fluorescence quenching

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ABSTRACT

Peptidoglycan glycosyltransferases (GTase) of family 51 are essential enzymes for the synthesis of the glycan chains of the bacterial cell wall. They are considered potential antibacterial target, but discovery of inhibitors was hampered so far by the lack of efficient and affordable screening assay. Here we used *Staphylococcus aureus* MtgA to introduce a single tryptophan reporter residue in selected positions flanking the substrates binding cavity of the protein. We selected a mutant (Y181W) that shows strong fluorescence quenching in the presence of moenomycin A and two lipid II analogs inhibitors. The assay provides a simple method to study GTase-ligand interactions and can be used as primary high throughput screening of GTase inhibitors without the need for lipid II substrate or reporter ligands.

1. Introduction

Antimicrobial resistance (AMR) is now recognized as a major global health crisis [1]. To face this problem, new bacterial targets should be explored to identify novel antibiotic classes. The peptidoglycan (PG) is an essential component of the bacterial cell wall [2]. Its synthesis is targeted by β-lactam antibiotics (penicillins, cephalosporins), the most prescribed drugs used in the treatment of bacterial infections. These antibiotics inhibit the transpeptidation step catalyzed by penicillin-binding protein (PBPs) [3]. Besides this transpeptidase (TPase) activity, the PBPs are also responsible for the polymerization of glycan chains catalyzed by the TPase-associated glycosyltransferase (GTase) domain (GT51family in the CAZy database, http://www.cazy.org/ [4]) of the bifunctional PBPs [5]. Both reactions are essential to build the protective peptidoglycan sacculus of major bacteria. The GTase module is composed of a lysozyme-like globular domain and a small hydrophobic domain (called jaw domain). The interface between these two subdomains harbors the enzymatic cavity and can be divided into two substrate binding sites: a donor site for the lipid-bound growing glycan chain, which is also the moenomycin A (Moe A) binding site, and an acceptor site for lipid II. Chain elongation is achieved by the addition of disaccharide unit MurNAc-GlcNAc of lipid II.

MoeA (Fig. 1) is a potent antibiotic which specifically bind to the GTase active site and competitively inhibits PG synthesis [6]. Its use has not been approved in human but it was used in animal feedstock for decades without reported resistance [7]. This exceptional feature makes the MoeA binding pocket an excellent antibiotic target. However, the discovery of GTase inhibitors was hampered by the lack of access to convenient screening assay. Current assays for screening of GTase inhibitors either rely on labelled (fluorescent or radioactive) lipid II substrate or on fluorescent derivative of MoeA inhibitor which are difficult to obtain in sufficient quantities because their preparation is tedious and time-consuming. Therefore, a simple and rapid primary assay that does not rely on these reagents and can allow the identification of a ligand binding to the active site of enzymes with GTase activity will open the way for the screening of much larger compound libraries.

Intrinsic protein fluorescence spectroscopy has proved to be a useful technique to study ligand binding. Change in tryptophan fluorescence emission is usually used as a reporter to measure ligand binding or conformational changes, due to the high sensitivity of the indole ring to local environment variations. The monofunctional MtgA of *S. aureus* was selected as a model enzyme because this protein is devoid of tryptophan residue [8]. Targeted

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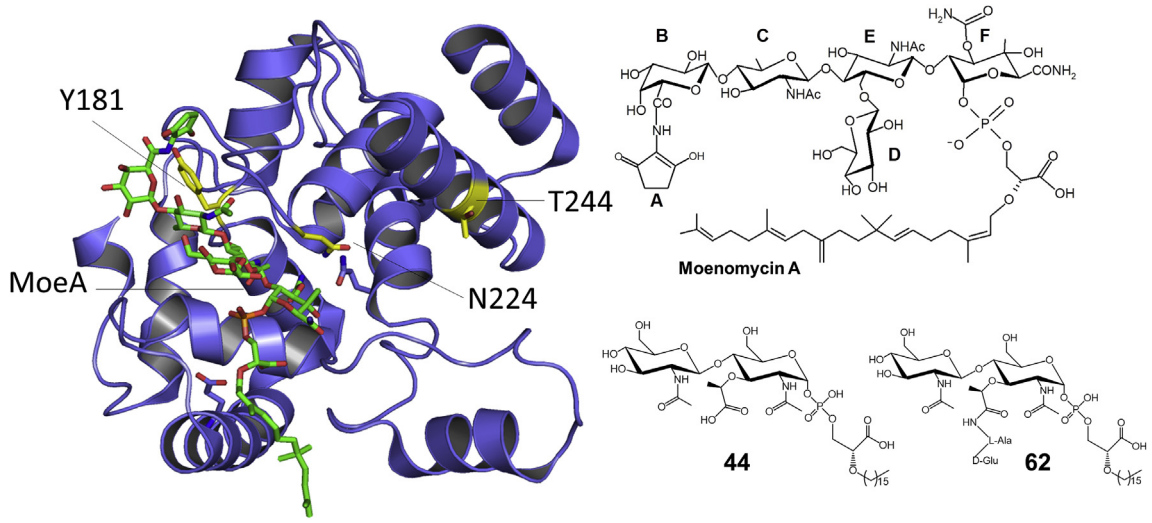

Fig. 1. (A) Structure of *S. aureus* MtgA in complex with moenomycin A (PDB 3HZS). Amino acids mutated to Trp are labelled and shown in yellow sticks. Moenomycin A (in green sticks) is bound to the donor site. (B) Structure of Moenomycin A, and lipid II analogs 44 and 62 used for binding studies with MtgA and mutants. EF-phosphoglycerate moiety is the proposed MoeA pharmacophore.

substitution of amino acids located around the active site of this protein allowed the selection of a single tryptophan mutant showing high variations of fluorescence emission intensities (50–70%) upon addition of known ligands, thus validating our approach.

2. Materiel and methods

2.1. Cloning and site-directed mutagenesis

Single mutations were generated using QuikChange Site-Directed Mutagenesis method (Agilent Technologies) using the oligonucleotides in Table 1 and the plasmid pDML2004 as template [8]. All constructs were checked by sequencing.

2.2. Proteins purification

S. aureus His tagged MtgA (D68-R269), without transmembrane segment, called HisMtgA, was expressed in *E. coli* and purified by Ni-NTA affinity chromatography as described previously [8], with the exception that the purification buffer contained in addition 0.7% CHAPS. All the mutants were produced and purified under the same conditions as the “wild type” HisMtgA. The glycosyltransferase activity of the mutants and the non-mutated proteins were tested as previously described [8].

2.3. Fluorescence spectroscopy

Fluorescence measurements were obtained by monitoring the changes in intrinsic fluorescence emission of the protein samples in a 10 mm path length quartz cell using a Jasco FP-8000 spectrofluorimeter equipped with thermostatically controlled cell holder (25 °C). The MtgA mutants (concentration ~0.26 μM) in the buffer 20 mM Tris-HCl pH 8, 500 mM NaCl, CHAPS 0.7% were titrated with increasing concentration of ligands (MoeA, or compounds 44 or 62) and the quenching of tryptophan fluorescence emission spectra were recorded between 305 et 450 nm, following excitation at 295 nm. Each spectrum was the average of 3 acquisitions and was corrected for the contribution of the experimental solution without protein. The data from the titration experiment of the mutant Y181W by MoeA or substrate analogs were analyzed using nonlinear regression in Graphpad Prism 6 software.

Table 1
Oligonucleotides used in this study.

Name of oligonucleotides	SEQUENCES
N224W-17	TAGTAAAGTCTGGGCACCTAGCGTATATAATATC
N224W-18	GATATTATATACGCTAGGTGCCCAGACTTTACTA
T244W-19	ACGCAACGTGTAAGCTGGAACTTAGAAAAAATG
T244W-20	CATTTTTTCTAAGTTCCAGCTTACACGTTGCGT
Y181W-21	TATTTAAATAATATTTGGTTTGGGGATAATC
Y181W-22	GATTATCCCCAAACCAAATATTATTTAAATA

2.4. Circular dichroism (CD)

Far-UV CD spectra (200–260 nm) were recorded with a Jasco J-810 spectropolarimeter at 20 °C in 25 mM Tris-HCl pH 8, 500 mM NaCl, CHAPS 0.7% using a 1 mm path length quartz Suprasil cell (Hellma), with protein concentration at *ca.* 1.3–2.8 μM. Four scans (10 nm/min, 1 nm bandwidth, 0.1 nm data pitch and 1 s DIT) were averaged, base lines were subtracted and no smoothing was applied. Data are presented as the molar ellipticity per residue ($[\Theta]_{MRW}$), calculated using the molar concentration of protein and number of residue (223 amino acids).

3. Results and discussion

3.1. Mutagenesis and selection of a single tryptophan mutant

The amino acid sequence of *S. aureus* MtgA does not contain any tryptophan residue; we took advantage of this property to introduce a single tryptophan residue in selected positions around the binding cavity for substrates, both in the donor and acceptor sites, of the truncated HisMtgA form (D68-R269) of the protein lacking the transmembrane segment (Fig. 1). The mutants have been expressed and purified in the same conditions used for the “wild-type” HisMtgA. Three mutants (Y181W, N224W and T244W) were selected that behaved as the non-mutated protein in terms of both stability and expression level (Fig. 2). Analysis of the GTase activity of the mutants using radioactive lipid II, showed that they are active and convert lipid II substrate into glycan chains. The relative