COMMUNICATION

ChemComm
www.rsc.org/chemcomm

A fluorescent analogue of UDP-*N*-acetylglucosamine: application for FRET assay of peptidoglycan translocase II (MurG)

Jian-Jun Li and Timothy D. H. Bugg*
Department of Chemistry, University of Warwick, Coventry, UK CV4 7AL.
E-mail: T.D.Bugg@warwick.ac.uk; Fax: 02476-524112; Tel: 02476-573018

Received (in Cambridge, UK) 22nd October 2003, Accepted 24th November 2003
First published as an Advance Article on the web 9th December 2003

A direct continuous fluorescence assay for translocase II MurG based on fluorescence resonance energy transfer (FRET) has been developed using a 6-substituted fluorescent analogue of UDP-*N*-acetylglucosamine.

The glycosyltransferase enzyme translocase II (MurG) catalyses the second step of the lipid cycle of bacterial peptidoglycan biosynthesis, namely the transfer of a GlcNAc residue from UDP-*N*-acetylglucosamine (UDP-GlcNAc) to undecaprenyl pyrophosphoryl MurNAc-pentapeptide (lipid intermediate I) to form undecaprenyl pyrophosphoryl GlcNAc-MurNAc-pentapeptide (lipid intermediate II) (Fig. 1).[1] Enzymes of this pathway are important targets for antibiotic chemotherapy.[1] MurG is known to be inhibited by the cyclic peptide ramoplanin.[2] The availability of an X-ray crystal structure for MurG makes it an attractive target for drug development,[3] but assay of this enzyme is difficult, due to the complexity of the substrates.

Since lipid I is not readily isolable from bacterial cells, and is difficult to handle, some lipid I analogues have been synthesized.[4–7] Generally, a radiochemical assay is the only method for measurement of MurG activity.[4,6] Recently a coupled assay has been reported, in which the release of UDP was detected by coupling with pyruvate kinase/lactate dehydrogenase.[5,7] In this communication, we report a direct continuous fluorescence assay for MurG based on fluorescence resonance energy transfer (FRET).

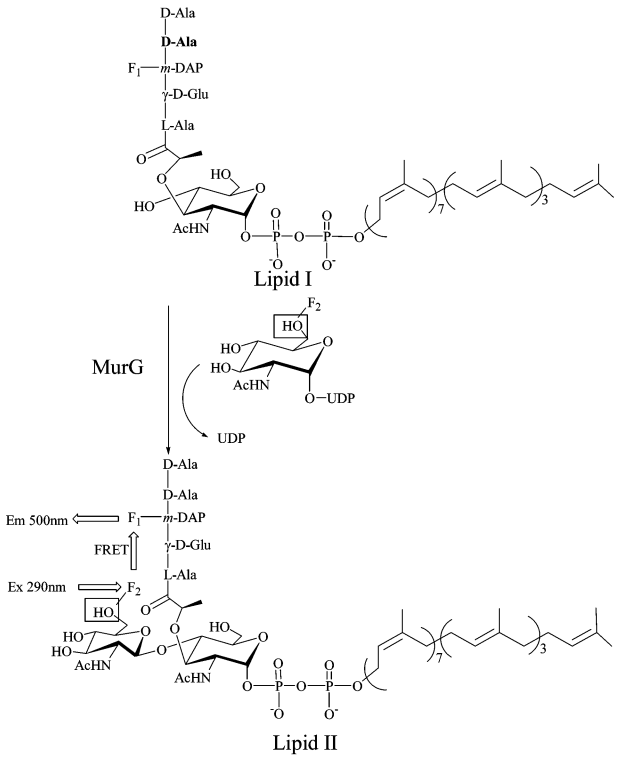


Fig. 1 MurG catalysed reaction. In the FRET assay, the dansyl fluorophore (F_1) acceptor is attached to position 3 of pentapeptide, the indole-3-acetic acid fluorophore (F_2) donor is attached to the C-6 position of UDP-GlcNAc.

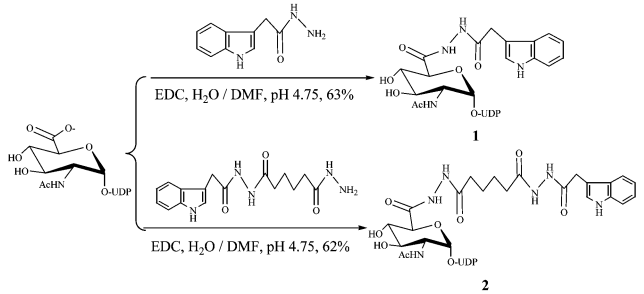
FRET involves the transfer of fluorescence energy from a donor to an acceptor in the substrate or product of the reaction. FRET has become an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.[8] We have previously used a UDPMurNAc-pentapeptide substrate modified with a dansyl fluorophore at position 3 (modified on N of *meso*-DAP) as a fluorescent substrate for the preceding enzyme translocase I (MraY).[9] We have therefore investigated the possibility of attaching a fluorophore to the UDPGlcNAc substrate of MurG, in order to set up a FRET assay for MurG. We have used an indole-3-acetic acid fluorophore (λ_{em} 345 nm), which is able to transfer fluorescence energy effectively to the dansyl fluorophore (λ_{ex} 340 nm, λ_{em} 500 nm).

Functionalisation of UDP-GlcNAc was achieved by Pt/O_2 oxidation of the C-6 hydroxyl of UDP-GlcNAc to a carboxylic acid, using the method of Yamazaki *et al.*[10] 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide (EDC) coupling of amines to the C-6 carboxylic acid was unsuccessful, but coupling of hydrazides at pH 4.75 proceeded smoothly, using the method of Pouyani *et al.*[11] Indole-3-acetic hydrazide was successfully attached to oxidized UDP-GlcNAc in 63% yield (Scheme 1). Using adipic hydrazide, a further indole-acetic acid derivative containing a 10-atom linker was also synthesized.

Escherichia coli MurG was purified from overexpressing construct pET3a carrying the *murG* gene.[4] UDPMurNAc-L-Ala-D-Glu-*m*-DAP (N^{ε}-dansyl)-D-Ala-D-Ala was prepared as previously described,[9] and was converted to dansyl-labelled lipid I having C_{35} prenyl chain[12] by treatment with *E. coli* translocase I, followed by extraction into n-butanol.[13] The fluorescent UDP-GlcNAc analogues[14] were tested as substrates for MurG, in the presence of dansyl-labelled lipid I, in 0.2 M Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.2% CHAPS. Time-dependent fluorescence changes were observed (λ_{ex} 290 nm, λ_{em} 500 nm), dependent upon both substrate and enzyme, as shown in Fig. 2.

A linear relationship between enzyme concentration and rate of increase in fluorescence was observed (Fig. 3). No fluorescence changes were observed in the absence of enzyme. Turnover of lipid I to lipid II was further confirmed by measurement of UDP release, using a coupled assay,[5,7] and by thin layer chromatography. Interestingly, the two fluorescent analogues demonstrated different behaviour: **1** showed 1.5-fold higher rates of fluorescence increase than **2**, implying a more efficient energy transfer in **1**, due to the shorter linker.

Using the dansyl-labelled lipid I and **1** as substrates, the kinetic parameters for processing by MurG were determined using a



Scheme 1 Synthesis of fluorescent analogues of UDP-GlcNAc.

DOI: 10.1039/b313316h

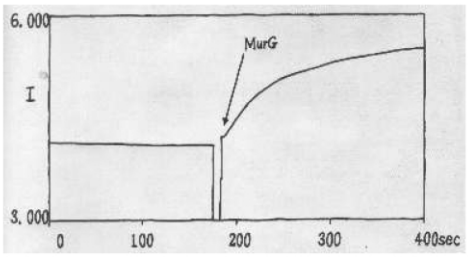


Fig. 2 Progress of MurG catalysed reaction upon addition of 3.0 μg MurG to 3.0 μM dansyl lipid I and 2.7 μM **1**.

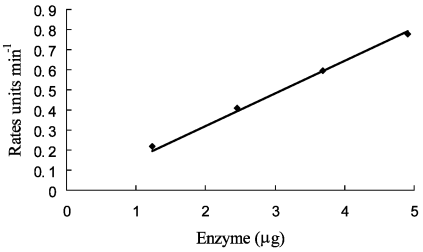


Fig. 3 Relative rate of increase in fluorescence against enzyme concentration.

Lineweaver–Burk plot. The measured K_m value for **1** was 0.06 μM, much lower than the reported K_m value for unmodified UDP-GlcNAc (58 μM[4]), in which case radiochemical assay was used. The measured k_{cat} value was 153 Fl units/mg protein/min. Measurement of UDP release using the coupled assay gave a rate of turnover of 0.025 μmoles/mg protein/min. Fluorescence changes could be totally inhibited by 20 μM ramoplanin, and 40% inhibited by 0.02 μM ramoplanin.

A number of other detergents were examined for activity in this assay. Fluorescence changes were only observed using zwitterionic detergents (CHAPS, SB3–10), at detergent concentrations below the critical micellar concentration (CMC). Therefore it appears that the reaction is not taking place in a detergent micelle, but that probably the low concentration of detergent is required to solubilise the lipid I substrate.

In conclusion, UDP-GlcNAc was successfully modified through the C-6 position with a fluorescent label. Assays with *E. coli* MurG have shown that the enzyme can accept the modified UDP-GlcNAc as substrate, and energy transfer is observed under certain conditions with a dansyl-labelled lipid I substrate. To our knowledge, this is the first direct continuous fluorescence assay for MurG.[15] The availability of fluorescently modified UDP-GlcNAc will enable fluorescent assays to be established for other glycosyltransferases that use UDP-GlcNAc.

We would like to thank GlaxoSmithKline Pharmaceuticals for financial support, and Dr M. Burnham and Dr K. O'Dwyer for helpful comments. We thank Professor S. Walker for the provision of expression construct pET3a carrying the *murG* gene, and we thank Dr S. Donadio (Vicuron Pharmaceuticals, Milan, Italy) for the provision of a sample of ramoplanin.

Notes and references

1 T. D. H. Bugg, in *Comprehensive Natural Products Chemistry*, ed. M. Pinto, Elsevier, Oxford, 1999, **vol. 3**, p. 241.
2 (*a*) E. A. Somner and P. E. Reynolds, *Antimicrob. Agents Chemother.*, 1990, **34**, 413; (*b*) P. E. Reynolds and E. A. Somner, *Drugs Exptl. Clin. Res.*, 1990, **16**, 385.
3 S. Ha, D. Walker, Y. Shi and S. Walker, *Protein Sci.*, 2000, **9**, 1045.
4 S. Ha, E. Chang, M. C. Lo, H. B. Men, P. Park, M. Ge and S. Walker, *J. Am. Chem. Soc.*, 1999, **121**, 8415.
5 L. Chen, H. B. Men, S. Ha, X. Y. Ye, L. Brunner, Y. N. Hu and S. Walker, *Biochemistry*, 2002, **41**, 6824.
6 G. Auger, J. van Heijenoort, D. Mengin-Lecreulx and D. Blanot, *FEMS Microbiol. Lett.*, 2003, **219**, 115.
7 H. Liu, T. K. Ritter, R. Sadamoto, P. S. Sears, M. Wu and C.-H. Wong, *ChemBioChem*, 2003, **4**, 603.
8 R. P. Haughland, *Handbook of Fluorescent Probes and Research Chemicals*, 6th Edn., Molecular Probes, Eugene, OR, USA, 1996, p. 46.
9 P. E. Brandish, M. K. Burnham, J. T. Lonsdale, R. Southgate, M. Inukai and T. D. H. Bugg, *J. Biol. Chem.*, 1996, **271**, 7609.
10 T. Yamazaki, C. D. Warren, A. Herscovics and R. W. Jeanloz, *Can. J. Chem.*, 1981, **59**, 2247.
11 T. Pouyani and G. D. Prestwich, *Bioconjugate Chem.*, 1994, **5**, 339.
12 Auger *et al.* and Chen *et al.* found lipid I bearing a C_{55} prenyl chain is a poor substrate for MurG. See refs. 5 and 6.
13 J. S. Anderson, M. Matsuhashi, M. A. Haskin and J. L. Strominger, *J. Biol. Chem.*, 1967, **242**, 3180.
14 The fluorescent analogues of UDP-GlcNAc **1** and **2** were characterised by ^{1}H NMR and MS. **1**, δ_H (400 MHz, D_2O), 7.75 (d, 1H, J = 8.3 Hz, H-6 uracil), 7.61 (d, 1H, J = 8 Hz, H-4 indole), 7.47 (d, 1H, J = 8.0 Hz, H-7 indole), 7.32 (s, 1H, H-2 indole), 7.21 (dd, 1H, J = 7.3, 7.0 Hz, H-6 indole), 7.13 (dd, J = 7.1, 7.0 Hz, H-5 indole), 5.82 (d, 1H, J = 3.5 Hz, H-1 ribose), 5.81 (d, 1H, J = 8.3 Hz, H-5 uracil), 5.51 (dd, 1H, J_{PH} = 7.4 Hz, J_{HH} = 3.2 Hz, H-1 hexose), 4.38 (d, 1H, J = 10.0 Hz, H-5 hexose), 4.17 (m, 2H, H-2, 3 ribose), 4.11 (m, 2H, H-5a, 5b ribose), 4.06 (m, 1H, H-4 ribose), 4.01 (dt, 1H, J = 10.3, 3.0 Hz, H-2 hexose), 3.82 (s, 2H, CH_2-indole), 3.81 (t, 1H, J = 9.8 Hz, H-3 hexose), 3.70 (t, 1H, J = 9.4 Hz, H-4 hexose), 2.03 (s, 3H, NAc). ESI-MS (M − 1) 791.16, expected 792.14; **2**, δ_H (400 MHz, D_2O), 7.85 (d, 1H, J = 8.8 Hz, H-6 uracil), 7.59 (d, 1H, J = 7.8 Hz, H-4 indole), 7.45 (d, 1H, J = 8.0 Hz, H-7 indole), 7.30 (s, 1H, H-2 indole), 7.20 (dd, 1H, J = 7.0, 7.0 Hz, H-6 indole), 7.12 (dd, 1H, J = 7.8, 7.0 Hz, H-5 indole), 5.87 (d, 1H, J = 4.2 Hz, H-1 ribose), 5.85 (d, 1H, J = 8.0 Hz, H-5 uracil), 5.51 (dd, 1H, J_{PH} = 7.3, J_{HH} = 3.0 Hz, H-1 hexose), 4.36 (d, 1H, J = 9.8 Hz, H-5 hexose), 4.26 (m, 2H, H-2, 3 ribose), 4.20 (m, 2H, H-5a, 5b ribose), 4.12 (m, 1H, H-4 ribose), 4.00 (dt, 1H, J = 10.5, 3.0 Hz, H-2 hexose), 3.80 (t, 1H, J = 9.7 Hz, H-3 hexose), 3.79 (s, 2H, CH_2-indole), 3.69 (t, 1H, J = 9.5 Hz, H-4 hexose), 2.31–2.25 (m, 4H, CH_2CH_2CH_2CH_2), 2.01 (s, 3H, NAc), 1.62–1.57 (m, 4H, CH_2CH_2CH_2CH_2). ESI-MS (M − 1) 933.25, expected 934.21.
15 During the preparation of this manuscript, a fluorescent binding assay for MurG has been published. See: J. S. Ham, Y. Hu, L. Chen, B. Gross and S. Walker, *J. Am. Chem. Soc.*, 2003, **125**, 11168.