Site-specific N-terminal labelling of proteins *in vitro* and *in vivo* using *N*-myristoyl transferase and bioorthogonal ligation chemistry†

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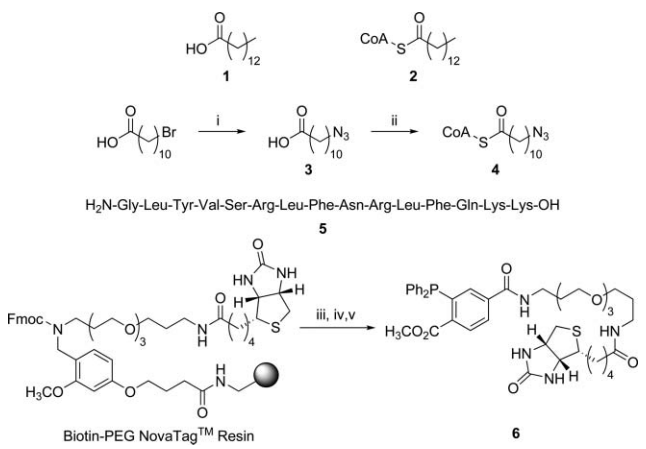
***N*-Myristoyl transferase-mediated modification with azide-bearing substrates is introduced as a highly selective and practical method for *in vitro* and *in vivo* N-terminal labelling of a recombinant protein using bioorthogonal ligation chemistry.**

Site-specific labelling of proteins with chemical and fluorescent probes is a prerequisite for many established and emerging techniques in chemical and cell biology.[1] Protein labelling technology has been revolutionised by the discovery of highly selective, water-compatible reactions[2] that permit bioorthogonal ligation of a wide variety of synthetic probes at one or more sites defined by a small chemical tag such as an azide, alkyne or aldehyde. Whilst bioorthogonal ligation chemistry has proven to be quite robust,[3] there remains a need for new methods that allow the introduction of tags into a protein in a site-specific manner. Recent innovations in this area, including expanded codon usage for incorporation of tagged amino acids[4] and site-directed post-translational modification,[1,5] can require relatively complex biochemical and/or synthetic protocols that can limit their widespread use for labelling applications. Here, we describe a practical transferase-based technique that permits site-specific *in vitro* and *in vivo* generation of N-terminal azide-tagged recombinant protein, and subsequent labelling by bioorthogonal ligation chemistry.

N-Myristoylation at an N-terminal glycine is a constitutive co-translational modification that occurs in all eukaryotic cells, and contributes to regulation of signalling and trafficking by modulating protein–membrane and protein–protein associations.[6] Myristoyl-CoA:protein *N*-myristoyl transferase (NMT), the enzyme that catalyses myristoylation, has been characterised in a wide range of organisms, from yeast to humans.[7] Comprehensive studies by Gordon *et al.* found that the specificity of *Saccharomyces cerevisiae* NMT (ScNMT) towards fatty acyl-CoA analogues is limited to compounds that closely mimic myristoyl-CoA, particularly with respect to chain length (~14 heteroatoms) and flexibility.[8] Nevertheless, early work suggested that ScNMT tolerates the introduction of some functionality in the acyl chain of the myristoyl-CoA substrate[8,9] and recent reports have described the use of modified myristate for non-radioactive labelling in mammalian cell lines.[20] Encouraged by this work, we examined the possibility of using azide-modified myristate analogues as a means for introducing a bioorthogonal ligation tag at the N-terminus of proteins bearing an N-terminal glycine. A well-characterised and widely-used NMT cloned from *Candida albicans* (CaNMT)[10] was selected for the present work: it exhibits excellent stability, activity and solubility, and may be over-expressed and purified without the need for an affinity tag.[11]

To test this approach we synthesised ω-azido undecanoyl-CoA **4** and a model peptide substrate **5**, corresponding to the N-terminal region of *Plasmodium falciparum* ADP ribosylation factor 1 (PfARF1),[11,12] carrying the canonical N-terminal myristoylation target motif Gly-Xaa-Xaa-Xaa-Ser (Scheme 1). Incubation of equimolar quantities of **4** and **5** with a catalytic quantity of CaNMT (0.25 mol%) in a suitable buffer for 18 h was sufficient for complete transfer of the ω-azido undecanoyl group from **4** to the target peptide as determined by HPLC (Fig. 1), and at a rate comparable to myristoyl-CoA **2** (data not shown).

Following these promising initial results, the tagging technique was applied to the labelling of a recombinant protein. An ω-azido



Scheme 1 Myristic acid (**1**), myristoyl-CoA (**2**) and the azido-tagged analogues of myristic acid (**3**) and CoA ester (**4**), PfARF1 peptide substrate (**5**) and capture reagent (**6**) synthesised and/or used in this study. *Reagents and conditions*: (i) NaN_3, DMSO; (ii) (a) CDI, THF, (b) CoASH, THF–$NaHCO_3$ (0.5 M); (iii) piperidine, DMF; (iv) 3-(diphenylphosphino)-4-(methoxycarbonyl)benzoic acid, HATU, DIPEA; (v) 95% $TFA_{(aq)}$. See ESI for full experimental details.

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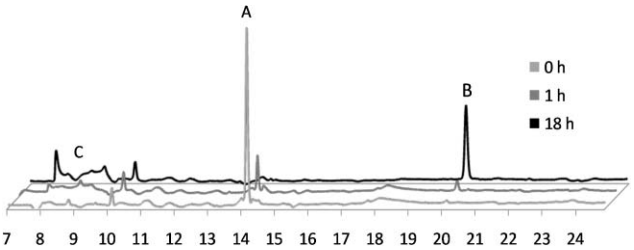

Fig. 1 HPLC traces showing transfer of azido-myristate **3** (*via* CoA ester **4**) to peptide **5** *in vitro* after reaction times of 0 h, 1 h and 18 h. (A) Peptide **5**; (B) azido-myristoylated **5**; (C) CoASH evolved during the reaction.

undecanoyl group was successfully transferred under similar conditions from **4** to full-length recombinant PfARF1[11,12] as shown by gel electrophoresis (Fig. 2). A capture reagent **6** bearing a biotin label, PEG linker and a phosphine capture group was conveniently synthesised using solid-phase chemistry on Biotin-PEG NovaTag™ resin (Scheme 1). Staudinger–Bertozzi ligation[3a] with **6** effectively transferred a biotin label to the tagged protein, as visualised with NeutrAvidin™–horseradish peroxidase (HRP) conjugate following gel electrophoresis and blotting to nitrocellulose membrane (Fig. 2B). Pull-down of biotinylated protein from the reaction mixture using Neutravidin™–agarose beads enabled the overall efficacy to be measured directly by virtue of a C-terminal His_6 affinity tag present in the PfARF1 construct; quantification by immunoblotting using anti-His–HRP conjugate demonstrated a transfer efficiency >99% (Fig. 2; lanes 3 *vs.* 5). There is no observable gel-shift of this protein upon myristoylation and capture, which is indicative of the minor influence of this N-terminal label on overall hydrophobicity.[5b]

By taking advantage of *E. coli* engineered to co-express CaNMT and a substrate protein,[13] this highly effective tagging technique may be performed in a single step *in vivo*, concurrent with protein over-expression. CaNMT and PfARF1 protein expression constructs bearing complementary resistance genes were co-transformed into *E. coli* BL21, and bacterial growth and subsequent expression of both proteins were induced under conditions of double antibiotic selection. Simultaneous feeding with **3** results in uptake, transformation into the active CoA analogue by endogenous *E. coli* acyl-CoA synthetase[8,9,13,14] and subsequent incorporation into PfARF1 by CaNMT. Bacteria were lysed, and the remarkable efficiency and specificity of this 'one-pot' *in vivo* process were demonstrated by labelling with reagent **6** and immunoblotting as described for *in vitro* experiments (Fig. 3).†§ Pull-down with NeutrAvidin™-agarose beads demonstrated a >99% efficiency of the overall process, relative to expressed PfARF1 (Fig. 3; lanes 1 *vs.* 2). The strict requirement for an N-terminal glycine,[10] and thus the presence of a single tag, was demonstrated by the absence of labelling in bacteria co-expressing CaNMT and PfARF1[G2A], a PfARF1 mutant bearing an N-terminal alanine (Fig. 3B, lanes 3 and 4).[11] It also appears that endogenous activity of *E. coli* methionine aminopeptidase (EcMetAP)[15] is sufficient for removal of the N-terminal initiator methionine in PfARF1 to reveal the requisite N-terminal glycine residue in quantitative yield. The utility of this *in vivo* protocol is further enhanced by the need for only the free acid: in contrast to acyl-CoA analogue **4**, ω-azido undecanoic acid **3** is readily synthesised in high yield in a single step from inexpensive 11-bromoundecanoic acid (Scheme 1), and may be used in feeding experiments without chromatographic purification.

Taken together, these results demonstrate that CaNMT is an effective tool for *in vitro* and *in vivo* transfer of an azide-modified acid to the N-terminus of a polypeptide derived from a species entirely unrelated to *C. albicans*. Previous studies have shown that the protein substrate specificity of NMT *in vitro* or in bacterial co-expression systems is dependent on the presence of a short N-terminal canonical myristoylation motif of general sequence GXXX[T|F|S],[16] whereas the *in vivo* specificity in eukaryotic cells is complicated by the presence of NMT isoforms and varying levels of expression and localisation of both enzyme and substrates.[10] In common with recombinant N-terminal labelling (*e.g.* His_6 tag), the addition of a myristoylation motif to proteins in which the N-terminal region is not involved in folding or other interactions does not usually impede its normal function,[17] and azido-myristoylation[18] with *N*-myristoyl transferase presents a potentially useful alternative to native chemical ligation[19] for N-terminal

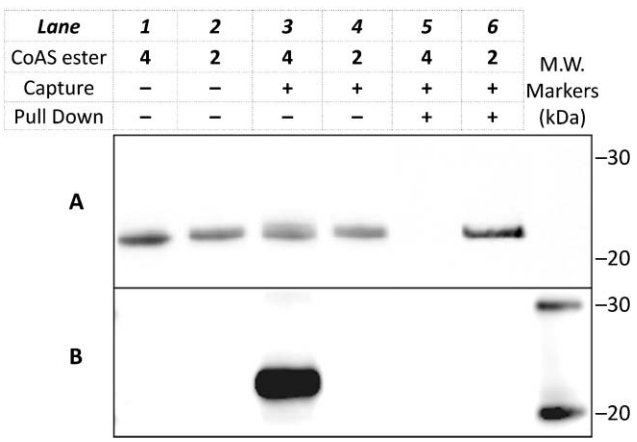

Fig. 2 *In vitro* transfer of **4** to PfARF1. **A**: Anti-His–HRP blot. **B**: NeutrAvidin™–HRP blot showing transfer of biotin after capture with reagent **6** (lane 3) and pull-down with NeutrAvidin™–agarose (lane 5). Control experiments using myristoyl-CoA **2** are shown for comparison.

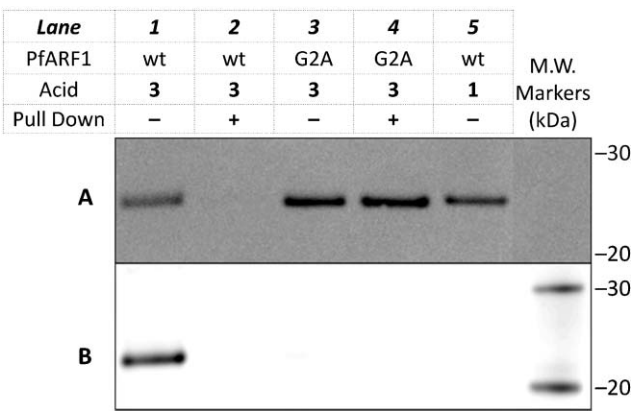

Fig. 3 *In vivo* transfer to PfARF1 and capture from cell lysates. **A**: Anti-His–HRP blot. **B**: NeutrAvidin™–HRP blot of pull-down showing transfer of biotin only to wild-type PfARF1 co-expressed with CaNMT in the presence of analogue **3**. wt = wild type PfARF1, G2A = PfARF1[G2A].

labelling of recombinant proteins under non-denaturing conditions.

The widespread adoption of a novel protein labelling technique is dependent on the simplicity and efficacy of the protocol.[5a] We show here that the requisite tagged substrates and labelling reagents are readily synthesised in a few high-yielding steps and that transfer can be performed concurrent with recombinant expression with very high efficiency, a combination that will allow rapid adoption for N-terminal labelling of proteins. Enzymatic tagging *via N*-myristoylation is also orthogonal to each of the alternative site-specific enzymatic tagging methods reported to date,[1,5] suggesting that the methods might be combined for sequential or simultaneous site-specific labelling with different probes. Furthermore, our work provides strong support for recent results that indicate azido-myristoylation may be used to tag proteins metabolically for proteomic applications.[20] Work is ongoing in our labs to exploit this approach for general protein labelling and targeted chemical proteomics in a variety of cell lines.

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§ Lysates were pre-depleted of all endogenous biotinylated proteins by pull-down using NeutrAvidin™–agarose before treatment with **6** (see ESI†).

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