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Selective Labeling of Polypeptides Using Protein Farnesyltransferase via Rapid Oxime Ligation

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Abstract

An aldehyde-containing alternative substrate for protein farnesyltransferase was prepared and shown to be enzymatically incorporated into a peptide and a protein. The protein was subsequently immobilized onto aminooxy-functionalized agarose beads or labeled with a fluorophore. This method for protein modification provides an alternative to the commonly employed Cu(I)-catalyzed click reaction

Chemical modification of proteins is important for many applications in biology and biotechnology. Selective functionalization of proteins is challenging because of the large number of reactive functional groups typically present in polypeptides. Our laboratory and others have recently exploited the high specificity of the enzyme, protein farnesyltransferase (PFTase), to site-specifically modify peptides and proteins.[1–4] In nature, PFTase, catalyzes the transfer of a farnesyl isoprenoid group from farnesyl pyrophosphate (FPP, **1**, Fig. 1) to a sulfur atom present in a cysteine residue. That residue must be located in a tetrapeptide sequence (denoted as a CAAX-box) positioned at the C-terminus of a protein or peptide to be a PFTase substrate. Interestingly, CAAX-box sequences such as CVIA can be appended to the C-terminus of many proteins rendering them efficient substrates for PFTase. Since PFTase can tolerate many simple modifications to the isoprenoid substrate,[5,6] it can be used to introduce a diverse range of functionality into proteins at their C-termini. Chemoselective reaction with the resulting protein can then be used for a wide range of applications. Although a number of reactions have been developed,[7–11] to date, the Cu(I) catalyzed click reaction has been the most widely used bioorthogonal process.[12] While useful, that reaction employs Cu(I) which is toxic to cells and can erode enzymatic activity.

Ligation reactions between aldehydes or ketones with hydrazine derivatives or alkoxyamines are attractive alternatives but these reactions suffer from slow kinetics. Recently, Dawson and coworkers reported the dramatic acceleration of oxime formation in presence of aniline[13]; several applications of this reaction have subsequently been described.[14]

Here, we describe the synthesis of an aldehyde-containing analogue of FPP denoted as FAPP (**2**) and demonstrate that it is efficiently incorporated by PFTase. Model reactions with peptide substrates were used to confirm the structure of the prenylated product and monitor the rate of oxime formation. To investigate the utility of **2** for protein modification, a GFP variant incorporating a CAAX-box was employed. PFTase was used to incorporate

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the modified isoprenoid into the protein. The resulting aldehyde-modified polypeptide was then either immobilized onto alkoxyamine-containing agarose beads or further functionalized with a second fluorophore via oxime formation.

Farnesyl aldehyde pyrophosphate (FAPP, **2**) was prepared in six steps starting from farnesol (Scheme S1). Tetrahydropyran (THP)-protected farnesol was initially regioselectively oxidized at C-12 to a terminal alcohol followed by further oxidation to the corresponding aldehyde. Removal of the THP group, conversion to an allylic bromide and displacement with $[(n\text{Bu})_4\text{N}]_3\text{HP}_2\text{O}_7$ followed by purification by ion-exchange chromatography and reversed-phase HPLC (RP-HPLC) yielded FAPP (**2**) whose structure was confirmed by ^{1}H-NMR, ^{31}P-NMR, and HR-ESI-MS.

Compound **2**, was first evaluated as a substrate for PFTase (Fig. 2A) by employing a continuous fluorescence-based enzyme assay. Varying concentrations of **2** were incubated with a constant concentration of the fluorescent peptide substrate, *N*-dansyl-GCVIA (**3**) and PFTase (see Fig. S1 and S2). From those experiments K_M and k_{cat} were found to be 2.2 μM and 0.065 μM min^{-1}, respectively. In comparison, similar experiments employing FPP (**1**) yielded values for K_M and k_{cat} of 0.42 μM and 0.26 μM min^{-1}, respectively. These values indicate a 5-fold increase in K_M, a 4-fold decrease in k_{cat} and an overall 20-fold drop in catalytic efficiency. While, significant, this attenuation can be limited to a 4-fold reduction in rate by performing preparative reactions at saturating substrate concentrations. To verify the results obtained from the kinetic analysis and confirm the structure of the prenylated peptide, the formation of peptide **4** was monitored by RP-HPLC analysis. The peptide substrate **3** (2.4 μM) was incubated with **2** (25 μM) and PFTase at 30 °C. Complete conversion was observed after 40 min (Fig. 1B, chromatogram b). The reaction mixture was concentrated by solid phase extraction with a Sep-Pak cartridge and purified by RP-HPLC. LC-MS analysis of the purified material gave an $[M+H]^+$ of 913.5 consistent with the proposed structure of **4** (Fig. S3). Next the ligation reaction between aminooxy alexafluor **5** and aldehyde-functionalized peptide **4** was evaluated. Compound **4** (7.5 μM) was incubated with **5** (200 μM) in PB (0.1 M, pH 7.0) at rt and the reaction was initiated by addition of aniline (100 mM). To monitor the reaction, aliquots were withdrawn at 40 sec intervals and flash frozen. Subsequent RP-HPLC analysis showed that the ligation reaction was complete within the first 40 sec which is impressive for a bimolecular reaction at physiological pH in which the reagents are present at μM concentrations (Fig. 2C). LC-MS analysis of the reaction mixture gave an ion of 698.2 as the predominant species, which is consistent with $[M+2H]^{2+}$ for **6** (Fig. S3). Only a trace amount of unreacted starting aldehyde **4** was observed suggesting an equilibrium constant of 10^8 M for the ligation reaction. In contrast to the promising results noted above for oxime formation, attempts to achieve ligation via hydrazone formation between Texas-red hydrazine and **4** were not successful. RP-HPLC analysis of such a reaction mixture showed only a trace of possible product being formed. These results suggest that the equilibrium constant for hydrazone formation may not be sufficiently large to convert a significant percentage of aldehyde **4** when the reactants are present in μM concentrations.

With the ability of **2** to be incorporated by PFTase and subsequently derivatized via oxime formation established, we next evaluated the utility of this alternative substrate for selective protein modification. Accordingly, **2** was incubated with eGFP-CVIA (**7**), in the presence of PFTase for 2 h at 30 °C (Scheme 1). The reaction time was based on our earlier observation that the peptide substrate **3** could be prenylated in less than 40 min. Concentration by ultracentrifugation followed by size exclusion chromatography to remove unreacted **2** yielded aldehyde-functionalized eGFP-CVIA (**8**). To determine the efficiency of prenylation, thiol titration was performed on both unmodified protein **7** and the prenylated product **8.** DTNB titration of **7** revealed the presence of 2.8 thiols/protein in good agreement

with the expected value of 3. A similar titration of **8** indicated the presence of 1.7 thiols/protein. These results indicate the loss of 1.1 thiols/protein after reaction and are consistent with complete modification of the cysteine residue present within the CVIA sequence after prenylation with **2.** Next, we examined two possible applications for the aldehyde-modified protein produced above. First, its utility for protein immobilization was examined (Scheme 1). Aminooxy-functionalized agarose beads (**9**) were prepared by coupling a bifunctional aminooxy-PEG azide with alkyne-agarose beads via click chemistry. Beads (**9**) were incubated with aldehyde-functionalized eGFP-CVIA (**7**) at rt in the presence of 100 mM aniline. The reaction was vortexed and the fluorescence of supernatant was measured as a function of time. Results show that equilibrium is reached in less than 15 min. In that time, the beads became highly fluorescent (Fig. 3A); less fluorescent beads were observed in the absence of aniline catalyst (Fig. 3B) and no fluorescent beads were seen using GFP lacking the aldehyde moiety (Fig. 3C). Based on the amount of **8** remaining in the supernatant, the efficiency of covalent immobilization (Fig. S5) was estimated to be ~30%.

Finally, oxime ligation was used to site-specifically label eGFP-CVIA with a second fluorophore. Aminooxy alexafluor-488 (**5**) (125 μM) was treated with **8** (25 μM) in presence of 100 mM aniline for 30 min at rt. Labeled protein (**11**) was purified from excess **5** by extensive dialysis at 4 °C. The UV spectrum obtained for coupled product (**11**) showed a significant increase in absorbance near 500 nm when compared to the unmodified protein **8** (Fig. 4A). The difference spectrum for this pair matches closely the spectrum of alexafluor-488 (**5**) thereby confirming successful ligation (Fig. 4B). Quantitative comparison of the UV difference spectrum with the spectrum of alexafluor-488 suggests an efficiency of 60% for oxime ligation in this case.

Conclusions

In this report, we demonstrate that PFTase can be used to introduce an aldehyde group near the C-terminus of a polypeptide and that the resulting protein can be immobilized or further functionalized via aniline-promoted oxime formation. Given that CAAX-box sequences can be appended to the C-termini of almost any protein, the method reported here should be useful for a wide range of applications in protein chemistry. This copper free labeling approach may be particularly useful for live cell studies or for applications where enzymatic activity must be preserved. It should also be noted that this labeling chemistry and the Cu(I)-catalyzed click reaction are orthogonal. This opens up the possibility of performing multiple modifications on proteins using different, bioorthogonal chemistries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Notes and references

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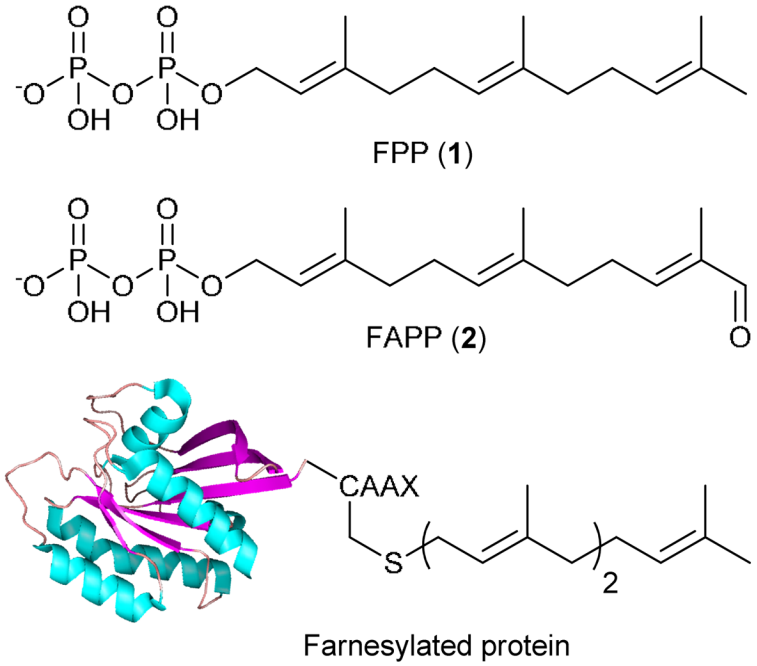


Fig. 1.
Farnesyl pyrophosphate (**1**), farnesyl aldehyde pyrophosphate (FAPP, **2**) and a farnesylated protein showing a CAAX-box (CVIA) positioned at the C-terminus of a protein.

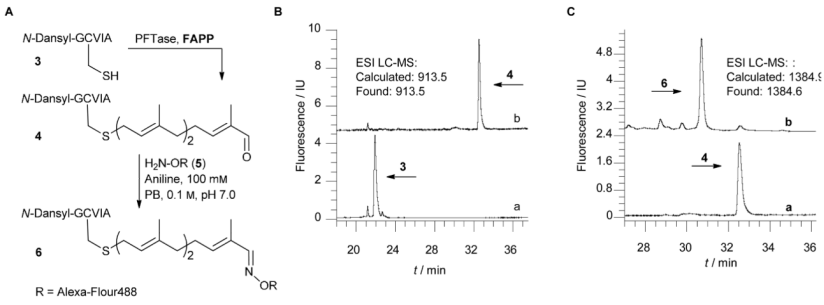


Fig. 2.

Prenylation of a CAAX-box containing peptide with FAPP (**2**) and subsequent aniline-catalyzed oxime ligation with aminooxy alexaflour-488. Panel A) Structures of reactants and products. Panel B) RP-HPLC analysis of the PFTase catalyzed prenylation of **3** with **2** to yield **4.** Chromatogram a: reaction mixture prior to the addition of enzyme; chromatogram b: reaction mixture after 40 min at 30 °C showing conversion to the prenylated peptide **4.** Panel C) RP-HPLC analysis of the oxime ligation reaction. Reaction was initiated by the addition of aniline. Chromatogram a: reaction mixture prior to the addition of alexaflour-488 (**5**); chromatogram b: reaction mixture 40 sec after the addition of alexaflour-488 (**5**) and aniline to the reaction mixture at rt showing conversion to the oxime **6.** LC/MS analysis of the prenylated peptide and oxime ligated product confirmed quantitative formation of **4** and **6.**

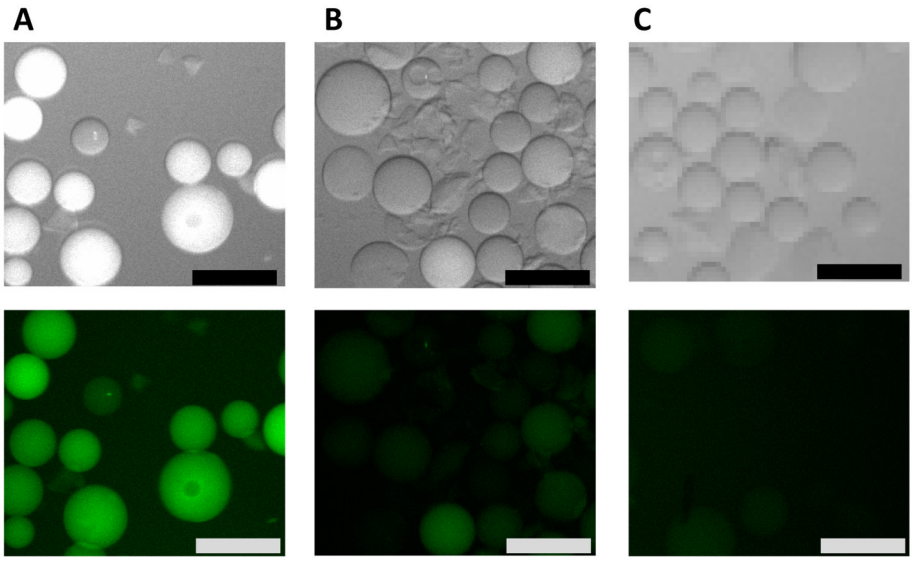


Fig. 3.

Immobilization of aldehyde-labeled eGFP-CVIA (**8**) onto aminooxy-functionalized agarose beads (**9**) to yield (**10**): Panel A) Reaction mixture in the presence of aniline (100 mM). Panel B) Reaction mixture in the absence of aniline. Panel C) Control immobilization reaction containing unmodified eGFP-CVIA (7) in the presence of aniline (100 mM). All reactions were carried out in the presence of protein (20 μM), and PB (100 mM, pH 7.0). Bright-field images are above and fluorescent microscope images below. Scale bars in the lower right-hand corners represent 200 μm.

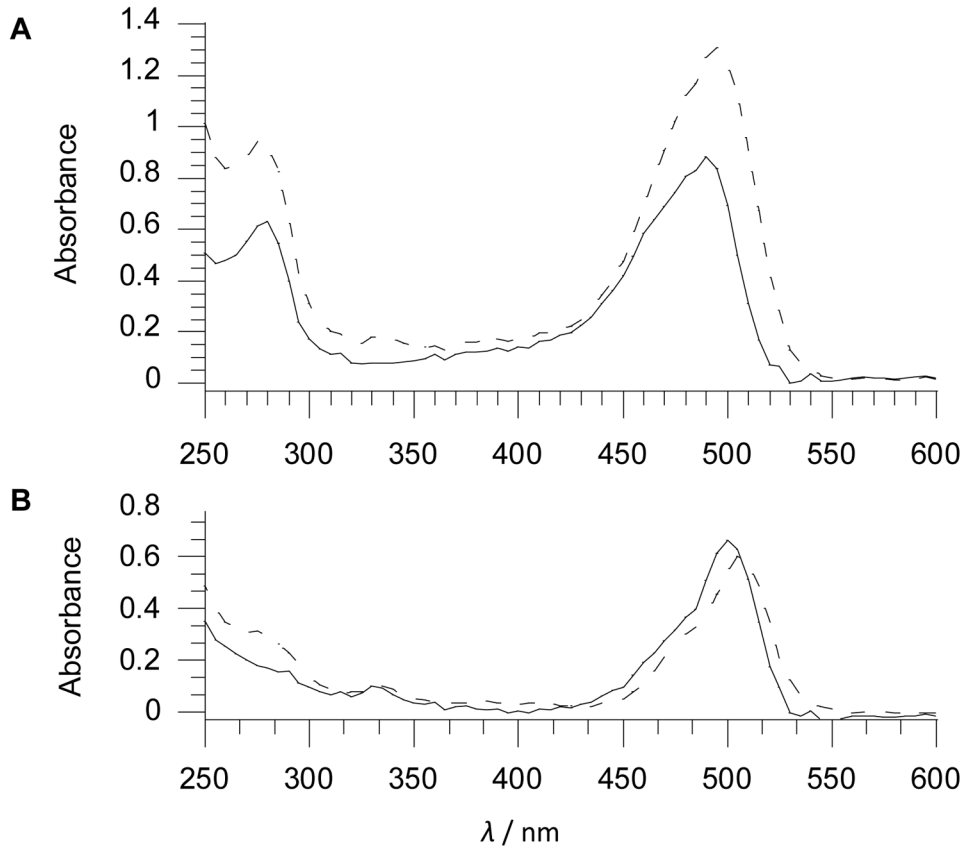
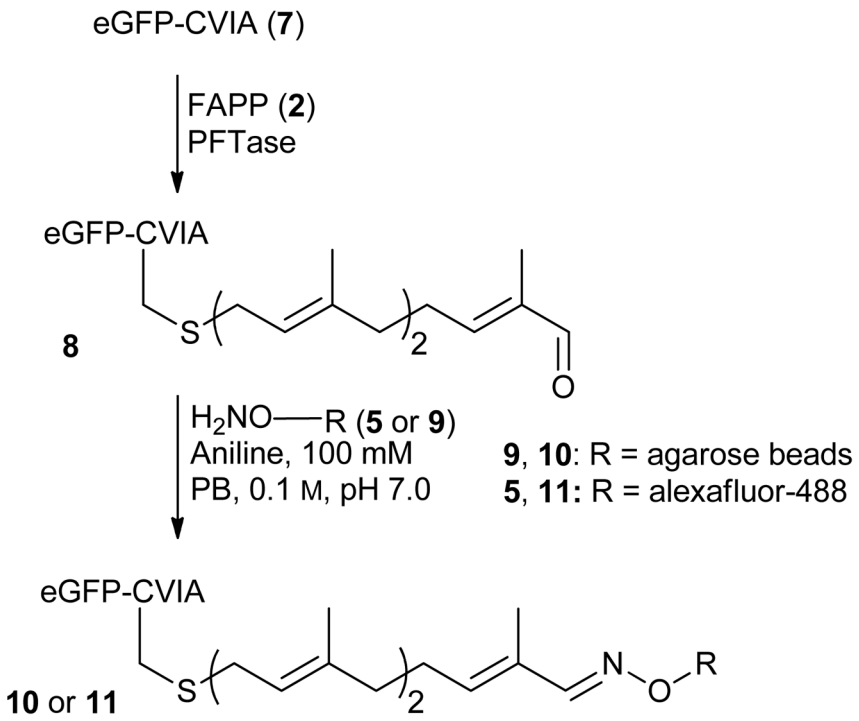


Fig. 4.

Selective labeling of aldehyde-labeled eGFP–CVIA (**8**) with alexaflour-488 (**5**) to yield **11.** Compound **8** (25 μM) was incubated with **5** (125 μM) and aniline (100 mM). After incubation at 25 °C for 30 min, samples were dialyzed against PB to remove unreacted **5.** Samples were then adjusted to contain equal protein concentration (17 μM) and the UV spectra were obtained. Panel A) Dashed line: Spectrum of **11** obtained from the treatment of **8** with **5;** Solid line: Spectrum of **8.** Panel B) Dashed line: Difference spectrum (Spectrum of **11** – Spectrum of **8**); Solid line: Spectrum of **5** (10 μM).



Scheme 1.
Prenylation of eGFP-CVIA with FAPP and subsequent immobilization on aminooxy functionalized agarose beads or labeling with aminooxy alexaflour-488 by oxime ligation.