Fluorescent Multiplex Analysis of Carrier Protein Post-Translational Modification

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Multiplex analysis has proven to be a powerful tool for dissecting genetic identities within complex biological mixtures, as illustrated by applications in in situ hybridization,[1] polymorphic PCR,[2] and gene-expression profiling.[3] Given the progression of proteomic analyses,[4,5] comparable systems will be a valuable asset for protein-based applications.[6] An ideal system for the application of multiplex analysis to proteins is the promiscuous post-translational modification of carrier-protein domains from thioester-mediated biosyntheses. This transformation, catalyzed by 4′-phosphopantetheinyltransferases (PPTases), can be modified with a variety of reporter-labeled prosthetic groups to yield labeled proteins,[7] wherein each chemical species transfers with a unique kinetic profile. Here we introduce a three-color fluorescent multiplex analysis of carrier-protein domains that may be applied to the discovery of primary and secondary biosynthetic pathways and as functional markers for fusion-protein systems involving multiple species.

Carrier-protein domains comprise a small yet diverse group of proteins essential to primary and secondary biosynthetic pathways, including fatty acid, polyketide, and non-ribosomal peptide synthesis.[8] The carrier proteins involved in these pathways function as scaffolds to mediate modular synthesis through a domain bearing a 4′-phosphopantetheine arm.[9] This prosthetic group is incorporated through transfer of 4′-phosphopantetheine from coenzyme A (CoA) to a conserved serine residue by a PPTase (Figure 1). The three-component reaction between CoA, carrier protein, and PPTase has been shown to accept a variety of CoA–thioester substrates.[10] Recently, we further investigated this tolerance by covalently modifying carrier-protein domains with reporter molecules, as illustrated by the conversion of apo-carrier protein to crypto-carrier protein (Figure 1).[7] This modification has been subsequently utilized in fusion-protein labeling with in vivo applications.[11]

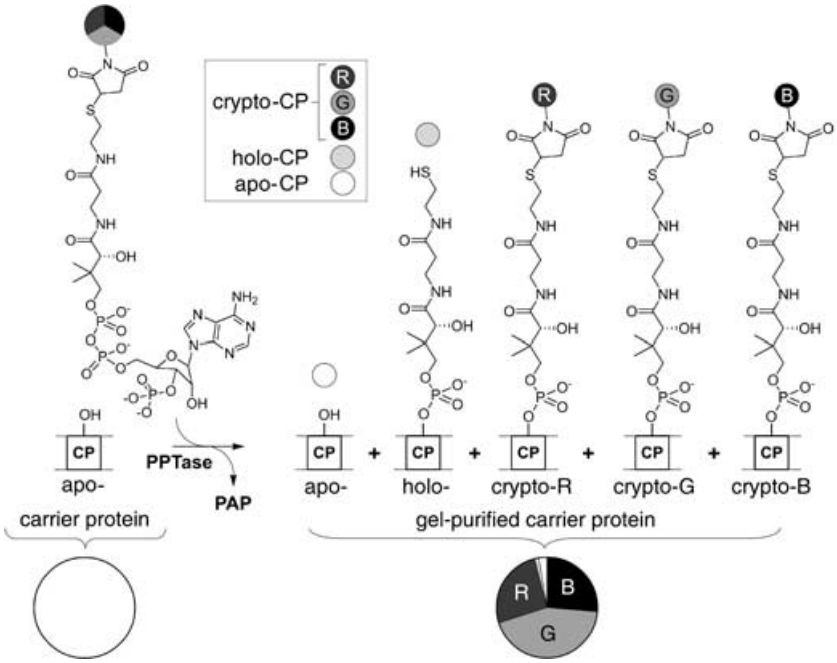

Figure 1. Multiplexed post-translational modification of carrier proteins. In natural systems, a PPTase transfers a 4′-phosphopantetheine residue from CoA to a conserved serine residue on each apo-carrier protein domain to create the holo-carrier protein and 3′-phosphoadenosine-5′-phosphate (PAP). When exposed to reporter-modified CoA, the PPTase diverts the natural pathway to yield the reporter-modified crypto-carrier protein. The crypto- nomenclature indicates that the carrier protein is now blocked from further reaction at the thiol terminus and can be coded with a fluorescent tag.

Early in our investigations, we noticed that the kinetics of carrier-protein modification was dependent on the CoA derivative used.[7] Based on this evidence, a multiplex assay was constructed by using orthogonal sets of fluorescent CoA analogues **1**–**5** (Figure 2).[12] As illustrated in Figure 1, the incubation of a carrier protein with sets of dyes (red, green, and blue) and PPTase creates three different fluorescently modified proteins. The relative population of each state was determined spectrophotometrically and conveniently displayed by using a pie graph.

The application of this method to vibriobactin synthase carrier protein from *Vibrio cholerae*, VibB, is depicted in Figure 3a. Purified VibB was labeled individually with surfactin PPTase (Sfp)[13] and CoA analogues **1**–**3** to provide a red, green, and blue carrier protein. Treatment of VibB with Sfp and an equimolar mixture of **1**–**3** (dye set **A**) produced VibB with 29%, 41%, and 30% of the tags from **1**, **2**, and **3**, respectively. Comparable analyses in other carrier-protein domains (fatty acid synthase acyl carrier protein from *E. coli*, ACP; oxytetracycline

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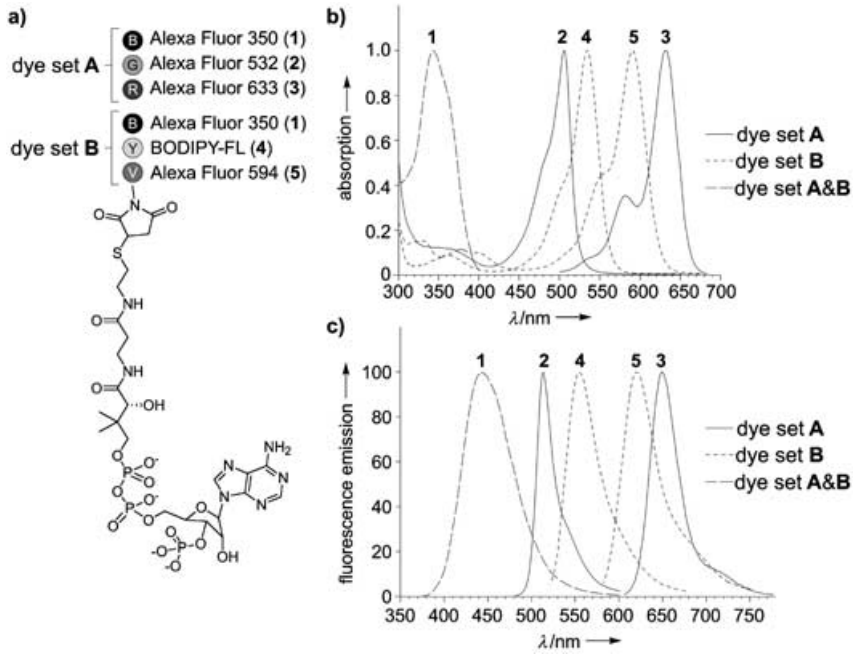

Figure 2. Sets of fluorescent analogues. a) Two sets of analogues, dye sets **A** and **B**, were prepared by selecting combinations of CoA analogues **1**–**5** such that each set provided orthogonal absorption and fluorescence spectra. a) Absorption and b) fluorescence maxima were standardized to 1.0 and 100, respectively, by adjustment of concentration. Extinction coefficients and fluorescent data for each dye are provided in the Supporting Information.

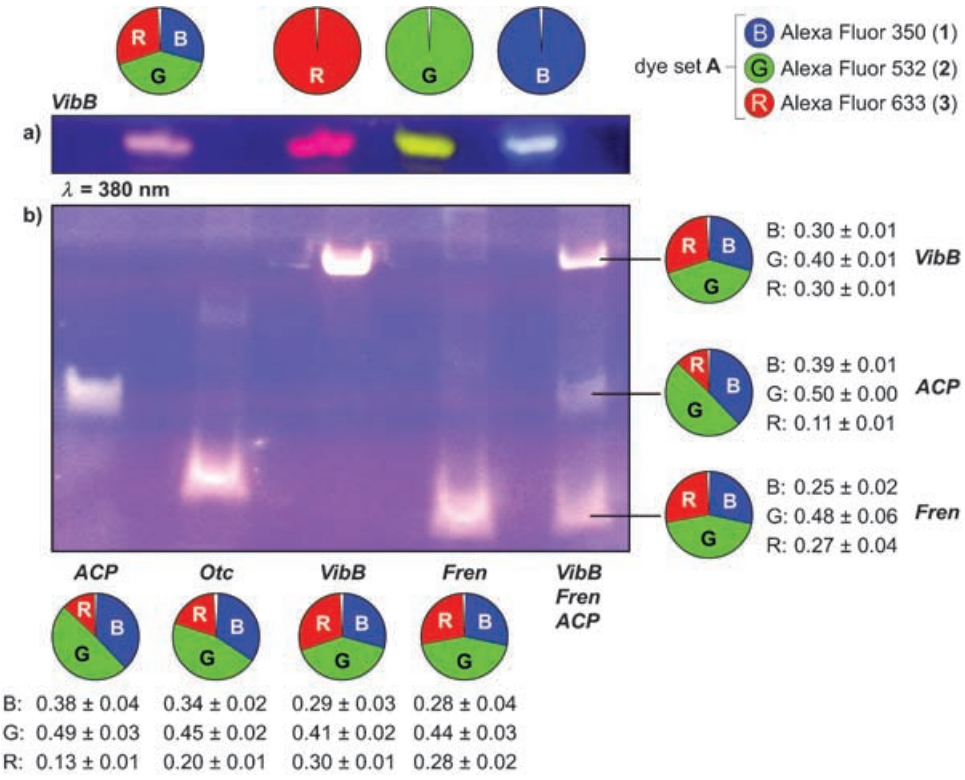

Figure 3. Three-color carrier protein labeling. a) Carrier protein VibB underwent tagging when treated with dye set **A** containing CoA analogues **1**–**3**. SDS-PAGE gel depicting the emission from the dye-labeled crypto-carrier protein state upon excitation at $\lambda = 380$ nm. b) SDS-PAGE analysis of ACP, Otc, VibB, Fren, and a mixture of multiple carrier proteins after treatment with Sfp and dye set **A**. The relative dye labeling of carrier proteins is represented by pie charts.

synthase carrier protein from *Streptomyces rimosus*, Otc; and frenolicin synthase carrier protein from *Streptomyces roseofulvus*, Fren) verified that the reaction between CP, PPTase, and CoA could differentiate between the carrier proteins assayed, as illustrated by the relative populations of tags **1**–**3** (Figure 3b).

The utility of this method is highlighted by its ability to selectively identify carrier proteins within mixtures (lane 5, Figure 3). Here an equimolar mixture of ACP, VibB, and Fren was treated with dye set **A** and Sfp. The three carrier proteins were separated by molecular weight by SDS-PAGE, and the uptake of reporter-labeled CoA analogues **1**–**3** was determined spectrophotometrically. Interestingly, the uptake of **1**–**3** within each carrier protein remained within $\pm 1.6\%$ of that found when labeling each carrier protein individually.

In a final set of experiments, we asked how different PPTases or CoA derivatives would change the profile of dye labeling. Having shown this new assay to be effective in identifying carrier proteins either alone or in mixtures, we wanted to demonstrate that the observations were not restricted to the use of dye set **A**. We altered the reaction conditions by preparing a second dye set (**B**) by mixing equimolar aliquots of **1**, **4**, and **5** (Figure 4a). At the same time, we introduced two additional PPTases to the assay. Acyl carrier-protein synthase (ACPS) from *B. subtilis* and pseudomonas carrier-protein synthase (PCPS) from *P. aeruginosa* were cloned and expressed,[13,14] and the influence of both dye sets and PPTase was screened simultaneously against four carrier-protein domains (Figure 4b–c). For ACP, the multiplex reaction with ACPS induces

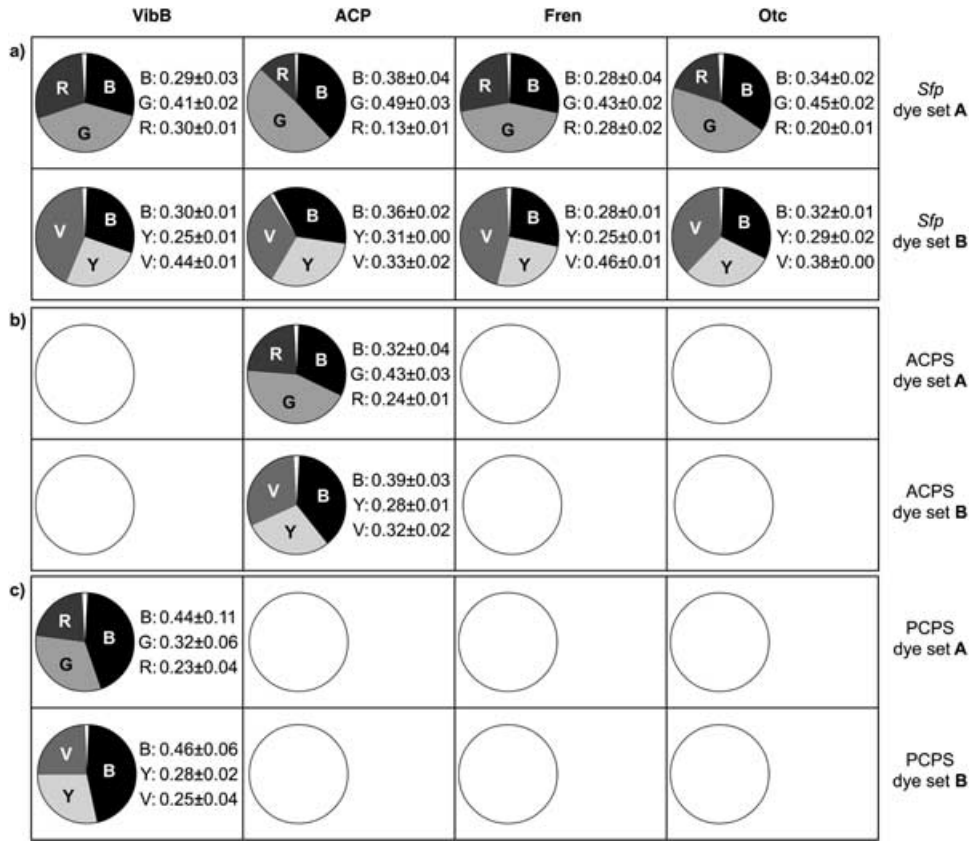

Figure 4. Labeling of carrier proteins with three PPTases (Sfp, ACPS, and PCPS) and two dye sets (**A** and **B**). Relative dye labeling with a) Sfp (*B. subtilis*), b) ACPS (*B. subtilis*), and c) PCPS (*P. aeruginosa*). Relative dye labeling of carrier proteins is represented by pie charts. ○ indicates no reaction, as given by a lack of crypto-state formation. Assignments of dye sets are given in Figure 2a.

a modest shift relative to Sfp (Figure 4a vs. b). For VibB, the effect of changing the PPTase is more pronounced, as shown by comparison of Figure 4a and c. The characteristic labeling pattern is further varied by the choice of dye set, as demonstrated by comparison of pie graphs within Figure 4b and c.

This assay provides initial insight into molecular-level interactions between proteins from different natural-product systems. Carrier proteins found in metabolic pathways can be functionally visualized and classified, even in the absence of genetic information. We have expanded on earlier work that identified reactivity in recombinant proteins from different systems[8] by investigating this reactivity on the molecular level. Recent publications have also demonstrated the use of carrier proteins as fusion partners for functional labeling in vitro and in cellular systems.[10] By combining the techniques presented here, such fusion systems could be adapted to track multiple proteins simultaneously. This multiple analysis of the three-component reaction between carrier protein, modifying enzyme (PPTase), and label (fluorescent CoA analogue) provides a robust system for the selective coding of these proteins. In addition, the extension of this assay to secondary metabolic systems, fusion systems, and truncated or modified carrier-protein domains might provide a robust tool to selectively decipher complex biological networks.

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