Special Issue Article

Journal of PeptideScience

Received: 14 October 2013 Revised: 20 November 2013 Accepted: 21 November 2013 Published online in Wiley Online Library: 9 January 2014

(wileyonlinelibrary.com) DOI 10.1002/psc.2600

Chemo-enzymatic three-fragment assembly of semisynthetic proteins‡

Lena Schmohl and Dirk Schwarzer*

Here, we report the development of a method for three-fragment assemblies of semisynthetic proteins by combining sortase-mediated ligation with site-specific bioconjugation catalyzed by the 4′-phosphopantetheine transferase Sfp. This method enables the introduction of synthetic peptides into central regions of proteins without the need to purify intermediates. The assembled proteins are linked at the N-terminal junction with a 4′-phosphopantetheine moiety and with a peptide bond at the C-terminal ligation site. We have demonstrated the applicability of this method by assembling a semisynthetic model protein derived from fluorescence resonance energy transfer-based reporters from three fragments in a one-pot reaction.

Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords: protein semisynthesis; sortase-mediated ligation; phosphopantetheine transferase; FRET reporter

Introduction

Modern techniques of protein semisynthesis represent powerful tools in protein chemistry because they allow the site-specific introduction of posttranslational modifications (PTMs), fluorophores, or other biophysical probes into proteins [1,2]. Native chemical ligation and expressed protein ligation enable the ligation of synthetic modified peptides to the N-termini and C-termini of proteins and have been instrumental for elucidating the structure and function relationship of many proteins [3,4]. In recent years, enzymatic ligation approaches based on the bacterial transpeptidase sortase A have gained considerable attention, and this enzyme has been used for a wide range of applications in protein chemistry [5–8]. In the natural context, sortases are responsible for anchoring surface proteins to the peptidoglycan layer of gram-positive bacteria including *Staphylococcus aureus* [9]. During catalysis, sortase A recognizes a highly conserved LPxTG sorting motif in one of the ligation partners, cleaves the sequence at the threonine residue under concomitant formation of an enzyme-bound thioester. In what follows, the thioester is ligated to the N-terminus of the second ligation partner, which needs to possess at least one N-terminal glycine residue (Figure 1A) [10].

Most protein semisynthesis approaches reported today are limited to the ligation of synthetic peptides to either the N-terminus or the C-terminus of a recombinant protein. The introduction of synthetic peptides into central regions of a protein remains highly challenging because such approaches require two consecutive ligation steps including additional protection strategies or purification of the intermediates. Orthogonal ligation strategies that allow the simultaneous ligation of three fragments in a one-pot reaction would greatly facilitate such approaches, and first developments into this direction have been reported for native chemical ligation [11]. In the case of enzymatic ligations, the sortases from *S. aureus* and *Streptococcus pyogenes*, which recognize different sorting motifs, allow consecutive ligations [12,13]. In order to develop an enzymatic strategy for the three-fragment assembly of a semisynthetic protein, we reasoned that a combination of two enzymes that differ in substrate selectivity and the mode of catalysis would be a suitable basis for orthogonal semisynthesis of proteins. Enzymes that form native peptide bonds at the backbone are rare in nature. However, in several cases, semisynthetic proteins might be functional when the two fragments are selectively linked via amino acid (AA) side chains. In such cases, enzymes that have been established for the bioconjugation of AA side chains can be combined with sortase A in an orthogonal protein assembly strategy. A class of enzymes that has been successfully established for the specific labeling of proteins with fluorophores and other biophysical probes are 4′-phosphopantetheine transferases (PPTases). These enzymes modify the acyl carrier proteins of fatty acid and polyketide synthases and the peptidyl carrier proteins of nonribosomal peptide synthetases with the 4′-phosphopantetheine (Ppant) cofactor [14]. During catalysis, PPTases transfer the Ppant cofactor from coenzyme A (CoA) to a conserved serine residue of the respective carrier proteins. Importantly, PPTases are promiscuous with regard to modifications attached to the Ppant's thiol group, and this feature has been exploited for several protein modification approaches. In these cases, the attached probes are readily transferred onto carrier proteins that can be fused to any protein of interest [15–17]. The development of short tags like the S6-tag, which is only 12 AAs long and serves as an efficient substrate for the PPTase Sfp, has further extended the application range of this protein labeling approach (Figure 1B) [18–20]. Based on the differences in catalysis and selectivity, we reasoned that sortase-mediated ligation and PPTase-catalyzed protein labeling can be combined for orthogonal three-fragment assemblies.

* *Correspondence to: Dirk Schwarzer, Interfaculty Institute of Biochemistry, University of Tuebingen, Hoppe-Seyler-Str. 4, D-72076 Tuebingen, Germany. E-mail: dirk.schwarzer@uni-tuebingen.de*

‡ *This article is published in Journal of Peptide Science as part of the Special Issue devoted to contributions presented at the Chemical Protein Synthesis Meeting, April 3-6, 2013, Vienna, edited by Christian Becker (University of Vienna, Austria).*

Interfaculty Institute of Biochemistry, University of Tuebingen, Hoppe-Seyler-Str. 4, D-72076 Tuebingen, Germany

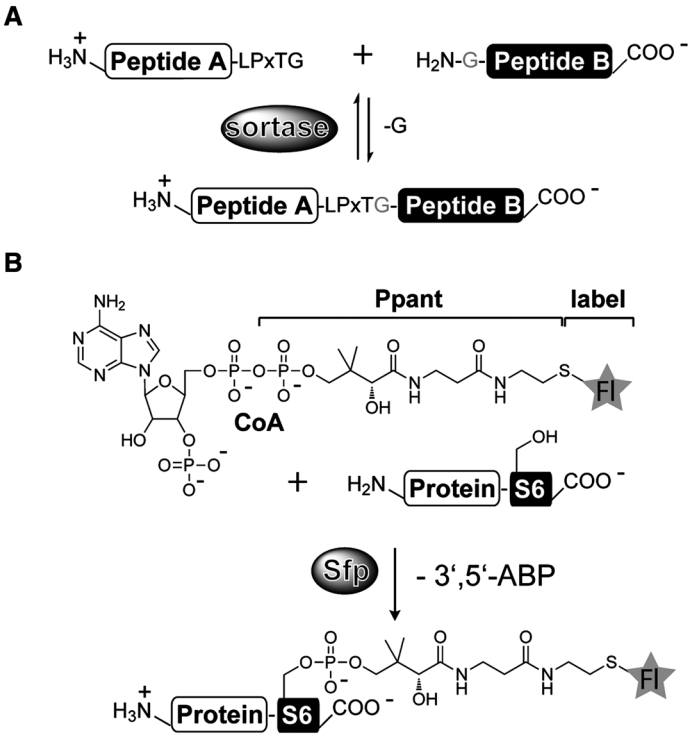

Figure 1. Enzymatic protein modification. (A) General scheme of sortase-mediated ligation reactions and (B) site-specific protein labeling catalyzed by the PPTase Sfp. A CoA derivative selectively modified with a fluorophore (Fl) is transferred onto a serine residue of the S6 motif. At the same time, 3',5' adenosine bisphosphate (3',5' ABP) is released.

Among the possible protein constructs that could benefit from such approaches are fluorescence resonance energy transfer (FRET)-based reporters for PTMs [21]. In general, FRET reporters are very powerful tools for biology, and several chemical biology investigations have focused on the development of strategies for the site-specific labeling of proteins with fluorophores [12,15,17,22]. These reporters typically consist of a PTM site (e.g. recognition site for a protein kinase) and a dedicated PTM-binding domain sandwiched between two fluorescent proteins (FPs) that serve as the donor and acceptor pairs for FRET [23]. Modification of the substrate sequence induces an intramolecular rearrangement of the reporter due to the interaction between the PTM and the PTM-binding domain that ultimately results in a change of the FRET efficiency. The development of such reporters can profit from three-fragment assemblies for two reasons: (i) Because changes in FRET efficiencies can hardly be predicted, several constructs need to be tested for optimal signal to noise ratios. In the case of semisynthetic reporters, various FPs and peptides can be assembled in parallel facilitating simple access to combinatorial libraries of reporter constructs. (ii) Reporters generated from three-fragment assemblies possess a synthetic peptide as central domain, which includes the PTM. In contrast to recombinant constructs, assembled reporters can be generated in premodified form, rendering them suitable substrates for enzymes that remove the PTMs.

Here, we report the development of a chemo-enzymatic strategy for the three-fragment assembly of semisynthetic proteins in a one-pot reaction. Our strategy is based on the orthogonal reactions of sortase A and the PPTase Sfp. We demonstrated the applicability of this method for the assembly of a semisynthetic model protein derived from a FRET reporter for protein kinases.

Materials and Methods

General Methods and Reagents

Amino acid derivatives were purchased from GLS (Shanghai, China), coupling reagents were purchased from Merck Novabiochem (Darmstadt, Germany), and Tentagel RAM resin was purchased from Rapp Polymere (Tübingen, Germany). All other chemicals and oligonucleotides were purchased from Sigma-Aldrich (Steinheim, Germany). Analytical RP-HPLC was performed on a Shimadzu (Duisburg, Germany) LC-10AT HPLC system with a Nucleosil C18 EC column (5 μm, 4.6 × 250 mm, Machery-Nagel (Düren, Germany)), with 0.1% TFA in water (A) and 80% ACN, 0.1% TFA in water (B), as eluents. The analytical gradient was 5–95% B over 45 min with a flow rate of 1.5 ml/min. Preparative and semipreparative purifications were conducted on a Varian ProStar 210 HPLC system equipped with a Dynamax C18 column (10 μm, 21.4 × 250 mm, Varian (Darmstadt, Germany)) and a flow rate of 13 ml/min or Reprosil 100 C18 (5 μm, 10 × 250 mm, Dr. Maisch GmbH (Ammerbuch-Entringen, Germany)) and a flow rate of 4 ml/min. ESI-MS analysis was conducted on a Mariner ESI-TOF (Applied Biosystems (Foster City, CA, USA)).

Solid-phase Peptide Synthesis

All peptides were synthesized by Fmoc-based solid-phase peptide chemistry using a ResPep synthesizer (Intavis AG Bioanalytical Instruments (Köln, Germany)). The synthesis was performed on a TentaGel S RAM resin (0.24 mmol/g). AA side chains were protected as follows: Arg(Pbf), His(Trt), Lys(Boc), Lys(Mtt), Ser(tBu), Ser(PO(OBzl)OH), and Thr(tBu). Coupling reactions were performed with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate as the activation agent and NMM in DMF/

Journal of PeptideScience

NMP as base. Each successive AA was doubly coupled in fivefold molar excess. Removal of the Fmoc group was carried out with 20% piperidine in DMF.

The first 19 coupling steps were performed by automated SPPS. The Mtt group of the N-terminal Fmoc-Lys(Mtt) was removed by treating the resin with 200 ml of 1% TFA in DCM in flow wash. Afterward, 5,6-carboxyfluorescein was coupled followed by the introduction of the last two residues by hand.

Bromoacetylation and Synthesis of Peptide-coenzyme A Conjugates

The resin-bound peptide was washed with 400 mM *N*-methylmorpholine in DMF. Afterward, 4 eq bromoacetic acid *N*-hydroxysuccinimide ester in DCM was added, and the resin was stirred for 1 h. No base was added to the reaction mixture. This step was repeated once. The peptides were cleaved from the resin with TFA/phenol/triisopropylsilane/H_2O (140 : 5 : 4 : 5) for 3 h and purified by preparative HPLC.

Bromoacetylated peptides (7 mg) were solved in 1 ml of 1 M triethylammonium bicarbonate buffer at pH 8.5, and 5 eq CoA trilithium salt was added [24]. The reaction mixture was stirred under argon at room temperature for 24 h. Afterward, the mixture was acidified with TFA, and the peptide-CoA conjugates were purified by semipreparative HPLC and analyzed by ESI-TOF-MS (Applied Biosystem (Foster City, CA, USA)).

Peptide **2a**:, CoA-Ahx-Ahx-K(Fl)-Ahx-ARKSAPTG-Ahx-LPKTGGRR-NH2; Mass (calc):, 3398.57 mass (obs): 3398.91;
Peptide **2b**:, CoA-Ahx-Ahx-K(Fl)-Ahx-ARKS(p)APTG-Ahx-LPKTGGRR-NH2; Mass (calc):, 3478.55 mass (obs): 3479.64

Construction of DNA Vectors

GST-14-3-3ζ-S6

The gene fragment encoding 14-3-3ζ (mouse) was amplified by PCR with the following oligonucleotides: 5′-TTTACTAGTATGGATAA-AAATGAGCTGGTG-3′ and 5′-AAACCTAGGTCAACCGGATCCA TTTT-CCCCTCCTTCTCCTG-3′. After restriction digestion with SpeI and AvrII, the DNA fragment was ligated with a pET42b vector (Novagen (Darmstadt, Germany)) that was digested in the same way yielding the vector pET42b-14-3-3ζ. The DNA encoding the S6 sequence (GDSLSWLLRLLN) and an additional linker (GSGGSGAKLSQL) was inserted into the pET42b-14-3-3ζ. This linker maintained a similar distance between the 14-3-3 domain and the phosphorylation site as in a previously reported kinase reporter [23]. The AA sequence of the linker results from the cloning strategy and was inserted into the plasmid as follows: The vector was subjected to restriction digestion with BamHI and AvrII. The following prephosphorylated oligonucleotides encoding the S6 motif were mixed, heated to 95 °C, and gradually cooled to room temperature (-5 K every 3 min): 5′-GATCCGGTTCTAAGCTTGGAGACTCCCT-3′, 5′-pGTCTTGGCTGCTGAGGCT-GCTGAACTGAC-3′, 5′-CAAGACAGGGAGTCTCCAA GCTTAGAACCG-3′, and 5′-CTAGGT-CAGTTCAGCAGCCTCAGCAGC-3′. The annealed oligonucleotides were subsequently cloned into the digested pET42b-14-3-3ζ vector to yield pET42b-14-3-3ζ-short-S6. Afterward, pET42b-14-3-3ζ-short-S6 was digested with HindIII and BamHI, and oligonucleotides 5′-GATCTGGTGGATCCGGTGCCAAGCTTTCTC-3′ and 5′-AGCTGAGAA-AGCTTGGCACCGGATCCACCA-3′ were inserted as described earlier in order to provide an additional linker sequence. This procedure yielded the expression plasmid pET42b-14-3-3ζ-S6.

G_5-mCherry-H_6

The DNA fragment encoding mCherry was amplified by PCR with the following oligonucleotides: 5′-TTTGGATCCGTGAGCAAGGG-CGAGGAG-3′ and 5′-TTTCTCGAGCTTGTACAGCTCGTC-3′. After restriction digest with BamHI and XhoI, the PCR product was cloned into pET21-G_5-SrtA that was digested in the same way to yield the expression plasmid pET21-G_5-mCherry [7].

Expression and Purification of Proteins

GST-14-3-3ζ-S6

Escherichia coli DE21 was transformed with pET42b-14-3-3ζ-S6 and cultured at 37 °C in LB (Luria-Bertani broth) with 30 μg/ml kanamycin. Expression was induced with 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6. After 20 h incubation at 25 °C, the cells were harvested by centrifugation (5000 g, 15 min at 4 °C); resuspended in 20 mM Tris, 500 mM NaCl, and 1 mM EDTA (pH 7.9); and lysed by an EmulsiFlex-C5 homogenizer (Avestin (Mannheim, Germany)). The lysate was centrifuged at 20 000 g for 30 min at 4 °C and purified on glutathione SepharoseTM 4B (purchased from GE Healthcare (München, Germany)) and dialyzed against 20 mM Tris and 1 mM EDTA (pH 8.0). Purified proteins were stored at −20 °C after adding 10% glycerol until usage.

G_5-mCherry

After transformation of *E. coli* Rosetta (DE3)pLysS with pET21a-G_5-mCherry-H_6, the cells were cultured at 37 °C in LB with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. At an OD_{600} of 0.6, IPTG was added to a final concentration of 0.5 mM to induce expression. The temperature was reduced to 25 °C, and the culture was incubated for 20 h [25]. Afterward, the cells were harvested and lysed in 20 mM Tris, 500 mM NaCl, 100 μM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) (pH 7.9) followed by purification on Ni-NTA agarose (Qiagen GmbH(Hilden, Germany)). The protein was dialyzed against 25 mM Tris and 100 mM NaCl (pH 8.4) and then further purified by HiTrap Q XL anion exchange column from GE Healthcare (buffer A: 20 mM Tris (pH 8.4); buffer B: 20 mM Tris, 1 M NaCl (pH 8.4)). The purified G_5-mCherry-H_6 was dialyzed against 15 mM Tris (pH 7.5). Purified proteins were stored at −20 °C after adding 10% glycerol until usage.

Sfp

E. coli DE21 was transformed with the pET28a-sfp plasmid, and the cells were cultured at 37 °C in LB with 30 μg/ml kanamycin until an OD_{600} of 0.6 was reached. Expression was induced with a final concentration of 1 mM IPTG for 6 h at 25 °C. The cells were lysed in 20 mM Tris and 500 mM NaCl (pH 7.9), purified by Ni-NTA agarose, dialyzed against 10 mM Tris and 1 mM EDTA (pH 7.5), and frozen with additional 10% glycerol. Purified proteins were stored at −20 °C until usage.

Sortase A

E. coli DE21 was transformed with a pET23b-SrtA, and expression was induced at an OD_{600} of 0.6 with 0.5 mM IPTG for 20 h at 20 °C. Cells were lysed in 20 mM Tris and 0.1% Triton X-100 (pH 6.0) and purified by Ni-NTA. The purified protein was dialyzed against 50 mM Tris, 150 mM NaCl and 5 mM $CaCl_2$ at pH 7.5 and frozen at −20 °C after adding 10% glycerol until usage.

Assembly Reactions

Ligation

The reaction buffer for all ligation studies was 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (pH 7.5). For the kinetic studies, the ligation of either **1** or **3** to the peptide **2a** was carried out at 37 °C with 50 μM enzyme (sortase A or Sfp), 100 μM protein (mCherry or 14-3-3ζ), and 200 μM peptide **2a**. The three-fragment assembly of the peptides and proteins was performed at 37 °C by incubating the components as follows: 50 μM Sfp, 50 μM sortase A, and 100 μM 14-3-3ζ (**1**); 200 μM mCherry (**3**); and 100 μM peptide **2a** or **2b** in a final volume of 10 μl. At selected time points, 1 μl samples were collected and analyzed by SDS-PAGE.

In order to purify the assembled products, the reaction was performed as described earlier in a volume of 1.4 ml for 2 h. In what follows, the reaction mixture was transferred to a dialysis tube. The reaction was continued for 96 h under constant dialysis against reaction buffer. Afterward, the products were purified on glutathione Sepharose followed by Ni-NTA agarose. On average, we obtained 125 ± 50 μg of **6a** or **6b** after purification.

Analysis of the Assembly Reactions and Western Blot Analysis

The reaction mixture was separated on either 10% or 12% SDS-polyacrylamide gels and analyzed by UV irradiation at 312 nm (FujiFilm (Düsseldorf, Germany) LAS-3000). Afterward, the gels were stained with Coomassie Brilliant Blue G250 (Sigma-Aldrich, Steinheim, Germany). The densitometric analysis of the SDS-PAGE was performed with the ImageJ software.

Western blot analyses were performed as follows: The proteins were blotted onto a PVDF (polyvinylidene fluoride) membrane (Carl Roth GmbH (Karlsruhe, Germany)). As primary antibodies, anti-mCherry 1 : 2000 (Acris Antibodies GmbH (Herford, Germany)) and anti-14-3-3ζ 1 : 1000 (Santa Cruz (Heidelberg, Germany)) were used. The secondary antibodies, both purchased from Santa Cruz, were donkey anti-goat HRP (1 : 5000) and goat anti-rabbit HRP (1 : 5000). The blots were developed with an ECL western blotting kit (Thermo Scientific GmbH (Schwerte, Germany)).

Fluorescence Measurement

The FRET measurements were performed on a QuantaMaster™7 fluorimeter (Photon Technology International (Seefeld, Germany)) with 1 μM ligation product **6a** and **6b** or a mixture of 1 μM mCherry, 1 μM 14-3-3ζ, and 1 μM peptide **2a** or **2b** in 50 mM Tris, 100 mM NaCl, and 1 mM $MnCl_2$ at pH 7.5 in a final volume of 200 μl at 25 °C. The slit width both for excitation and emission was 5 nm. The samples were excited at 450 nm, and the fluorescence emission was detected from 470 to 700 nm at 90°. For the calculation of energy transfer, the ratio of mCherry (607 ± 2 nm) to 5,6-carboxyfluorescein intensity (522 ± 2 nm) was determined.

Results and Discussion

Strategy

Typical FRET-based reporters possess two FPs at the N-termini and C-termini, sandwiching a modification site and a PTM-binding domain. This concept has been used previously for the development of a reporter for histone phosphorylation [23]. Histone H3 possesses six phosphorylation sites including Ser28 that is modified during mitosis and interphase [26]. The aforementioned reporter consists of a short sequence derived from histone H3 covering the modification site of S28 and a 14-3-3 protein that binds to phosphorylated serine residues. In addition, the cyan fluorescent protein and the yellow fluorescent protein at the N-termini and C-termini are used as FRET pairs. This reporter served as basis for the design of the semisynthetic model protein. Our strategy is illustrated in Figure 2: The N-terminal fragment **1** is a recombinant 14-3-3ζ protein containing a glutathione *S*-transferase (GST) tag at the N-terminus and the S6 sequence at the C-terminus. The latter sequence facilitates the Sfp-catalyzed transfer of the modified Ppant from CoA derivatives onto the first serine of the S6 motif (GDSLSWLLRLLN) [19]. The central fragment (**2**) is conjugated with CoA at the N-terminus and possesses a 5,6-carboxyfluorescein moiety followed by a short sequence derived from histone H3 (residues 25–32) that can be phosphorylated by the Aurora B kinase at the central S28 residue [27,28]. The C-terminus of **2** contains the sorting motif (here LPKTG). Peptide **2** was synthesized in nonphosphorylated (**2a**) and S28-phosphorylated (**2b**) forms. The C-terminal fragment **3** is the FP mCherry that was N-terminally extended by five glycine residues and C-terminally elongated by a hexa-histidine (H_6) tag.

The added Sfp should catalyze the formation of intermediate **4** linked by Ppant from **1** and **2**. Sortase A ligates **2** and **3** forming

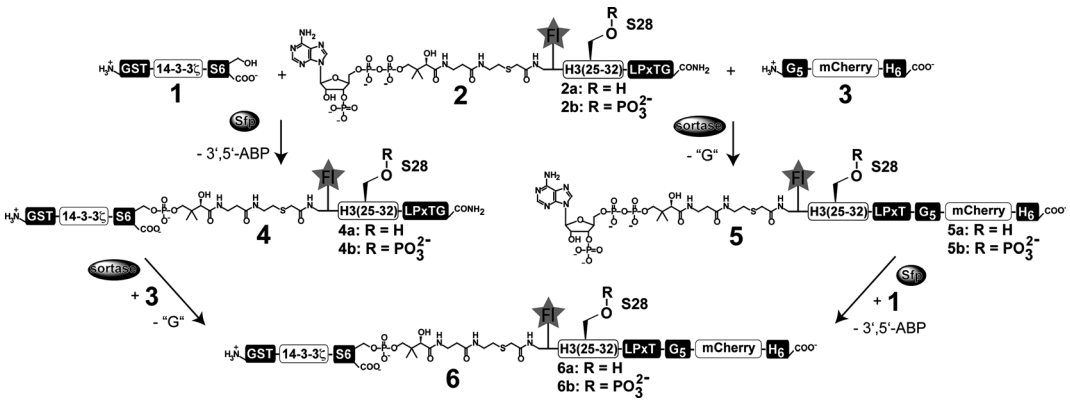

Figure 2. Strategy for the three-fragment assembly of the semisynthetic target protein. The synthetic central fragment (**2**) is conjugated to the phosphoserine binding 14-3-3ζ protein (**1**) by the PPTase Sfp. Ppant bridges the N-terminus of the central fragment **2** with the S6 motif at the C-terminus of 14-3-3ζ (**1**) in **4**. The fluorescent mCherry protein (**3**) is ligated to the C-terminus of the synthetic central fragment (**2**) by sortase A resulting in **5**. Based on the orthogonality of these enzymes, the reaction can be performed in a one-pot reaction forming **6**. Fragment **2** is synthesized in a nonphosphorylated form (**2a**) or with a phosphorylated serine at S28 (numbering according to histone H3) (**2b**). The intermediates and products **4a**, **5a**, and **6a**, and **4b**, **5b**, and **6b** correspond to the nonmodified and phosphorylated forms, respectively.

Journal of PeptideScience

intermediate **5**. Simultaneous incubation of **1**, **2**, and **3** with Sfp and sortase A should result in the simultaneous assembly of target protein **6** in a one-pot reaction.

This initial design varies from the aforementioned recombinant reporter construct and does not contain an FP at the N-terminus but a synthetic fluorophore (5,6-carboxyfluorescein) in the synthetic fragment **2**, which is positioned between the 14-3-3 domain and the phosphorylation site in the assembled product **6** [23]. The synthetic fluorophore in **2** allows simple monitoring of the individual ligation and bioconjugation reactions by SDS-PAGE. Furthermore, it cannot be excluded that an internal FRET donor can also be used to readout the interaction between 14-3-3 and the modified S28 due to changes in the orientation of 5,6-carboxyfluorescein and the C-terminal FP in the bound state. Based on this, we chose mCherry as the C-terminal fragment because the excitation wavelength of this FP overlaps with the emission spectrum of 5,6-carboxyfluorescein. Finally, if efficient FRET between the internal 5,6-carboxyfluorescein and mCherry can be measured in **6**, this approach can be extended to a triple-FRET cascade possessing a third fluorophore at the N-terminus.

Synthesis of Peptide-CoA Conjugates and Generation of Recombinant Fragments

Peptides **2a** (CoA-Ahx-Ahx-K(Fl)-Ahx-ARKSAPTG-Ahx-LPKTGGRR-NH_2) and **2b** (CoA-Ahx-Ahx-K(Fl)-Ahx-ARKpSAPTG-Ahx-LPKTGGRR-NH_2) were synthesized in two steps. At first, the peptides were synthesized on solid support by automated SPPS, and 5,6-carboxyfluorescein was installed at a lysine side chain. Afterward, the resin-bound peptide was bromoacetylated at the N-terminus and cleaved. In what follows, the bromoacetylated peptides were modified with CoA in solution based on a protocol for lysine-CoA conjugates that serve as inhibitors for histone acetyltransferases [24]. After HPLC purification, the peptide-CoA conjugates were obtained in an average isolated yield of 35% and >90% purity (Figure S1; see Supporting Information).

Genes encoding GST-14-3-3ζ-S6 (**1**) and G_5-mCherry-H_6 (**3**) were cloned and expressed in *E. coli*. Expressed proteins were purified by affinity chromatography on either glutathione agarose (**1**) or Ni^{2+}-NTA affinity resin (**3**).

Establishing the Orthogonal 'Three-fragment Assembly' in a One-pot Reaction

With all building blocks available, we started establishing the assembly reactions. At first, we analyzed the ligation of 100 μM mCherry (**3**) and 200 μM of the synthetic central fragment **2a**. Both components were mixed in a ratio of 1 : 2 (**3** : **2a**) with 50 μM of sortase A from *S. aureus*. At selected intervals, samples were collected and analyzed by SDS-PAGE. As illustrated in Figure 4A, the ligation product **5a** possesses a different electrophoretic mobility than the starting material **3** and can be clearly identified by the fluorescence of the ligated 5,6-carboxyfluorescein moiety. Because of the reversible nature of sortase-mediated ligations, the reaction does not proceed beyond 50% conversion when educts are added in near stoichiometric concentrations (Figure 3A).

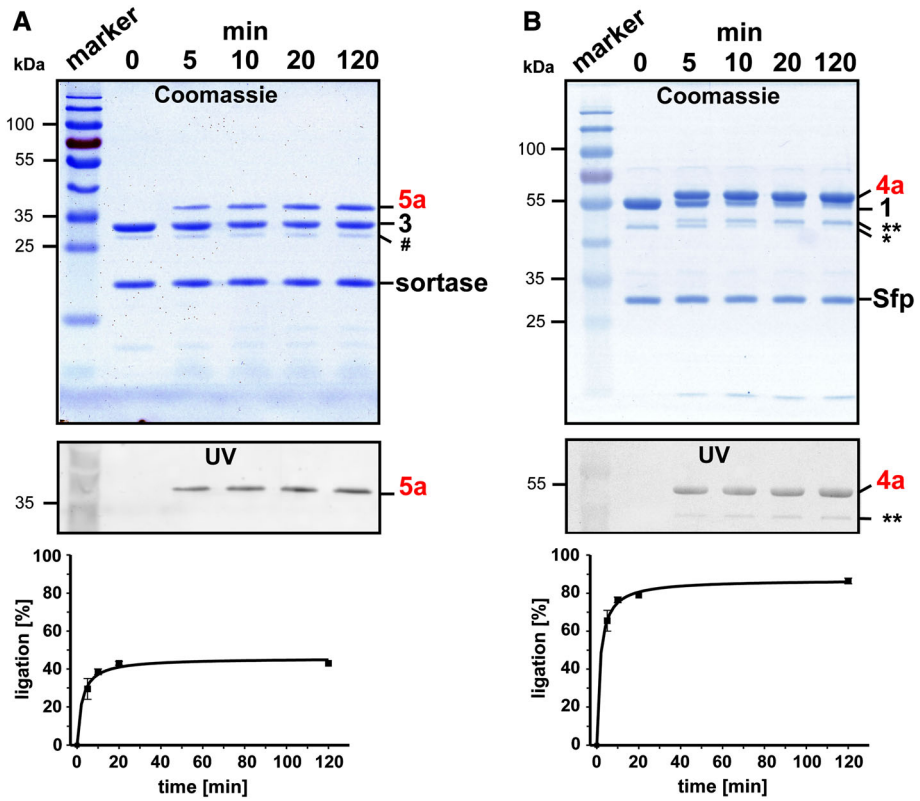

Figure 3. Characterization of the individual ligation and conjugation steps. (A) Sortase A catalyzes the ligation of **2a** and **3**. The formed product **5a** possesses a different electrophoretic mobility than **2a** and **3** and can be identified by its fluorescence under UV illumination. Densitometric analysis of the Coomassie Brilliant Blue stained SDS-polyacrylamide gel was used to determine the ligation yield in a time-dependent manner. (B) The PPTase Sfp catalyzes the conjugation of **1** and **2a**. The 5,6-carboxyfluorescein moiety of product **4a** fluoresces upon UV illumination, and the SDS-PAGE was subjected to densitometric analysis to quantify the ligation yield. #Protein impurities; *degradation product of **1**; **degradation product of **1** modified with **2a**.

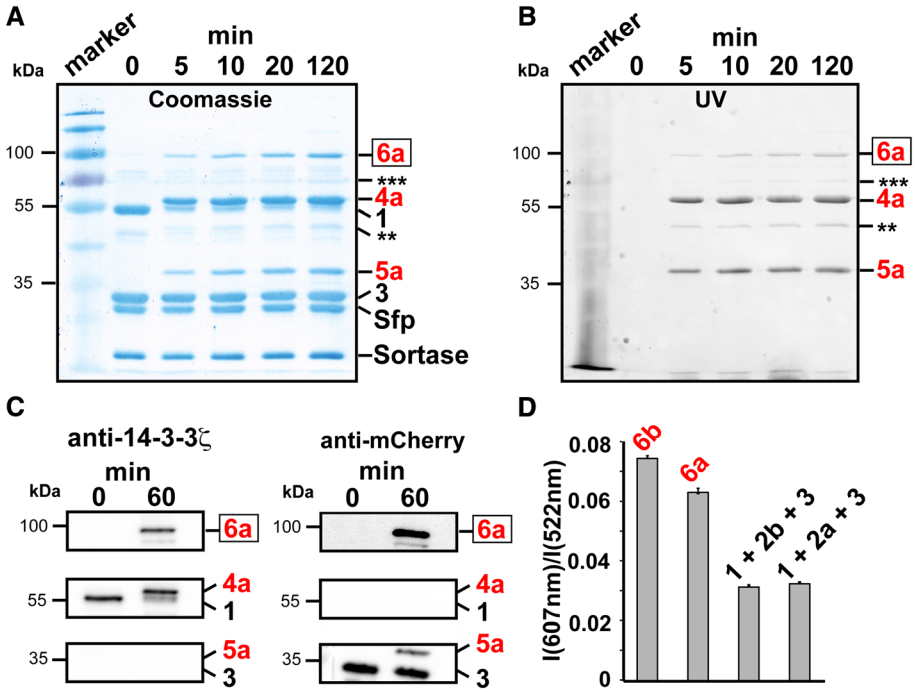

Figure 4. Three-fragment assembly. (A) Time course of the three-fragment assembly of **1**, **2a**, and **3** analyzed by SDS-PAGE. (B) UV illumination of the same SDS-polyacrylamide gel shows the formed product **6a**, the intermediates **4a** and **5a**, and the starting material **2a**. (C) Western blot analysis of the three-fragment assembly before the reaction (0 min) and after 1 h of reaction time (60 min). Only the assembled product **6a** is stained with antibodies raised against 14-3-3ζ and mCherry. The intermediates and starting materials were only detected by one of the antibodies. (D) FRET measurements of assembled **6a** and **6b** in comparison with the nonassembled starting materials **1**/**2a**/**3** and **1**/**2b**/**3**. Samples were excited at 450 nm, and the emissions at 522 nm (5,6-carboxyfluorescein) and 607 nm (mCherry) were recorded. **Degradation product of **1** modified with **2a**; ***degradation product of **1** assembled into **6a**.

The Sfp-catalyzed linkage of 100 μM GST-14-3-3ζ-S6 (**1**) and 200 μM synthetic peptide **2a** by 50 μM of Sfp yielded product **4a** and proceeded more efficiently than the sortase-mediated ligation. Furthermore, a ligation yield of 80% was achieved within 20 min of reaction time. In contrast to sortases, PPTases cannot cleave their products, rendering this methodology very useful for protein chemistry (Figure 3B).

Next, we set out to establish both reactions simultaneously in order to form the desired target protein construct **6a** in a one-pot reaction (Figure 4A and Figure S2). To this end, we mixed **1**, **2a**, and **3** in an optimized ratio of 100 μM : 100 μM : 200 μM (**1** : **2a** : **3**) with 50 μM of Sfp and sortase A and followed the reaction over time. The individual ligation products **4a** and **5a** formed in the initial phase of the three-fragment assembly. After 5 min of reaction time, a new band with the expected mass of target protein **6a** was detected (calculated mass: 84 kDa). Upon UV illumination, the band fluoresces indicating that it contains **2a** (Figure 4B). To further validate the identity of putative target protein **6a**, we subjected the ligation reaction to western blot analyses with antibodies against mCherry and 14-3-3ζ. As expected, the band at 84 kDa was immunostained with both antibodies while **1** and **4a** were only detected with the anti-14-3-3ζ antibody. In agreement with these observations, the starting material **3** and intermediate **5a** were only detected with anti-mCherry antibody (Figure 4C). In summary, the western blot analyses indicated that the desired target protein **6a** was assembled successfully in a one-pot reaction.

Encouraged by these results, we performed the assembly of **6a** and **6b** on larger scale under conditions described earlier and purified the products from the reaction mixtures. To this end, the reaction time was extended to 96 h under continuous dialysis in order to remove the leaving group of the sortase-mediated ligation and shift the conversion beyond 50%. The following purification of **6a** and **6b** was facilitated by the affinity tags (GST and H_6).

In what follows, we analyzed **6a** and **6b** by fluorescence spectroscopy in order to investigate if efficient FRET between 5,6-carboxyfluorescein and mCherry can occur. To this end, we excited the 5,6-carboxyfluorescein moiety of **6a** and **6b** and nonassembled mixtures of the starting materials **1**/**2a**/**3** and **1**/**2b**/**3** at 450 nm. Emissions at 522 nm (5,6-carboxyfluorescein) and 607 nm (mCherry) were measured, and the ratio of the intensities I(607 nm)/I(522 nm) was used as an indicator for the FRET efficiency. As illustrated in Figure 4D and Figure S3, proteins **6a** and **6b** displayed marginal differences in the FRET efficiency, which might indicate a readout of a conformational change resulting from the intramolecular interaction between 14-3-3ζ and pSer28 in **6b**. However, further biophysical investigations are required in order to confirm the nature of this difference in the FRET efficiency. Most importantly, **6a** and **6b** showed a robust twofold increase in the mCherry/fluorescein emission ratio compared with the nonassembled controls (Figure 4D), which indicates efficient FRET in the semisynthetic model proteins. This observation confirms that the design can be extended to triple-FRET probe when an additional FP is linked as fluorescence donor to the N-terminus of the reporter construct.

In summary, these experiments demonstrate that fluorescent-based reporter constructs can be assembled in nonmodified and premodified forms from recombinant and synthetic fragments. Future developments will focus on the optimization of this prototype reporter constructs. Such optimization can include variation of the linker length in the synthetic central fragment and the position and type of fluorophores used for the FRET measurements. Once a set of different synthetic and recombinant fragments is generated, they can be freely combined in parallel and large numbers.

Journal of PeptideScience

In general, this strategy is not limited to FRET-based reporters. Sortase-mediated ligations are widely used for the selective modification of proteins and protein semisynthesis. In contrast, the Sfp-catalyzed bioconjugation is used less frequent. The latter technique establishes a nonpeptide Ppant linkage, which can be a major disadvantage for protein semisynthesis. Therefore, this method may not be suitable for all protein semisynthesis approaches. In our experimental setup, Sfp-catalyzed bioconjugation was used to link the N-terminus of the central fragment **2** to an AA side chain at the C-terminus of the N-terminal fragment **1**. This was achieved by linking the CoA moiety to the N-terminus of the central fragment **2**. However, CoA can also be conjugated to a lysine side chain enabling the linkage of two peptides via two AA side chains, which extends the application range of this approach [20]. A major advantage of the Sfp-catalyzed bioconjugation is the near quantitative modification because Ppant can be considered as an irreversible PTM. Sfp and other known Ppant transferases do not possess Ppant phosphodiesterase activity, and as of today, it remains uncertain if such phosphodiesterases exist at all [29]. Based on this, the Ppant linkage should be highly stable in cellular lysates and living cells that is an important benefit for cellular applications.

Conclusion

In conclusion, we have established an orthogonal strategy for chemoenzymatic three-fragment assemblies of semisynthetic proteins based on the transpeptidase sortase A and the 4′-phosphopantetheine transferase Sfp. This method enables the introduction of synthetic peptides into central regions of proteins without the need to purify intermediates. We have demonstrated the applicability of this methodology for the assembly of a prototype FRET reporter for kinases and phosphatases. The general strategy should simplify the optimization process of this type of reporters. Most importantly, the developed three-fragment assembly strategy is general and can be used for generating a wide range of different semisynthetic proteins that do not require a native peptide bond at all ligation sites.

Acknowledgements

We thank Dr Thorsten Stafforst and Dr Ayan Samanta for support with FRET measurements. We are further grateful to Alexander Dose, Jan Oliver Jost, Julia Sindlinger, and Diego Aparicio Pelaz for critical reading of the manuscript. This work was supported by the priority program SPP 1623 of the Deutsche Forschungsgemeinschaft (SCHW 1163/4-1).

References

1 Hackenberger CPR; Schwarzer D.Chemoselective ligation and modification strategies for peptides and proteins. *Angew. Chem. Int. Ed.* 2008; **47**: 10030–10074.
2 Kent SBH. Total chemical synthesis of proteins. *Chem. Soc. Rev.* 2009; **38**: 338–351.
3 Dawson PE, Muir TW, Clarklewis I, Kent SBH. Synthesis of proteins by native chemical ligation. *Science* 1994; **266**: 776–779.
4 Muir TW, Sondhi D, Cole PA. Expressed protein ligation: A general method for protein engineering. *Proc. Natl. Acad. Sci. U.S.A.* 1998; **95**: 6705–6710.
5 Popp MWL, Ploegh HL. Making and breaking peptide bonds: protein engineering using sortase. *Angew. Chem. Int. Ed.* 2011; **50**: 5024–5032.
6 Tsukiji S, Nagamune T. Sortase-mediated ligation: a gift from gram-positive bacteria to protein engineering. *Chembiochem* 2009; **10**: 787–798.
7 Piotukh K, Geltinger B, Heinrich N, Gerth F, Beyermann M, Freund C, Schwarzer D. Directed evolution of sortase a mutants with altered substrate selectivity profiles. *J. Am. Chem. Soc.* 2011; **133**: 17536–17539.
8 Teschke T, Geltinger B, Dose A, Freund C, Schwarzer D. Probing the recognition of post-translational modifications by combining sortase-mediated ligation and phage-assisted selection. *Acs Chem. Biol.* 2013; **8**: 1692–1697.
9 Ton-That H, Marraffini LA, Schneewind O. Protein sorting to the cell wall envelope of Gram-positive bacteria. *Bba-Mol. Cell. Res.* 2004; **1694**: 269–278.
10 Clancy KW, Melvin JA, McCafferty DG. Sortase transpeptidases: insights into mechanism, substrate specificity, and inhibition. *Biopolymers* 2010; **94**: 385–396.
11 Bang D, Pentelute BL, Kent SBH. Kinetically controlled ligation for the convergent chemical synthesis of proteins; *Angew. Chem. Int. Ed.* 2006; **45**: 3985–3988.
12 Antos JM, Chew GL, Guimaraes CP, Yoder NC, Grotenbreg GM, Popp MWL, Ploegh HL. Site-specific N- and C-terminal labeling of a single polypeptide using sortases of different specificity. *J. Am. Chem. Soc.* 2009; **131**: 10800–10801.
13 Race PR, Bentley ML, Melvin JA, Crow A, Hughes RK, Smith WD, Sessions RB, Kehoe MA, McCafferty DG, Banfield MJ. Crystal structure of streptococcus pyogenes sortase A: implications for sortase mechanism. *J. Biol. Chem.* 2009; **284**: 6924–6933.
14 Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, Reid R, Khosla C, Walsh CT. A new enzyme superfamily – the phosphopantetheinyl transferases. *Chem. Biol.* 1996; **3**: 923–936.
15 Yin J, Liu F, Li XH, Walsh CT. Labeling proteins with small molecules by site-specific posttranslational modification. *J. Am. Chem. Soc.* 2004; **126**: 7754–7755.
16 Zou YK, Yin J. Phosphopantetheinyl transferase catalyzed site-specific protein labeling with ADP conjugated chemical probes. *J. Am. Chem. Soc.* 2009; **131**: 7548–7549.
17 George N, Pick H, Vogel H, Johnsson N, Johnsson K. Specific labeling of cell surface proteins with chemically diverse compounds. *J. Am. Chem. Soc.* 2004; **126**: 8896–8897.
18 Yin J, Straight PD, McLoughlin SM, Zhou Z, Lin AJ, Golan DE, Kelleher NL, Kolter R, Walsh CT. Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. *Proc. Natl. Acad. Sci. U.S.A.* 2005; **102**: 15815–15820.
19 Zhou Z, Cironi P, Lin AJ, Xu YQ, Hrvatin S, Golan DE, Silver PA, Walsh CT, Yin J. Genetically encoded short peptide tags for orthogonal protein labeling by Sfp and AcpS phosphopantetheinyl transferases. *Acs Chem. Biol.* 2007; **2**: 337–346.
20 Chen I, Dorr BM, Liu DR. A general strategy for the evolution of bond-forming enzymes using yeast display. *Proc. Natl. Acad. Sci. U.S.A.* 2011; **108**: 11399–11404.
21 Newman RH, Fosbrink MD, Zhang J. Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells. *Chem. Rev.* 2011; **111**: 3614–3666.
22 Yi L, Sun H, Itzen A, Triola G, Waldmann H, Goody RS, Wu YW. One-pot dual-labeling of a protein by two chemoselective reactions. *Angew. Chem. Int. Ed.* 2011; **50**: 8287–8290.
23 Lin CW, Ting AY. A genetically encoded fluorescent reporter of histone phosphorylation in living cells. *Angew. Chem. Int. Ed.* 2004; **43**: 2940–2943.
24 Lau OD, Kundu TK, Soccio RE, Ait-Si-Ali S, Khalil EM, Vassilev A, Wolffe AP, Nakatani Y, Roeder RG, Cole PA. HATs off: selective synthetic inhibitors of the histone acetyltransferases p300 and PCAF. *Mol. Cell* 2000; **5**: 589–595.
25 Matsumoto T, Tanaka T, Kondo A. Sortase A-catalyzed site-specific coimmobilization on microparticles via streptavidin. *Langmuir* 2012; **28**: 3553–3557.
26 Cerutti H, Casas-Mollano JA. Histone H3 phosphorylation universal code or lineage specific dialects? *Epigenetics* 2009; **4**: 71–75.
27 Wei Y, Yu LL, Bowen J, Gorovsky MA, Allis CD. Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* 1999; **97**: 99–109.
28 Liokatis S, Stutzer A, Elsasser SJ, Theillet FX, Klingberg R, van Rossum B, Schwarzer D, Allis CD, Fischle W, Selenko P. Phosphorylation of histone H3 Ser10 establishes a hierarchy for subsequent intramolecular modification events. *Nat. Struct. Mol. Biol.* 2012; **19**: 819–823.
29 Nakanishi M, Yatome C, Ishida N, Kitade Y. Putative ACP phosphodiesterase gene (acpD) encodes an azoreductase. *J. Biol. Chem.* 2001; **276**: 46394–46399.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site

