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Selective Staudinger Modification of Proteins Containing *p*-Azidophenylalanine

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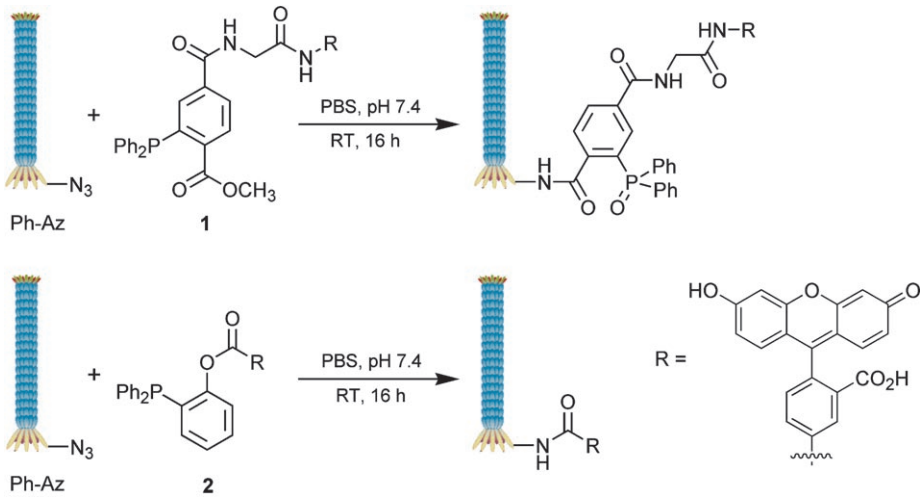
Various methods have been developed to selectively modify proteins with synthetic agents and probes, and to covalently attach proteins to surfaces. These include semisynthesis,[1] the use of electrophilic reagents that selectively label cysteine and lysine residues,[2,3] and the selective introduction of amino acids with reactive side chains into proteins by in vitro biosynthesis with chemically aminoacylated tRNAs.[4,5] Recently, we showed that one can genetically encode unnatural amino acids with reactive side chains directly in prokaryotic and eukaryotic organisms efficiently and with high fidelity.[6–10] Amino acids with side chains containing keto, acetylenic, and azido groups have been incorporated into proteins in response to unique three-base (nonsense) and four-base (frameshift) codons. These amino acids were subsequently modified selectively with exogenous agents by oxime formation or [2+3] cycloaddition reactions. We now report that this approach can be extended to the modification of proteins with selectively incorporated aryl azide amino acids by using phosphine-derived probes and the Staudinger ligation reaction.

The Staudinger ligation has been used previously to modify cell surface carbohydrates in both cellular and in vivo systems.[11,12] The reaction proceeds with excellent yields under aqueous conditions and is highly selective for azides. The Staudinger ligation has also been used to selectively modify proteins that contain azidohomoalanine substituted for methionine residues.[13] The selectivity of this approach, however, is intrinsically limited since all methionine residues throughout the proteins and proteome are substituted with azidohomoalanine, often in competition with the native amino acid. To overcome these limitations, we have taken advantage of a genetically encoded azide-containing amino acid that is incorporated at defined sites in a protein in response to a nonsense codon. The azide is subsequently modified by a Staudinger ligation to selectively incorporate exogenous reagents into the protein. Previously we demonstrated that the unnatural amino acid, *p*-azidophenylalanine (pAzPhe), which is essentially unreactive toward biomolecules, can be selectively incorporated into proteins in *E. coli* by using a heterologous suppressor tRNA–aminoacyl tRNA synthetase pair with altered specificity.[14] Here we show that the Staudinger ligation can be used to modify this amino acid efficiently and selectively with spectroscopic probes in either proteins or phage-displayed peptides.

A phage-display system was used in which the streptavidin-binding peptide (SBP) AGXTLLAHPQ was displayed pentavalently as a fusion to the pIII protein of M13 filamentous phage.[15] The N-terminal AG sequence facilitates cleavage of the signal peptide; the third residue, X, encoded by the amber nonsense codon TAG, designates the unnatural amino acid to be incorporated. The phage Ph-Az (encoding SBP with pAzPhe at residue X) was prepared in *E. coli* strain TTS/RS in the presence of 2 mM pAzPhe with good efficiency and high fidelity.[15] *E. coli* TTS/RS contains a plasmid that constitutively expresses a *Methanococcus jannaschii* mutant amber suppressor $tRNA^{Tyr}_{CUA}$ ($mutRNA^{Tyr}_{CUA}$) and a mutant *M. jannaschii* tyrosyl-tRNA synthetase (MjTyrRS) which specifically charges $mutRNA^{Tyr}_{CUA}$ with pAzPhe.[6,14,15] As a negative control, another SBP-displayed phage (Ph-Q) was prepared in *E. coli* XL1-Blue, a natural amber suppression strain that incorporates glutamine at residue X.

The fluorescein-derived phosphines **1** and **2** (Scheme 1) were used for the Staudinger ligation reaction since they can be easily detected. Phosphine **1** should react with phage Ph-Az to form an aza-ylide intermediate,[16] followed by intramolecular cyclization[11] to ultimately yield a fluorescein-labeled phage product (Scheme 1). The conjugation of **2** with Ph-Az should undergo a traceless Staudinger ligation by a similar reaction mechanism to yield a fluorescein-labeled phage without an intervening triphenylphosphine oxide group. Conjugation



Scheme 1. Schematic representation of the Staudinger conjugation of phage Ph-Az with phosphines **1** and **2**.

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reactions were carried out between phage Ph-Az and triphenylphosphines **1** and **2** with approximately 10^{11} phage particles and 0.01 mM phosphine in 10 mM phosphate buffered saline solution (PBS, pH 7.4); similar reactions were carried out with phage Ph-Q as a negative control. The ligation reactions were carried out at ambient temperature for 16 hours, and the mixtures were then dialyzed and subjected to SDS-PAGE and Western blot analysis (Figure 1). Only the fluorescein conjugate

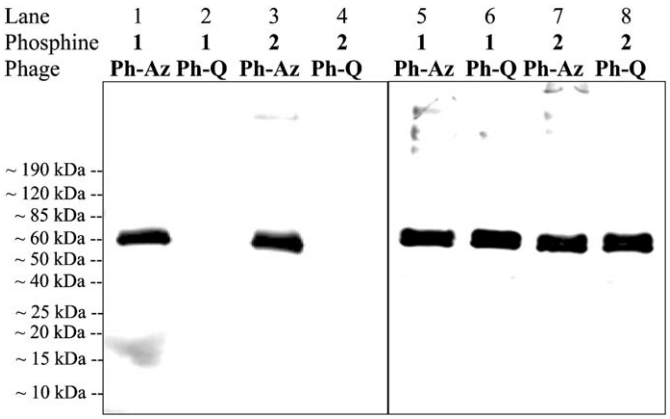

Figure 1. Anti-fluorescein (lanes 1–4) and anti-pIII (lanes 5–8) Western blot analysis of phages Ph-Az and Ph-Q after Staudinger ligation with phosphines **1** and **2**.

from the ligation of Ph-Az with either phosphine **1** or **2** was observed as a single band; it was not observed in the control reactions with Ph-Q. This band was further identified as the pIII minor coat protein by anti-pIII Western blot analysis. These results clearly show a high degree of selectivity between phosphine **1** or **2** and the azide-containing phage peptide.

To show that the Staudinger coupling reaction does not lead to a loss in protein activity, phage viability was determined by titering phage Ph-Az before and after the Staudinger ligation with **1** or **2**. The observed number of viable phage particles from a Staudinger reaction mixture was $(1.7\pm1)\times10^{11}$ plaque-forming units per milliliter (PFU mL^{-1}), compared to $(2.5\pm1)\times10^{11}$ PFU mL^{-1} determined from control solutions without phosphine **1** or **2**. In a model phage-selection experiment, a similar number of phage particles prepared from the aforementioned Staudinger ligation of phosphine **1** with Ph-Az and Ph-Q were incubated in separate wells that had been precoated with anti-fluorescein antibody. After iterative washing, the bound phage was eluted with 0.05% BSA–FITC conjugate and titered. The recovery rate of fluorescein-labeled phage derived from the ligation of Ph-Az with **1** (0.12%) is 120-fold greater than that of the control phage derived from Ph-Q (0.001%). These results demonstrate that the Staudinger ligation reaction does not significantly affect phage viability. It is important to note that phage Ph-Az is nonviable after exposure to the reaction conditions in the [2+3] cycloaddition with a terminal alkyne group and copper catalyst.[15,17] It was found that the copper catalyst is predominantly responsible for the viability loss; however, addition of high concentrations of EDTA during the dialysis step did not notably improve phage viability.

To further characterize the Staudinger ligation products and determine the conjugation efficiency, a representative Z-domain protein[18] containing pAzPhe at residue 7 was expressed in an *E. coli* strain with mutRNA$^{Tyr}_{CUA}$ and the mutant MjTyrRS, which selectively charges pAzPhe.[6,10,14] This azide-containing Z-domain was purified and conjugated with phosphines **1** and **2**. After conjugation, the dialyzed reaction mixtures were analyzed by MALDI-TOF. The major peaks of the observed spectra match the expected Staudinger ligation products (Figure 2 and Supporting Information). No azide-contain-

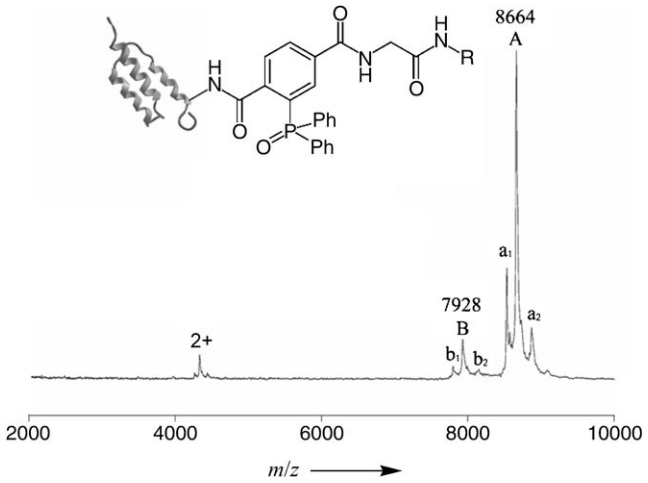

Figure 2. MALDI-TOF analysis of the reaction products from the Staudinger ligation of pAzPhe containing Z-domain protein with phosphines **1**. Peaks A and B can be assigned to the conjugation and reduction products, respectively; minor peaks a_2, b_2, a_1 and b_1 are derived from the matrix adducts and the exclusion of methionine from A and B.

ing Z-domain was observable (<1%); this indicates that the reaction proceeds in high yield. Doping experiments with authentic material demonstrated that the pAzPhe Z-domain mutant is stable (see Supporting Information). For the Staudinger ligation of phosphine **1**, the conjugation efficiency is estimated to be >90%, based on the integration ratio of the peaks in the MALDI-TOF spectrum. On the other hand, the traceless Staudinger ligation of phosphine **2** afforded a lower yield of ~50%. The lower conjugation efficiency of **2** might be due to a slower ligation rate, presumably in the intramolecular cyclization step,[19] and the ease of the hydrolysis of the phenol ester in **2**. These would lead to an amine product, as in the classical Staudinger reactions.[11,16]

In summary, we have shown that model Staudinger ligations between fluorescein-tethered phosphines and either a pAzPhe-containing phage-displayed peptide or a mutant Z-domain protein occur with excellent selectivity and efficiency. The Staudinger ligation does not affect phage viability, so enrichment can be performed without difficulty after the completion of ligation. This work provides another useful method for selectively modifying proteins without altering their function and should be useful for the generation of highly homogenous pe-

gylated proteins, surface immobilized proteins, or proteins modified with spectroscopic or affinity reagents.

Experimental Section

All chemicals were purchased from Aldrich. MALDI-TOF and ESI-TOF spectra were obtained from the Scripps Center for Mass Spectrometry. General procedures for the production of phage Ph-Az and Ph-Q, phage titering, Western blot analysis, and the expression of azido-containing Z-domain were reported previously.[14,15,18]

Synthesis: Compound **1** was synthesized according to published procedures.[11,20] Compound **2** was prepared by the coupling reaction of 2-(diphenylphosphino)phenol (74 mg, 0.27 mmol)[21] and 5(6)-carboxyfluorescein (100 mg, 0.27 mmol) in the presence of dicyclohexylcarbodiimide (62 mg, 0.3 mmol) in anhydrous DMF (1 mL) at ambient temperature for 12 h, and purified by using preparative TLC to give a red powder (3 mg, 2%); HRMS (ESI-TOF) for $C_{39}H_{24}O_7P_1$: calcd: 635.1265 $[M-1]^-$; found: 635.1248.

Ligation reactions of phage: A stock (0.5 mM) of each phosphine reactant in DMF was prepared and was diluted with reaction buffer to a final concentration of 0.01 mM and total volume of 50 μL. The ligation reactions were carried out in PBS (0.01 M, pH 7.4) with approximately 10^{11} phage particles at ambient temperature and with shaking for 16 h. The reaction mixture was then dialyzed against PBS and analyzed.

Enrichment experiment: Anti-fluorescein antibody (20 μg mL^{-1}, 250 μL per well) was coated in aqueous Na_2CO_3 (0.1 M, pH 9.6) in eight wells of an immunoplate (Fisher) at 37 °C for 4 h. Wells were washed (3×, 0.9% NaCl, 0.05% Tween 20), blocked with BSA (0.5%) overnight at 4 °C again, and then incubated at room temperature for 5 h with phage (100 μL) either from a ligation reaction or a control solution. After being washed, the phage was eluted from the plate with BSA–FITC conjugate (0.05%). The input (Ph-Az: 1.0×10^9 PFU; Ph-Q: 2.0×10^9 PFU) and output (Ph-Az: 1.2×10^6 PFU; Ph-Q: 1.0×10^4 PFU) phages were then titered.

Ligation reactions of mutant Z-domain protein: A stock (10 mM) of each phosphine reactant in DMF was prepared, and was diluted with reaction buffer containing mutant Z-domain protein (0.1 mM) to a final concentration of 1 mM and total volume of 10 μL. The ligation reactions were carried out in PBS at ambient temperature and with shaking for 16 h. The reaction mixture was then passed through a PD-10 column, eluted in water, and analyzed by MALDI-TOF spectroscopy.

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