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Adapter Reagents for Protein Site Specific Dye Labeling

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Abstract

Chemoselective protein labeling remains a significant challenge in chemical biology. Although many selective labeling chemistries have been reported, the practicalities of matching the reaction with appropriately functionalized proteins and labeling reagents is often a challenge. For example, we encountered the challenge of site specifically labeling the cellular form of the murine Prion protein with a fluorescent dye. To facilitate this labeling, a protein was expressed with site specific *p*-acetylphenylalanine. However, the utility of this aceto-phenone reactive group is hampered by the severe lack of commercially available aminooxy fluorophores. Here we outline a general strategy for the efficient solid phase synthesis of adapter reagents capable of converting maleimido-labels into aminooxy or azide functional groups that can be further tuned for desired length or solubility properties. The utility of the adapter strategy is demonstrated in the context of fluorescent labeling of the murine Prion protein through an adapted aminooxy-Alexa dye.

Keywords

ketoxime; bioconjugation; chemoselective

INTRODUCTION

The chemoselective formation of oximes has become a widely used tool for bioconjugation and the synthesis of complex biological macromolecules and polymers.^{1,2} Despite a growing list of specialized reagents, the researcher often encounters the frustration of limited availability and exorbitant pricing of dyes with the appropriate functionality to participate in the chosen labeling strategy. In the case of protein conjugation by aniline catalyzed oxime ligation, arylketones can be introduced into proteins using amber suppression approaches,³ in which the amber codon (TAG) substitutes for the amino acid codon, and a UAG-tRNA charged with *p*-acetylphenylalanine (*p*-AcPhe) inserts this noncoded residue in a site specific

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manner. Using this arylketone group, aminooxy functionalized labels can be chemoselectively attached to the *p*-AcPhe amino acid. This strategy has been the basis for several protein labeling studies.⁴⁻⁷

One problem with this conjugation approach is that aryl ketones are highly hindered and react slowly,⁸ even in the presence of reduced pH and aniline catalysis. Another complication, particularly with the aminooxy functionalized dyes, is prohibitive cost and limited availability. In addition, the relatively long reaction time required for conjugation creates solubility issues. Maintenance of protein and label solubility throughout the time course of the reaction is a common problem as the protein can precipitate in a time dependent manner, recovery will suffer if reaction kinetics are not faster than label and/or protein precipitation. Previously this problem was ameliorated for a hydrazone conjugation by drastically raising the concentration of dye to 38 mM.⁹ However, this approach relied on the apparently high intrinsic hydrophilicity of the cyanine dyes, and was not cost effective.

To circumvent these hurdles we have developed simple adapter molecules designed to both improve fluorophore hydrophilicity and to convert the reactivity profile of maleimido-fluorophores to the aminooxy functional group (Figure 1a). This approach should have widespread utility as maleimide dyes are universally available at a fraction of the cost of the equivalent aminooxy dye (which is rarely available). Numerous studies have incorporated the adapter concept,¹⁰⁻¹⁶ but the majority of these have produced reagents on a case-by-case *ad hoc* basis, often employing difficult techniques. Herein are described molecules the novice will synthesize with relative ease.

The utility of these probes is demonstrated using the challenging example of site specific modification of the amyloid forming murine prion protein (moPrP) 23-230 with fluorescent dyes. The prion protein (PrP) is directly responsible for the Transmissible Spongiform Encephalopathies (i.e. Creutzfeldt-Jakob disease, Mad Cow Disease, etc.) and has profound health implications.¹⁷ In mice its full cellular form is 208 amino acids long (23-230), with a 110 residue folded C-terminal domain (121-230). The conformation of this domain has been determined by NMR of the mouse variant¹⁸ and X-ray crystallography of the human protein.¹⁹ Interestingly the N-terminus of full length PrP is unstructured in the absence of metals (e.g. copper or zinc)²⁰ complicating its structural characterization. To more fully resolve the structure and dynamics of the cellular form, site specific labeling with spin labels has been investigated.²¹ However, maintaining solubility of labeled and unlabeled PrP protein compounds the inherent challenges in chemoselective protein labeling. Here we show how the generation of customized adapter molecules by solid phase peptide synthesis (SPPS) can facilitate bioconjugation of probes onto complex proteins such as PrP.

MATERIALS AND METHODS

General Methods and Reagents

N,N-Dimethylformamide (DMF), Methylene Chloride (DCM), aceto-nitrile (ACN), and *N,N*-diisopropylethyl amine (DIEA) were purchased from Fisher. Protected natural amino acids as well as unnatural, aminooxy acetic acid (Aoa) and aminohexanoic acid (Ahx), were purchased from CS Bio, Indofine, or Novabiochem. Dii-sopropylcarbodiimide was obtained

from Sigma-Aldrich. *O*-(1*H*-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HCTU) was obtained from Peptides International. Dimethylsulfoxide (DMSO) was purchased from Acros. Peptides were purified by reversed phase HPLC using linear gradients where solvent A is water [0.05% v/v trifluoroacetic acid (TFA)] and solvent B is 9:1 ACN:water (0.045% v/v TFA).

Synthetic Protocol for Aoa-Linker-Cys 2 by the 9-Fluorenylmethoxycarbonyl Method

Synthesis—About 362 mg (0.25 equivalents) Rink amide AM resin (Novabiochem) is weighed into a 25 mL fritted syringe and topped with 5 mL DMF. The resin is swelled for 5 minutes before removal of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group with 10 mL 20% 4-methylpiperidine (Alfa Aesar) in DMF. After 1 minute, the solution is drained and 10 mL fresh deprotecting solution is added, incubated for 1 minute, drained, and the resin washed with 50 mL DMF. To 585 mg (1 mmol) Fmoc-Cys(Trt)-OH in a separate vial is added 2.5 mL 0.4*M* HCTU in DMF. The solution is vortexed and bath sonicated until no visible solid remains. This solution is then added to the deprotected resin, stirred with a glass rod, and 180 μ L (1.03 mmol) DIEA is added. The mixture is allowed to react for 5 minutes, stirring every minute, before draining and washing the resin with 25 mL DMF. The cycle of Fmoc deprotection in 4-methylpiperidine followed by coupling with HCTU/DIEA is then repeated, first with Fmoc-Arg(Pbf)-OH (648 mg, 1 mmol), followed by Fmoc-Ahx-OH (353 mg, 1 mmol), and finally with tert-butoxycarbonyl (Boc)-Aoa-OH (Indofine) (191 mg, 1 mmol). A third deprotection step is added after the Fmoc-Ahx-OH addition, as Fmoc removal from Fmoc-Ahx-Arg(Pbf)-Cys(Trt)-Rink AM resin is sluggish. Note: to make N₃-linker-Cys 3, replace the Boc-Aoa-OH coupling with bromoacetylation followed by treatment with sodium azide. The swollen resin is then transferred to a fritted polyprep column (Bio-rad) and washed three times with DCM. After the final wash, vacuum is pulled through the resin for 2 minutes and the column bottom is plugged, leaving 589 mg (86% yield) of dry resin. The adapter reagent is cleaved from the solid support with 6 mL TFA (Sigma-Aldrich), 150 μ L triisopropyl silane (TIS) (Oakwood), and 150 μ L water, capping the column top and rotating for 90 minutes. The cleaved product is drained and the spent resin washed with an additional 1 mL TFA, combining the wash and filtrate in a 20 mL glass vial. Approximately 1/2 the volume is evaporated under a stream of Nitrogen gas, at which point a precipitate forms. The suspension is then added dropwise to 45 mL cold diethyl ether in a 50 mL falcon tube and centrifuged at 4000 rpm for 1 minute. The ether is decanted and the pellet dissolved in 25 mL 1:1 ACN:water containing 0.05% TFA and lyophilized twice to obtain Aoa-linker-Cys 2 as white solid (92 mg, 92% yield). Virtually all the additional crude impurities found in the Aoa-linker-Cys synthetic product are attributed to the aminoxy group reactivity, as seen in Supporting Information Figure S1 in supporting information when Aoa is replaced with glycine a greater than 85% pure product is obtained.

Purification—Immediately following lyophilization, the crude product is dissolved in 5 mL 6*M* guanidine hydrochloride (GuHCl) (>99% pure, ICN Biomedicals) 0.05% TFA, syringe filtered through a 25 mm 0.45- μ m filter, and loaded onto a Phenomenex Jupiter Proteo 90Å 150 mm $\times$ 21.2 mm 10- μ m RP-HPLC column at 15 mL/min flow rate. After baseline monitoring in 0% B for 10 minutes to remove guanidine, the product was eluted using a linear gradient of 0–20% B in 30 minutes. The major peak is collected and the

identity confirmed by mass spectrometry. Pure fractions are split between two pre-weighed 20 mL glass vials and lyophilized, yielding 30 mg pure adapter reagent (33% yield). Aminoxy adapter molecules are stored at -20°C until use. Azide adapter molecules are promptly conjugated to the chosen maleimide dye and then can be stored at -20°C .

Adapter/Maleimide Coupling: General Procedure—One mL of a 5 mM solution of Aoa-linker-Cys **2**, or N_3 -linker-Cys **3** molecule in 6M GuHCl 0.1M MES pH 5.2 was added to 1 mg of maleimide dye (**1a–1d**) for 30 minutes followed by RP-HPLC purification. For recovery calculation, 1% of the starting maleimide solution **1b**, was analyzed by UV/Vis spectroscopy. Following RP-HPLC purification, 1% of the resulting compound solution **4b**, was analyzed by UV/vis and compared to the starting solution. Adapter reagents were characterized by RP-HPLC and electrospray mass spectrometry as illustrated by analysis of **5a** (Supporting Information Figure S2) in supporting information. Molecular masses obtained by electrospray mass spectrometry were as follows: 1103 amu for **4a** (1103 g/mol calc. ave. iso.), 1160 amu for **4b** (1160 g/mol calc. ave. iso.), 1455 amu for **4c** (1455 g/mol calc. ave. iso.), and 1465 amu for **5c** (1465 g/mol calc. ave. iso.). The structure and molecular weight for **1d** are not provided by Invitrogen, however, the observed mass (**4d** = 1444 amu) and spectroscopic properties are consistent with an Alexa-type fluorophore of MW 982 g/mol.

pAc-Phe PrP Expression—Mutant murine PrP was produced using methods described previously (21). Briefly, the amber codon (TAG) was introduced into full length mouse PrP(23-230) sequence using the GeneArt Site Directed Mutagenesis kit (Invitrogen). This plasmid was then co-transformed into BL21(DE3) cells (Invitrogen) along with the pEVOL plasmid specific for *para*-acetylphenylalanine (pAc-Phe) incorporation.²² Growth media was supplemented with 400 mg/L pAcPhe and grown to an OD_{600} of 0.8 before induction with 300 mg/L, L-Arabinose and 1 mM IPTG.

After cell lysis, PrP was solubilized from inclusion bodies in 8M urea buffer at pH 8 and purified by Ni^{2+} -charged immobilized metal affinity chromatography with elution into 5M guanidinium buffer. The protein was further purified by reverse phase high-performance liquid chromatography using a C4 column and was identified by mass spectrometry.

Protein Labeling—To 100 μM MoPrP(23-230)N180pAcPhe in 25 mM MES pH 6 containing 10 mM anisidine (Sigma) was added a 10-fold molar excess of aminoxy-adapter-AlexaFluor 488 **4b**. The reaction was placed in the dark and allowed to react at 37°C for 22 hours. The reaction products were run on a 4–20% gradient Tris/Glycine SDS-PAGE gel (Biorad) and analyzed with a Typhoon Imager (Amersham) with direct excitation at 532 nm and fluorescent detection through 526 nm short-pass (488 fluorophore) and 580 nm band pass (ladder) emission filters. Total protein was visualized by Coomassie staining. For generation of the composite fluorescence image the GIMP software package was used. The CCD image produced by exposure with 580 nm filter was made transparent and remaining pixels colored and superimposed onto the 526 nm photo treated in identical fashion, with the exception of green color. The resulting picture was copied onto a black background and

brightness/contrast adjusted. The coomassie stained gel and composite fluorescence were from separate experiments.

Peptide Copper Azide Alkyne Cycloaddition Labeling—Separate stock solutions of each reagent were prepared in water or DMSO as described. We prepared a solution of 10 mM peptide KETAAAKFEKQHXSSTSA (X=propargyl-glycine) in DMSO, 50 mM N_3 -adapter-dye **5c** in DMSO, and 40 mM Cu(I)Br (Aldrich) in DMSO, 100 mM sodium ascorbate in water and 100 mM aminoguanidine in water. A 1:1:1 (10 μL each) mixture of copper, ascorbate, and aminoguanidine was made, and allowed to stand for 5 minutes. To this solution, alkyne peptide (100 μL) and azide-adapter-dye **5c** (10 μL) were added followed by addition of DIEA (1.5 %, 2 μL), and lutidine (0.75 %, 1 μL). Final reaction conditions were 7 mM peptide, 3.5 mM **5c**, 3 mM CuBr, 7 mM Ascorbate, 7 mM aminoguanidine, and 86% DMSO. The reaction proceeded for 30 minutes and the resulting mixture was analyzed by RP-HPLC/ESI-MS.

RESULTS

The site of *p*-AcPhe substitution can be influenced by solvent exposure of the parent amino acid and experimental objective (i.e. smFRET, copper quenching, anisotropy, etc.). To facilitate spectroscopic studies, asparagine 180 (Figure 1b), a site for potential N-linked glycosylation, was mutated to the ketone bearing unnatural amino acid *p*-AcPhe using amber suppression as has been previously published by the Millhauser group (21).

SPPS was chosen because of its synthetic flexibility, enabling the efficient assembly of customized linkers. For example, the linker length is easily varied by incorporation of glycine, β -alanine, etc., and a wide range of solubilizing amino acid residues are available. Originally adapters **2** and **3** were synthesized using Boc-SPPS with hydrogen fluoride cleavable methyl benz-hydrylamine resin, but the general strategy is easily amenable to the more common Fmoc/tBu protection strategy on a TFA labile Rink amide AM resin as described in Methods for adapter **2**. Maleimide reagents were either purchased from invitrogen or synthesized as described in SI. Analytical data for custom maleimides is presented in Supporting Information Figure S3.

An advantage of this SPPS approach is that customized linkers can be efficiently generated to provide the desired linker length and hydrophlicity. In this case, a moderate length linker was chosen to ensure an unhindered aminooxy reactive group while maintaining the utility of the dye as a Forster resonance energy transfer (FRET) probe. Indeed, there have been reports of FRET studies where the biomolecule has been labeled with GFP analogues,²³ a > 20 kDa protein, suggesting a wide tolerance of size to FRET efficiency. Reaction of aminooxy or azide compounds with maleimide reagents was performed in solution to maximize expediency and minimize expense (Figure 2). Typically the couplings were quantitative and the recovered yield was dictated by handling losses in RP-HPLC purification. For example, an 88% recovery of Aoa-adapter-488 **4b** from an Aoa-linker-Cys **2**/Alexafluor 488 C5 maleimide **1b** reaction was obtained after HPLC purification based on total sample absorbance at 488 nm (Supporting Information Figure S4).

Preservation of the natively folded helical conformer of PrP is paramount, in particular it is necessary to avoid the well-known scrapie-like folding intermediate that is prevalent at low pH,²⁴ this was accomplished by labeling with 100 μ M protein in 25 mM MES pH 6.0 for 22 hours at 37°C containing 10 mM anisidine catalyst²⁵ using just 1 mM adapter-dye reagent. Fluorescence detection of SDS-PAGE reveals a major band consistent with conjugation of the Alexafluor 488 dye (Figure 3). Fluorescence increased until 22 hours of reaction time (Figure S5) and LC-ESI-MS indicated that the labeling reaction proceeded to greater than 95% conversion (Figure 4).

The modular design and SPPS assembly of the adapter lends itself to the straightforward generation of new variants by changing the ligation group, spacing and solubility elements. To illustrate this flexibility, azide compounds of the form N₃-linker-Cys were created to demonstrate the generality of the method. Maleimide dye coupling reactions were identical to those used for amino oxy reagents and despite the presence of a Cys, no azide reduction was observed during isolation or conjugation. However, the crude synthetic product contained a substantial amount of disulfide homodimer, and if the purified reagent is not promptly conjugated to a maleimide, breakdown will occur. Copper assisted azide alkyne cycloaddition²⁶ of N₃-adapter-lissamine rhodamine B **5c** to a test peptide KETAAAKFEKQHXDSSTAA containing propargylglycine (X) yielded the expected molecular weight for the triazole linked product (Figure 5).

DISCUSSION

The chemoselective labeling of biological macromolecules and nanoparticles²⁷ with precious reagents such as fluorophores is broadly utilized throughout the biochemical, and materials sciences. The diversity of these applications is matched by the recent development of new reactive groups/ligation chemistries that have their distinct advantages when applied to macromolecular conjugation. In contrast, there is a more limited set of fluorophores suitable for conjugation that can be readily obtained by the researcher. Our adapter approach is designed to help the individual researcher customize commercially available reagents to better meet the specific requirements of the material or bioconjugate being investigated.

Formation of an oxime is optimally suited to low pH, where the guanidino group of arginine is exceptionally well solvated, however, other ligation chemistries function at elevated pH. In such a case carboxylic acids like the side chain of aspartate or glutamate may be desired and are readily substituted during SPPS. The experimenter, with little effort, could tune the modular properties of the adapter molecule to suit the needs of both protein and ligation chemistry. For example hydrazine, acrylate, strained alkynes, and dienophile based, conjugation chemistries²⁸ could be incorporated and linker length adjusted (*vide supra*).

To demonstrate the practical utility of these adapters, we applied them to the site specific fluorescent labeling of PrP. With a technique for making homogeneous preparations of dye labeled PrP in hand, experiments are in progress to chemo-selectively label at a second position for smFRET studies. Using the adapter approach one could envision making the linker region even more soluble by substituting aminocaproic acid with mini-polyethylene glycol, switching the linker-Cys position to generate a fluorophore with a shorter linker for

added sensitivity to protein anisotropic motion, and coupling maleimide DOTA GA (CheMatech) to chelate lanthanide ions for use in time resolved fluorescence,²⁹ double electron electron resonance, and magnetic resonance imaging.

While the present work focuses on the modification of *p*-AcPhe residues, there are multiple protocols for interjecting the aldehyde/ketone functionality into proteins compatible with these adapters. Solulink couples carboxybenzaldehyde derivatives to amino groups on proteins.³⁰ Redwood Bioscience introduces the formylglycine residue via an enzyme acting on a recognition sequence.³¹ N-terminal aldehydes and ketones can be introduced through the use of N-terminal transamination by pyridoxal phosphate,³² Cu⁺⁺/glyoxylate³³ or through periodate oxidation of N-terminal Ser/Thr residues.³⁴ Any of these methods can be used in concert with the aminooxy-adapter molecules described here for chemoselective labeling.

In summary, we have developed a water soluble adapter molecule which essentially transforms a cysteine reactive maleimide fluorophore to an aldehyde/ketone reactive aminooxy fluorophore. Adapter reagents are not restricted to these two chemistries as demonstrated by the azide adapter for use in copper azide alkyne cycloaddition (CuAAC) ligations/click chemistry applications. Alteration of dye attachment point by replacing cysteine with diaminopropionic acid presents the ability to employ the amine reactive fluorophores. The approach is general and many thousands of reporter molecules are conceivably adapted to the protein conjugation chemistry of choice derived from a few simple reagents and simple application of SPPS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

(a) Adapter reagents synthesized in this study. The maleimide dyes were coupled to the thiol of adapter reagent. Adapter dye **4b** was conjugated to prion protein, while **5c** was ligated to a test peptide. **1a** = 7-hydroxycoumarin maleimide, **1b** = Alexafluor 488 C5 maleimide, **1c** = lissamine rhodamine b maleimide, **1d** = Alexafluor 647 C2 maleimide. Compound **5a** was also prepared by reacting **1a** with **3**. Colors of **1a–1d** reflect fluorophore emission wavelengths. (b) Aniline catalyzed oxime ligation of mouse prion protein 123-225 Asn180pAcPhe, 23-122 and 226-230 are not shown because these residues are not in the original structure file. Model based on the solution NMR structure (1AG2.pdb). The structure of MoPrP is presented as a cartoon with *p*AcPhe180 as a stick model (Pymol, Delano Scientific).

FIGURE 2.

HPLC chromatogram of a crude, half hour reaction between Aminoxy-linker-Cys **2** and maleimide-647 **1d** forming **4d**, all of **1d** is consumed. Gradient 0–70% B in 30 minutes. Phenomenex Jupiter Proteo 4.6×150 mm, $4 \mu\text{m}$, $90 \text{ \AA}$.

- 1 west C precision
plus (biorad) ladder
2 wild type MoPrP 23-230
3 Aoa-adapter-488 (**4b**) labeled
MoPrP 23-230 Asn180pAcPhe

FIGURE 3.

SDS-PAGE analysis of 22 hour ligation reaction between MoPrP 23-230 Asn180pAcPhe and Aoa-adapter-488 **4b** (100 μ M protein, 1 mM adapter **4b**, 25 mM MES, pH 6, 10 mM anisidine).

FIGURE 4.

RP-HPLC trace of reaction between Aoa-adapter-488 **4b** and MoPrP 23-230 N180pAcPhe and esi-ms reconstruct of labeled protein (expected MW = 24,278 g/mol) and unlabeled protein (expected MW = 23,136 g/mol).

FIGURE 5.

Reaction scheme of CuAAC reaction between N_3 -adapter-lissamine rhodamine b, 5c and alkyne peptide KETAAAKFEKQHXSSTSA (X- propargylglycine). RP-HPLC trace and ESI-MS spectra of CuAAC reaction between N_3 -adapter-lissamine rhodamine b, 5c and alkyne peptide. (a) Alkyne peptide (expected MW = 2102 g/mol $MH_2^+ = 1052$, $MH_3^+ = 701.6$), and (b) "clicked" triazole product (expected MW = 3567 g/mol $MH_2^+ = 1784.5$, $MH_3^+ = 1190$, $MH_4^+ = 892.8$, $MH_5^+ = 714.4$).