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Site-specific Protein Bioconjugation via a Pyridoxal 5'-Phosphate-Mediated N-Terminal Transamination Reaction

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Abstract

The covalent attachment of chemical groups to proteins is a critically important tool for the study of protein function and the creation of protein-based materials. Methods of site-specific protein modification are necessary for the generation of well-defined bioconjugates possessing a new functional group in a single position in the amino acid sequence. This paper describes a pyridoxal 5'-phosphate (PLP) mediated transamination reaction that is specific for the N-terminus of a protein. The reaction oxidizes the N-terminal amine to a ketone or an aldehyde, which can form a stable oxime linkage with an alkoxyamine reagent of choice. Screening studies have identified the most reactive N-terminal residues, facilitating the use of site-directed mutagenesis to achieve high levels of conversion. Additionally, this reaction has been shown to work on a number of targets that are not easily accessed through heterologous expression, such as monoclonal antibodies.

Introduction

The generation of a bioconjugate that is modified in a single predicted site on the protein surface is a difficult task, but one that is often crucial for the success of a given application. Because it is frequently difficult to target a unique instance of a particular amino acid side chain, we have instead pursued the use of an N-terminal transamination reaction effected by the common biological cofactor pyridoxal-5'-phosphate (PLP) (Gilmore and Scheck et al., 2006). As shown in Figure 1, incubation with PLP converts the N-terminus of a protein into a ketone or an aldehyde group. This newly installed functionality can be further derivatized through the formation of a stable oxime bond using a diverse array of alkoxyamine probes. This bioconjugation scheme has been used for the site-specific modification of proteins with fluorophores (Scheck and Francis, 2007), polymer chains (Esser-Kahn and Francis, 2008), polymer initiators (Heredia and Maynard, 2007; Gao et al., 2009), and surfaces (Christman et al., 2007; Lempens et al., 2009). Due to its specificity for the N-terminal amino group, the PLP-mediated transamination reaction can be used in conjunction with other bioconjugation techniques, such as cysteine modification or expressed protein ligation, for dual protein modification (Esser-Kahn and Francis, 2008; Dedeo et al., 2009). PLP-mediated bioconjugation proceeds at mild pH and temperature, and is tolerant of a number of buffers and conditions. Peptide screening studies have shown that this reaction proceeds with high yield for a number of N-terminal residues (ala, gly, asn, glu, and asp, in particular), and to a lesser degree for others. As such, the reaction has often proven successful for native protein sequences, and in cases where it is not, standard molecular biology techniques can typically

be used to change the N-terminal sequence to obtain increased levels of conversion (Scheck et al., 2008).

Strategic Planning

As proteins with a variety of sequences, both known and unknown, have been successfully modified using PLP-mediated bioconjugation, a new target should initially be evaluated empirically. However, if the protein is recombinantly expressed, its suitability can be assessed according to the following guidelines. First, the identity and accessibility of the N-terminal residues should be taken into consideration. In general, we have found that buried and/or highly hydrophobic N-terminal sequences react poorly, most likely because they do not participate in imine formation with PLP. In these cases, we have found that the installation of one to three “spacer” residues can lead to enhanced reactivity (for a specific example, see Scheck et al., 2008). The reaction is also not appropriate for proteins (such as actin) that have been acylated at the N-terminal amino group through post-translational modifications.

The influence of the N-terminal residue on the reaction outcome has been illuminated by recent studies (Scheck et al., 2008). As shown in Figure 2, a library of tetrapeptides including all 20 natural amino acids as the N-terminal residue was exposed to the transamination/oxime formation sequence under identical reaction conditions (10 mM PLP, 18 h, rt). The resulting products included the desired oxime product, keto-peptides that formed but did not participate in subsequent oxime formation, and covalent adducts of PLP to the N-terminal group. As shown in the figure, the N-terminal residues can be grouped by yield into the categories of *high conversion*, *intermediate conversion* or *byproduct formation*. The *high conversion* category includes alanine, glycine, aspartate, glutamate, and asparagine, which were found to result in the highest yields of oxime product. Cysteine, arginine, threonine, tyrosine, leucine, serine, methionine, phenylalanine, and valine all gave intermediate levels of conversion, and we have found that proteins bearing these N-terminal groups are less predictable in terms of the product yields. The *byproduct formation* category includes various species of byproducts found from N-terminal glutamine, histidine, tryptophan, lysine and proline residues. Glutamine was found to transaminate to produce a ketone, but this structure was resistant to further oxime formation. Tryptophan and histidine undergo a previously reported Pictet-Spengler reaction (Li et al., 2000). Lysine and proline each form unique types of PLP adducts, and in these cases the formation of the covalent PLP adducts does not preclude oxime formation (although they have been found to be slightly less reactive than the keto groups formed on the N-termini). The lysine-terminal PLP adduct forms from transamination followed by the loss of water to form a cyclic imine. The nucleophilic enamine tautomer of this structure undergoes aldol addition to additional molecules of PLP to form a covalent adduct. The proline-terminal adduct observed is the product of an unexpected ring-opening reaction, which leads to the formation of a covalent PLP adduct.

The intended application and empirical analysis of new target proteins will dictate the need for mutagenesis. In many cases, sufficient reactivity can be achieved using the native terminus, and in some cases lower levels of conversion are tolerable for the application (e.g.

surface attachment or radiolabeling). In other cases, standard molecular biology protocols can be used to extend and change the N-terminus of proteins that terminate with *intermediate conversion* or *byproduct formation* residues. As noted above, extending the N-terminus by one to three residues at the same time as converting the N-terminal residue to one of high reactivity may improve the outcome by increasing the accessibility of the N-terminus. Similarly, if a protein that already terminates in a *high conversion* residue is found to have low yield, mutagenesis to extend the N-terminus and increase accessibility may help to improve the reactivity. If the location of the modification on the protein is crucial, circular permutantation has been used in one case to change the location of the N-terminus (Dedeo et al., 2009). It has been found that the second and third residues can also influence the reactivity of the N-terminal residue, and studies are underway to clarify these synergistic effects.

Basic Protocol

Protein Labeling via a Site-Specific N-terminal Transamination Reaction

This protocol divides the process of conjugating a chemical group of interest to the N-terminus of a protein into two stages. The first step is the PLP-mediated transamination, which installs a reactive keto group that can be used for further derivitization. The second step of the protocol describes typical conditions for the reaction of this site with a small molecule alkoxyamine to form an oxime linkage, which is stable under physiological conditions. A similar strategy may be used to attach hydrazide-functionalized compounds to form a hydrazone linkage, although these groups are more labile (Kalia and Raines, 2008). The analysis technique used to verify the modification will vary according to the chemical moiety being conjugated to the protein: the attachment of small molecules can be confirmed using mass spectrometry, while the attachment of fluorescent or high molecular weight compounds can be detected by UV-Vis spectrometry, HPLC, or SDS-PAGE. If the bioconjugation probe is in short supply or is difficult to detect using these analytical techniques, a proxy alkoxyamine reagent (such as commercially-available benzyloxyamine or aminooxyacetic acid) can be used during the optimization of the transamination segment of the protocol.

Materials—Target protein, dissolved in pH 6.5 phosphate buffer

PLP stock solution, freshly prepared and adjusted to pH 6.5

37 °C water bath

Materials for size exclusion separation, such as dialysis tubing or cartridges, centrifugal filters for buffer exchange, or gel filtration cartridges for size exclusion purification

Materials for reverse phase separation in the case of low-molecular weight proteins

Alkoxyamine solution

Analysis of protein modification may involve:

ESI- or MALDI-TOF mass spectrometry

Additional reagents and equipment for SDS-PAGE, UV-Vis, or HPLC

Stage 1: PLP-mediated N-terminal transamination

1. Combine the protein solution and the PLP stock solution in a 1.5 mL Eppendorf tube to give a final concentration of 10 mM PLP and 10 to 500 μ M protein at pH 6.5.

Carefully check the pH of this solution. If the pH of the PLP stock was not adjusted (see Reagents and Solutions section) this solution may be overly acidic, leading to protein precipitation or suboptimal levels of conversion.

2. Incubate at 37 °C for 4–20 h.

These conditions represent the standard conditions for most proteins, and can be used as a starting point for optimization with each new protein target. Factors that may be varied include the concentration of PLP, the reaction temperature, the incubation time, and the pH. The reaction may be complete in as little as an hour, typically when a higher concentration of PLP (up to 100 mM) is used. The stability of the protein may dictate the range of acceptable temperature and pH conditions. Consult the Troubleshooting section for details on how these parameters may affect reaction outcome.

3. Remove PLP using one of a variety of size exclusion methods (such as dialysis, buffer exchange, or gel filtration), capitalizing on the difference in the mass of the protein relative to PLP. If the target protein or peptide has a low molecular weight, reverse phase separation can be used.

If excess PLP is not removed, the aldehyde of the PLP molecule will quench the keto-reactive group in the next step, significantly lowering its effective concentration.

Stage 2: Derivatization via oxime-formation

1. Using a freshly prepared solution of keto-protein, combine with the alkoxyamine solution such that the alkoxyamine is in 10–1000x molar excess of the protein.

Since oxime formation reactions occur at an optimal pH of 4.5 (Jencks, 1959; Carey and Sundberg, 1977), prepare the alkoxyamine solution such that the final pH will be as acidic as possible within the stability requirements of the protein. For larger proteins, we commonly carry out this reaction step between pH 5.5 and 6.5. If the protein is not stable at acidic pH, or if it is not possible to use a large excess of alkoxyamine, then aniline or anisidine catalysis of oxime formation may be used to increase the reaction rate (Dirksen et al., 2006; Dirksen and Dawson, 2008).

2. Incubate at room temperature for 18–24 h.
3. Analyze conversion and remove excess alkoxyamine, if necessary.

If analyzing by mass spectrometry, small amounts of PLP adduct species may be observed. For identification of common product masses, including those with PLP, consult the Troubleshooting section.

Reagents and Solutions

Protein Stock Solution—The protein stock solution should be prepared in the buffer in which the transamination reaction will be run. The standard buffer is 10 to 50 mM potassium phosphate at pH 6.5; however other buffers, such as Tris and HEPES, have been found to work as well (Gilmore and Scheck et al., 2006). The presence of glycerol and other polyalcohols should be avoided, as they may form acetals with the PLP aldehyde group and thus lower its effective concentration. Note that many spin concentrators are packed in glycerol solutions, and can serve as unexpected sources of this impurity.

PLP Stock Solution—The PLP stock solution should be prepared immediately before use, as this compound has been reported to degrade in aqueous solution (Ball, 2006). The PLP solution should be made in the same buffer in which the transamination reaction will be run, typically 10 to 50 mM phosphate buffer at pH 6.5. After the addition of PLP to the buffer it is important to check and adjust the pH, as the phosphate group of PLP may significantly alter the pH of the buffer solution. It is often convenient to make the PLP stock solution at two times the desired concentration for the reaction, assuming that it will be added to the protein solution in a 1:1 volume ratio. For a transamination reaction run with 10 mM PLP, the following guidelines may be followed to make 1 mL of a 2× (20 mM) PLP stock solution: *Add 5.3 mg of pyridoxal 5'-phosphate monohydrate from Sigma to 1 mL of 25 mM phosphate buffer, pH 6.5, followed by the addition of 24 μ L of 1 M NaOH and brief sonication and vortexing. Check the pH with pH paper or a microelectrode and adjust to 6.5 if necessary.*

Alkoxyamine Solution—The alkoxyamine should be prepared as a concentrated stock solution. The storage conditions will vary with each individual reagent, but exposure to adventitious carbonyl groups, such as acetone, should be strictly avoided. Commercially available alkoxyamines include small molecules and dyes, and the nomenclature may include the names “hydroxylamine”, “aminooxy” or “alkoxyamine”. Small molecule alkoxyamines our group has used to determine conversion of a protein by mass spectrometry include benylalkoxyamine (*O*-benzylhydroxylamine hydrochloride) and (aminooxy) acetic acid (*O*-(carboxymethyl) hydroxylamine hemihydrochloride). Poly(ethylene glycol) alkoxyamines can be synthesized (Schlick et al., 2005) for measurement of protein conversion by SDS-PAGE shifts.

Commentary

Background Info

Protein bioconjugation is used to study proteins in their biological context through fluorophore attachment (Griffin et al., 1998; O'Hare et al., 2007; Cravatt et al., 2008), improve their efficacy as therapeutic agents by conjugation to polymer chains (Zalipsky, 1995; Baker et al., 2006), and enable the construction of new types of protein-based

materials (Wang et al., 2002; Christman et al., 2007; Esser-Kahn and Francis, 2008; Abedin et al., 2009). A number of reactions exist to modify the functional groups on amino acid side chains (Hermanson, 1996); however, the product mixtures that result from the indiscriminate modification of multiple sites on the protein surface are unsuitable for many applications. To create well-defined protein bioconjugates with a better chance of preserving protein stability and function, reactions that can modify a protein a single time at a specific site are essential.

Site-specific protein modification presents a considerable challenge, considering the hundreds of polar spectator groups that are present on any given protein surface. Selectivity must be achieved without the use of protective groups, and the reaction must proceed in aqueous solution under mild pH and temperature conditions. Amino acids that have a low natural occurrence on protein surfaces offer better opportunities for site-specific protein bioconjugation, as a single copy can often be introduced through genetic manipulation. Cysteine is widely used for this purpose, and can be modified using many commercially available maleimide and iodoacetamide reagents (Hermanson, 1996). In cases where a uniquely reactive cysteine cannot be introduced, reactions targeting tryptophan and tyrosine residues can serve as useful alternatives (Joshi et al., 2004; Tilley and Francis, 2006; Antos et al. 2009; Ban et al., 2010).

The C-terminus of proteins can also serve as a site for selective modification through Native Chemical Ligation. In this technique, proteins expressed or synthesized with a C-terminal thioester react chemoselectively with cysteine derivatives (Dawson et al., 1994; Muir, 2003). Additionally, a wide range of bioorthogonal chemistry is accessible by incorporating non-natural amino acids bearing alkynes and azides for Huisgen cycloaddition chemistry (Xie and Schultz, 2006; Strable et al., 2008), or aniline amino acid derivatives for oxidative coupling reactions (Hooker et al., 2006; Carrico et al., 2008; Tong et al., 2009).

To target a single protein in complex mixture, enzymatic labeling reactions targeting specific recognition sequences can be highly useful. This can be achieved using biotin ligase and formylglycine-generating enzymes, which have been used to install ketones and aldehydes, respectively, on their recognition sequences for subsequent reaction with hydrazine or alkoxyamine probes (Chen et al., 2005; Carrico et al., 2007). Another strategy is the direct conjugation of functionalized substrates to the recognition sequence, as seen with bacterial sortases that capitalize on the tolerance of the enzymes for non-natural oligoglycine substrates (Mao et al., 2004). Guanosine derivatives can also be used to modify specific domains of fusion proteins using the “SNAP” tagging strategy (Keppler et al., 2003). These reactions provide just a few examples of the rapidly growing set of enzymatic labeling techniques that are now available (O’Hare et al., 2007; Sletten and Bertozzi, 2009).

While the above strategies apply to proteins that can be genetically manipulated and heterologously expressed, chemical reactions that target the N-terminus can, in principle, be used on proteins from virtually any source. A number of chemoselective reactions exist for the modification of specific N-terminal residues, such as the sodium periodate cleavage of N-terminal serine or threonine residues, followed by derivatization of the resulting aldehyde group (Geoghegan and Stroh, 1992) or the condensation of aldehyde reagents with N-terminal tryptophan residues to form Pictet-Spengler products (Li et al., 2000). As a

complement to these strategies for specific N-termini, N-terminal transamination reactions can successfully convert a number of different residues into reactive keto groups. This can be accomplished using copper(II) ions and glyoxylic acid (Dixon and Fields, 1972; Dixon, 1984), or by using the PLP-mediated transamination reaction described in this protocol. The latter reaction proceeds under particularly mild reaction conditions and does not require the use of metal ions. It has been successfully used to modify a number of protein targets, including GFP (Gilmore and Scheck et al., 2006), monoclonal antibodies (Scheck and Francis, 2007), viral coat proteins (Scheck et al., 2008), metallothioneins (Esser-Kahn et al., 2008), and others.

Critical Parameters

It is important that the PLP solution is prepared according to the guidelines in the Reagents and Solutions section. Using a fresh solution of PLP, checking and adjusting the pH, and storing the solid PLP at 4 °C until use will yield optimal results. As noted above, glycerol and other polyhydroxylated compounds should be removed before the transamination step. It should be noted that many commercially available spin concentrators are packed in glycerol solutions. Adventitious ketones (such as acetone) should be avoided when handling the alkoxyamine compounds.

Troubleshooting

Regardless of N-terminal residue identity, it is possible to vary the reaction conditions for each protein target to optimize conversion. A proxy alkoxyamine that is commercially available and/or readily analyzed can be used while the optimal conditions are being screened for a new target protein. See the Reagents and Solutions section for examples. To confirm the compatibility of a protein to the overall reaction conditions, it is often beneficial to run a control reaction that lacks PLP, but in which all of the other parameters (including the addition of alkoxyamine) are held constant.

During the PLP-mediated transamination reaction, PLP aldol addition products can form from the reversible keto-enol tautomerization of the keto-protein product, as seen in Figure 3. Both species have been observed to form oximes upon exposure to the alkoxyamine reagent in the next step; however the presence of both intermediates creates a complex product mixture. If the goal is simply ligation of the alkoxyamine reagent to the protein the presence of the PLP adduct species may be tolerable, but if the PLP adduct is detrimental, the following changes in the reaction conditions can often be used to lower the amount that is formed.

As would be expected for a bimolecular aldol reaction, we have found that the yield of the PLP addition product increases with higher PLP concentration and longer reaction times—two parameters that also influence the transamination yield. Thus, a balance between these reaction pathways must be struck. Higher concentrations of PLP (30 – 100 mM) with shorter reaction times (1 – 6 h) or lower concentrations of PLP (5 – 10 mM) with longer reaction times (8 – 20 h) are conditions that will help maximize the yield of the desired oxime product while keeping PLP addition products to a minimum. The optimal strategy will

depend on the specific protein, so exploring multiple sets of conditions is recommended during screening.

The following table lists suggested strategies for overcoming potential issues encountered during PLP-mediated bioconjugation.

Problem	Possible Cause	Solution
Poor conversion to oxime product	Low yield of transamination reaction	<ul style="list-style-type: none"> • Increase the concentration of PLP, screening from 10 mM to 100 mM • Increase the incubation time or the temperature of the transamination reaction
	Low yield of oxime formation reaction	<ul style="list-style-type: none"> • Increase the equivalents of alkoxyamine • Lower the pH during oxime formation step • Use aniline catalysis, as reported by Dirksen et al., 2006 • Check quality of alkoxyamine reagent by NMR
High conversion to undesired PLP adduct	Byproduct formation	<ul style="list-style-type: none"> • Use lower concentrations of PLP • Use shorter reaction times • To identify common byproducts, see the table of common mass adducts
Nominal reactivity	Inaccessible or unreactive N-terminal residues	Consider mutagenesis
Protein precipitation	PLP solution is too acidic	Check and adjust pH of PLP solution before mixing with protein
	Protein insolubility	The transamination reaction can be run under buffer, pH, and temperature conditions that vary from those listed in the Basic Protocol. Alter these parameters as dictated by the stability of the target protein

When analyzing the mass spectrum of a PLP-modified protein, the following table can be used to identify the observed species. In some cases, a species may produce more than one of these mass changes (e.g. a PLP aldol addition product may also form an oxime).

M = Unmodified protein mass. Alk = Alkoxyamine mass.

Observed Mass	N-terminus	Possible Product
M – 1	Any	Keto-protein
M + Alk – 19	Any	Oxime
M – 44	Asp	Decarboxylation (still can form oxime)
M – 16	Ser	Beta elimination (still can form oxime)
M – 32	Cys	Beta elimination (still can form oxime)

M + 247	Any	PLP aldol addition
M + 229	Any	PLP aldol addition with dehydration
M + 229	His, Trp	Pictet-Spengler addition of PLP
M – 19	Lys	Cyclic enamine
M + 293	Pro	Ring opening with PLP addition

Anticipated Results

For many protein targets, optimization of the basic protocol can result in 50–90% conversion to desired oxime product. Site-directed mutagenesis may improve the conversion of initially unreactive target proteins. The mild reaction conditions are not expected to denature most proteins, and in most cases we have found that they retain their desired activity after modification.

Time Considerations

N-terminal protein modification using PLP-mediated transamination and subsequent oxime formation can be completed in three days with minimal hands-on time. On the first day, the PLP transamination reaction is started, and allowed to incubate overnight. On day two, the excess small molecule is removed and the oxime forming reaction is started. After overnight incubation the conversion can be analyzed and the protein conjugate is ready for use. The incubation times of both the PLP transamination and the oxime formation may be reduced in some cases. Heating may reduce the conversion time for the transamination reaction, and an excess of alkoxyamine will increase the rate of the oxime formation step. Considering the small amount of active time, it is quite feasible to modify multiple proteins in parallel or to screen multiple reaction conditions at once. A gradient program on a PCR thermocycler is convenient for screening conditions.

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Figure 1.

Reaction scheme. **(A)** In the first step, the protein is incubated with PLP, which transaminates the N-terminus to form a ketone or an aldehyde. **(B)** In the second step, the keto-protein reacts with an alkoxyamine probe to form an oxime-linked protein bioconjugate. The amine groups of lysine residues may reversibly form imines with PLP, but they do not proceed through the transamination process. Thus, the protein is modified in a single, specific location using this procedure.

Figure 2.

Peptide studies on tetrapeptide XKWA, where *X* includes all twenty natural amino acids, show the role of the N-terminal residue on reactivity toward the PLP-mediated transamination reaction. The products include the desired benzyloxime product (shown in blue), the species that has undergone the transamination reaction to the keto-peptide species but did not continue to form an oxime (shown in green), and various species resulting from covalent PLP addition to the N-terminus (all shown as orange). The N-terminal residues are grouped into the categories of *high conversion*, *intermediate conversion* and *byproduct formation*.

Figure 3.

In addition to the desired keto-protein product, the PLP-mediated transamination reaction can also result in the formation of an N-terminal adduct with PLP. Shown here are the observed product structures for an alanine-terminal protein. Both intermediates can form oximes upon exposure to the alkoxyamine reagent in the second step. However, this results in a more complex product mixture. To maximize keto-protein yield while minimizing the aldol adduct, the recommended strategies are to use a high concentration of PLP for short times, or a low concentration of PLP for longer reaction times.