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Authors

Evans, Eric GB
Millhauser, Glenn L

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Genetic Incorporation of the Unnatural Amino Acid *p*-Acetyl Phenylalanine into Proteins for Site-Directed Spin Labeling

Eric G.B. Evans and **Glenn L. Millhauser**[1]

Department of Chemistry and Biochemistry, University of California Santa Cruz, Santa Cruz, California, USA

Abstract

Site-directed spin labeling (SDSL) is a powerful tool for the characterization of protein structure and dynamics; however, its application in many systems is hampered by the reliance on unique and benign cysteine substitutions for the site-specific attachment of the spin label. An elegant solution to this problem involves the use of genetically encoded unnatural amino acids (UAAs) containing reactive functional groups that are chemically orthogonal to those of the 20 amino acids found naturally in proteins. These unique functional groups can then be selectively reacted with an appropriately functionalized spin probe. In this chapter, we detail the genetic incorporation of the ketone-bearing amino acid *p*-acetyl phenylalanine (pAcPhe) into recombinant proteins expressed in *E. coli.* Incorporation of pAcPhe is followed by chemoselective reaction of the ketone side chain with a hydroxylamine-functionalized nitroxide to afford the spin-labeled side chain "K1," and we present two protocols for successful K1 labeling of proteins bearing site-specific pAcPhe. We outline the basic requirements for pAcPhe incorporation and labeling, with an emphasis on practical aspects that must be considered by the researcher if high yields of UAA incorporation and efficient labeling reactions are to be achieved. To this end, we highlight recent advances that have led to increased yields of pAcPhe incorporation, and discuss the use of aniline-based catalysts allowing for facile conjugation of the hydroxylamine spin label under mild reaction conditions. To illustrate the utility of K1 labeling in proteins where traditional cysteine-based SDSL methods are problematic, we site-specifically K1 label the cellular prion protein at two positions in the C-terminal domain and determine the interspin distance using double electron–electron resonance EPR. Recent advances in UAA incorporation and ketone-based bioconjugation, in combination with the commercial availability of all requisite reagents, should make K1 labeling an increasingly viable alternative to cysteine-based methods for SDSL in proteins.

1. INTRODUCTION

The combination of site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy has become a powerful biophysical tool that continues to find widespread use in the study of protein structure and dynamics (Fanucci & Cafiso, 2006; Hubbell, López, Altenbach, & Yang, 2013). SDSL-EPR involves the site-specific incorporation of one or more spin labels, typically in the form of stable nitroxide radical side chains, into the primary sequence of a protein of interest, which can then be probed using a

[1]Corresponding author: glennm@ucsc.edu.

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variety of EPR techniques. SDSL-EPR can provide information on local backbone dynamics and secondary structure (Mchaourab, Lietzow, Hideg, & Hubbell, 1996; Miick, Todd, & Millhauser, 1991), tertiary contacts (Mchaourab, Kálai, Hideg, & Hubbell, 1999), membrane protein topography (Altenbach, Flitsch, Khorana, & Hubbell, 1989), induced conformational changes (Altenbach, Kusnetzow, Ernst, Hofmann, & Hubbell, 2008), and global protein flexibility and conformational dynamics (López, Oga, & Hubbell, 2012). In systems possessing two spin labels, or a spin label and a second paramagnetic center such as a transition metal ion, EPR can often provide structural information by way of interelectron distance measurements (Eaton & Eaton, 2002). Because the EPR experiment is not inherently limited by the tumbling rate of the macromolecule, as is the case with solution NMR spectroscopy, SDSL is often employed in the study of large macromolecular assemblies that are difficult to crystallize, and has found particularly extensive use in the elucidation of structural and dynamic properties of membrane proteins (Cafiso, 2014; Mchaourab, Steed, & Kazmier, 2011).

One of the main limitations of SDSL-EPR studies of proteins is the requirement for a chemically orthogonal functional group at the site of interest. This is most often achieved through the introduction of a cysteine residue using standard recombinant DNA mutagenesis followed by reaction with a thiol-specific nitroxide such as the methanethiosulfonate spin label (MTSL) (Mchaourab et al., 1999). However, there are many systems for which this strategy is not feasible. If the protein of interest contains a large number of native cysteines, they must be systematically mutated out, leaving only the cysteine at the site of interest available for labeling, a process that can require substantial effort. The mutation of a large number of native cysteines will also increase the likelihood that one of the substitutions, either alone or in combination with other cysteine mutations, will disrupt the protein's native structure, stability, or function. Moreover, many proteins rely on native cysteines for critical functions such as enzyme catalysis, redox reactivity, and binding of essential metal-ion cofactors (Fomenko, Xing, Adair, Thomas, & Gladyshev, 2007; Giles, Giles, & Jacob, 2003). It follows that modification or mutation of these key cysteines must be avoided. Finally, an increasing number of proteins are now recognized as possessing conserved disulfide bonds (Lu et al., 2015). Although thiol-specific probes will generally not react with cysteines that are involved in a disulfide linkage, the addition of extra cysteines into recombinant proteins can potentially lead to aberrant, nonphysiological disulfide formation.

The cellular prion protein (PrP) serves as one example of the limitations of traditional thiol-based labeling techniques. PrP contains a conserved disulfide bond between Cys 179 and Cys 214 (Fig. 1A) that is critical for proper folding of the protein (Maiti & Surewicz, 2001). As shown in Fig. 1B, replacement of Val 188 of full-length mouse PrP with a cysteine residue results in the appearance of several species upon oxidative refolding that are resolved by HPLC. Analysis by mass spectrometry reveals that these species correspond to distinct disulfide connectivities. Clearly, SDSL studies of such a mixture of disulfide-linked products would be misleading and biologically irrelevant.

Recent innovations allowing for the genetic incorporation of unnatural amino acids (UAAs) into recombinantly expressed proteins have provided an elegant alternative to cysteine-based labeling methods. Fleissner et al. reported the first example of SDSL employing UAAs with

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the incorporation of the ketone-bearing amino acid *p*-acetyl phenylalanine (pAcPhe) into T4 lysozyme, followed by reaction with a hydroxylamine-functionalized nitroxide to generate the covalently linked side chain spin label "K1" (Fig. 3A) (Fleissner et al., 2009). Subsequent studies have demonstrated the spin labeling of azide-bearing amino acids using the copper-free azide-alkyne cycloaddition "click" chemistry (Kálai, Fleissner, Jekő, Hubbell, & Hideg, 2011) and, more recently, the direct incorporation of an amino acid containing a stable nitroxide as part of the side chain, thereby eliminating altogether the need for a subsequent labeling reaction (Schmidt, Borbas, Drescher, & Summerer, 2014). However, due to the high fidelity and efficiency of pAcPhe incorporation, as well as the commercial availability of both the UAA and the hydroxylamine spin label, K1 incorporation remains the most common and widely applicable of the UAA spin-labeling methods to date.

In this chapter, we describe the method of site-specific incorporation of pAcPhe into recombinant proteins expressed in *E. coli* (Section 2). We then outline two conditions for the successful reaction of pAcPhe-containing proteins with the hydroxylamine-functionalized nitroxide HO-4120 (Section 3) to generate the spin-labeled side chain K1. To illustrate the utility of this approach, we site-specifically K1 label the cellular prion protein (PrP^C) at two positions in proximity to the native disulfide bond connecting helices 2 and 3 of the globular C-terminal domain (Fig. 1A). In the final section, we give sample preparation procedures for the measurement of inter-nitroxide distances by double electron–electron resonance (DEER) EPR, and provide an example of interspin distance determination on doubly K1-labeled PrP.

2. SITE-SPECIFIC INCORPORATION OF pAcPhe

The genetic incorporation of amino acids other than the 20 canonical, or "natural," amino acids into recombinant proteins expressed in bacteria was pioneered in the laboratory of Dr. Peter G. Schultz at the Scripps Research Institute (Wang, Brock, Herberich, & Schultz, 2001). The technique involves the suppression of the amber codon, introduced site-specifically as a TAG codon substitution, by an orthogonal tRNA/ aminoacyl-tRNA synthetase (aaRS) pair specific for the UAA of choice (method reviewed in Xie & Schultz, 2005). To date, at least 70 different UAAs have been incorporated into *E. coli*-expressed proteins (Liu & Schultz, 2010), and the methodology has been further expanded to yeast (Chin et al., 2003) and mammalian cell (Liu, Brock, Chen, Chen, & Schultz, 2007) expression systems. Among the first functional groups to be added to the genetic code of *E. coli* using this method was the ketone functionality of pAcPhe (Wang, Zhang, Brock, & Schultz, 2003). Ketones are not present in natural proteins and readily react with hydrazides and hydroxylamines in aqueous solution to form covalent hydrazones and oximes, respectively. These properties have led to the development of pAcPhe incorporation as an effective alternative to cysteine-based methods for the site-specific labeling of recombinant proteins with fluorescent probes (Brustad, Lemke, Schultz, & Deniz, 2008), polyethylene glycol, and biotin tags (Cho et al., 2011; Hutchins et al., 2011), small molecule drug conjugates (Axup et al., 2012), oligonucleotides (Kazane et al., 2012), glycosylation mimetics (Liu, Wang, Brock, Wong, & Schultz, 2003), and nitroxide spin labels (Fleissner et al., 2009).

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The general scheme for incorporation of pAcPhe into *E. coli*-expressed proteins is outlined in Fig. 2. The technique requires two expression plasmids. The first, or primary, plasmid encodes for the inducible expression of the protein of interest containing an amber codon (TAG) at the desired site of pAcPhe incorporation. The secondary plasmid encodes the orthogonal tRNA/aaRS pair evolved for pAcPhe specificity under both constitutive and inducible expression (Young, Ahmad, Yin, & Schultz, 2010). The two plasmids are cotransformed into a suitable *E. coli* strain and cells possessing both plasmids are selected for using orthogonal antibiotics. The cells are then grown in media supplemented with pAcPhe and mutant protein expression is accomplished by simultaneous induction of the two plasmids.

2.1 Expression Plasmids and Cells for pAcPhe Incorporation

The plasmids used for incorporation of pAcPhe into *E. coli*-expressed proteins are shown schematically in Fig. 2. For the secondary plasmid (Fig. 2, top right), which encodes for the orthogonal tRNA/aaRS pair, we employ the pEVOL plasmid containing the evolved tRNA/aaRS pair specific for pAcPhe addition (Young et al., 2010). While pEVOL has been shown to provide superior mutant protein yields, we have also had success using the analogous pSUPAR-pAcPhe plasmid construct (Cellitti et al., 2008; Fleissner et al., 2009). The selection marker for the secondary plasmid is resistance to chloramphenicol (Cam), and it is therefore imperative that the *E. coli* strain used does not already carry a plasmid encoding Cam resistance. Some common *E. coli* strains that carry Cam resistance markers include those designed for the expression of highly toxic proteins, which typically harbor pLysS, pLysE, or pLacI vectors, as well as cell lines designed for higher efficiency expression of rare codons, such as Rosetta® and CodonPlus®. As such, these cell lines should be avoided. We use the robust and extremely common BL21(DE3) *E. coli* strain (Invitrogen) for protein expression with good results.

Other than the requisite amber codon mutation at the desired position of pAcPhe incorporation, the primary plasmid encoding the gene of interest (Fig. 2, top left) typically requires little, if any, modification from the plasmid already employed for recombinant expression of the protein of interest. There are two main requirements. The first is that the expression vector must carry a selectable resistance marker for an antibiotic other than Cam. Most commonly, this is either ampicillin (Amp) or kanamycin (Kan). The second requirement is that the gene of interest must be terminated with a stop codon other than TAG (amber), namely either TAA (ochre) or TGA (opal). This should be checked by DNA sequencing and, if the terminating codon is TAG, it must be altered using site-directed mutagenesis to either TAA or TGA. In addition to these two main requirements, it may be beneficial to encode a C-terminal affinity tag for purification purposes. Because amber suppression is never 100% efficient, one invariably ends up with recombinant proteins that are truncated at the site of TAG insertion. Having an affinity tag that is C-terminal to the protein of interest allows for the facile purification of the full-length protein from truncated by-products.

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2.2 Choice of Labeling Sites

Aside from the general avoidance of active site or otherwise critical residues, which will vary from protein to protein, there are a few things to keep in mind when choosing sites for pAcPhe incorporation. As with cysteine-based spin labeling, it is preferable to choose positions that are solvent exposed. Selecting for surface sites decreases the likelihood that the mutation will disturb the native structure of the protein and ensures that the spin label has sufficient access to the side chain for conjugation. If three-dimensional structures or models are available for the protein of interest, it is recommended to computationally model the spin label in order to predict whether or not the proposed site will accommodate the spin label with minimal perturbation to the protein structure. For the K1 label, this can be easily performed using the *in silico* spin-labeling program mtsslWizard (Hagelueken, Ward, Naismith, & Schiemann, 2012), which is available as a PyMOL plugin.

Although the mechanism is not fully understood, the identity of the nucleotides flanking the amber codon in the mRNA transcript is known to influence the efficiency of amber suppression (Bossi, 1983). A recent study examined this phenomenon by randomly varying the nucleotide sequences in the two codons immediately preceding (5′) and following (3′) the amber stop codon, and observing the affect on the incorporation efficiency of several UAAs expressed in *E. coli* (Pott, Schmidt, & Summerer, 2014). For pAcPhe incorporation, the authors found that the most efficient suppression occurred with the amino acid sequence Asn-Asn-TAG-Thr-Lys, with efficiencies for this optimized sequence approaching that of natural Tyr (no amber suppression) incorporation. This study also confirmed, as reported previously (Bossi, 1983; Pedersen & Curran, 1991), that efficient amber suppression has a strong preference for a purine nucleotide, particularly adenosine (A), at the site immediately 3′ to the amber (TAG) codon. These empirical findings can serve as a guide for choosing mutation sites that are likely to result in highly efficient amber suppression, and in cases where structural precision is not important, insertion of the entire Asn-Asn-pAcPhe-Thr-Lys sequence into a loop region may be a viable strategy for achieving extremely high incorporation efficiencies. However, for most SDSL applications where high positional accuracy is desired, the researcher may simply wish to limit mutation sites to those in which the DNA base following the amber codon is A or G.

2.3 Materials

- 37 °C incubator/shaker
- 42 °C water bath
- UV-Vis spectrophotometer (e.g., Cary UV-Vis (Varian))
- Refrigerated centrifuge with swinging bucket style rotor
- Autoclave for sterilization
- pEVOL-pAcPhe plasmid (available from Professor P.G. Schultz, Scripps Research Institute, La Jolla, CA)

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- pET101 (Invitrogen), pJ414 (DNA 2.0), or similar expression plasmid containing the gene for the protein of interest (see Section 2.1 for specific details)
- Chemically competent DH5α (Invitrogen) or similar *E. coli* cells for plasmid propagation and isolation
- Chemically competent BL21(DE3) (Invitrogen) or similar *E. coli* cells for expression of recombinant proteins
- Chloramphenicol (Cam)—37 mg/mL (1000 ×) stock solution in ethanol, stored in aliquots at −20 °C.
- Ampicillin, sodium salt (Amp)—100 mg/mL (1000 ×) stock solution in H_2O, stored in aliquots at −20 °C.
- LB growth medium
- LB-agar medium
- Sterile culture plates
- *p*-Acetyl phenylalanine hydrochloride (pAcPhe) (Synchem, Inc.)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)—1*M* (1000 ×) stock solution in H_2O, sterile filtered through a 0.2 μm membrane, stored in aliquots at −20 °C.
- L-(+)-Arabinose—200 mg/mL (1000 ×) stock solution in H_2O, sterile filtered through a 0.2 μm membrane, stored in aliquots at −20 °C.

2.4 Expression of Proteins Containing Site-Specific pAcPhe

The steps outlined below were found to be optimal for the expression of the full-length mouse PrP, MoPrP(23–230); however, we anticipate that this procedure will be applicable to the vast majority of recombinantly expressed proteins in *E. coli.* In particular, the induction temperatures and times for a given protein of interest may be different than those for PrP. The fact that pAcPhe has been incorporated into a wide variety of proteins suggests that expression procedures can be modified to suit the optimal conditions of most *E. coli*-expressed proteins, and this is supported by the observation that pAcPhe incorporation efficiency is unaffected by expression temperatures in the range of 23–37 °C when using the pEVOL suppression plasmid (Young et al., 2010). It should be noted that doubly transformed *E. coli* growing in the presence of both Amp and Cam may display a slowed-growth phenotype, and it therefore may take more time than expected for colonies to develop and for cultures to reach the optimal cell density for induction. The following procedure was published in Spevacek et al. (2013) and is presented here with minor changes.

1. Mutagenesis and DNA sequencing (2–5 days)

   Using a PCR-based mutagenesis method such as the QuickChange site-directed mutagenesis kit (Stratagene), mutate the codon at the desired site of pAcPhe incorporation to TAG (see Section 2.2 for additional details). Isolate plasmid DNA from several transformed colonies and confirm the presence of the TAG mutation in the gene of interest by DNA sequencing. While the DNA is being sequenced,

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prepare LB-Agar plates containing 100 μg/mL Amp and 34 μg/mL Cam and store in the dark at 4 °C until use.

2. Cotransformation into BL21(DE3) cells (1–2 days)

   Transform 10–50 ng of primary plasmid containing site-specific TAG and 10–50 ng of pEVOL-pAcPhe, each in 1 μL volumes, into BL21(DE3) or similar *E. coli* cells using standard transformation procedures for your specific *E. coli* strain. Plate the transformation reaction onto LB-Agar plates containing both Amp (100 μg/mL) and Cam (34 μg/mL), and incubate at 37 °C until suitably large yet well-dispersed colonies are observed (overnight to 32 h).

3. Inoculation of Overnight Starter Culture (~12 h)

   Select a colony from the transformation reaction displaying dual Amp/Cam resistance and inoculate into 10 mL of sterile LB medium containing 100 μg/mL Amp and 34 μg/mL Cam. Grow the culture overnight at 37 °C with shaking at 200 rpm.

4. Protein expression (7–8 h)

   Supplement 1 L of sterile LB medium in a shaker flask with 1 mL Amp stock (100 μg/mL), 1 mL Cam stock (34 μg/mL), and 400 mg pAcPhe HCl (~2 m*M*). Inoculate the medium with the 10 mL overnight *E. coli* culture and grow at 37 °C, with shaking at 200 rpm, until the cells reach mid log phase as determined by an absorbance of approximately 0.8 at 600 nm. Induce recombinant protein expression with the addition of 1 mL L-arabinose stock (0.02%w/v) and 1 mL IPTG stock (1 m*M*) and shake for an additional 3–4 h at 37 °C.

5. Cell harvesting (30 min)

   Chill cells on ice for 10 min before pelleting by centrifugation at 3500 rpm for 15 min in a swinging bucket style centrifuge at 4 °C. Discard the spent medium from the supernatant and store the cell pellet at −80 °C until lysis and purification.

6. Lysis and purification

   Lyse the cells and purify the recombinant protein of interest using procedures identical to those normally used. The procedure used for purification of recombinant PrP mutants has been described previously (Spevacek et al., 2013). The ketone side chain of pAcPhe is stable to reagents commonly employed in the purification of proteins from *E. coli*, including reducing agents (DTT, TCEP), metal ions (i.e., Ni^{2+}), denaturants (guanidine, urea), and detergents (Triton X, Tween). Purification and handling of pAcPhe-containing proteins therefore requires no additional precautions or special procedures.

3. SPIN LABELING OF PROTEINS CONTAINING SITE-SPECIFIC pAcPhe

Chemo-selective spin labeling of the ketone moiety of pAcPhe-containing proteins is achieved by way of nucleophilic substitution with the hydroxylamine-functionalized spin label HO-4120 to generate the ketoxime-linked side chain K1 (Fig. 3A). Although both

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hydrazide and hydroxylamine reagents will react with ketones in aqueous solution to form covalent linkages, hydrazone generation by the former class of molecules is quite inefficient, most likely due to the higher susceptibility of hydrazones to hydrolysis as compared to their oxime counterparts (Kalia & Raines, 2008). Therefore, hydroxylamine-functionalized probes are generally preferred over hydrazides. Oxime formation is acid-catalyzed and, as such, mildly acidic reaction conditions are required for efficient conjugation. Successful labeling of pAcPhe-containing proteins with a variety of different hydroxylamine probes has indicated that optimal reaction occurs at 37 °C at pH values of 4.0–4.5 (Brustad et al., 2008; Cho et al., 2011; Fleissner et al., 2009). In addition, a protein concentration of 100 μ*M* or greater is usually required for efficient oxime ligation. However, as will be discussed later, the use of appropriate catalysts can reduce the concentration of protein required and allow for effective reactions to be carried out in more neutral pH environments and at lower temperatures.

As mentioned earlier, efficient ligation of pAcPhe by hydroxylamine probes requires pH values of 4.0–4.5. This pH dependence has been a limiting factor in the widespread use of protein-labeling techniques based on site-specific pAcPhe, as many proteins are intolerant of acidic conditions. This is especially true when combined with the increased temperatures and extended incubation times (18 h or more) required for the oxime reaction to reach completion. An important development in oxime ligation strategies for biological use was the discovery that aniline could significantly increase the rates of both oxime and hydrazone formation in aqueous solutions at ambient temperatures (Dirksen, Dirksen, Hackeng, & Dawson, 2006; Dirksen, Hackeng, & Dawson, 2006). Furthermore, the use of the substituted aniline derivative *p*-methoxyaniline (*p*-OMeAn) was shown to efficiently catalyze oxime formation at neutral pH, thereby extending the applicability of site-specific ketone labeling to proteins that are acid-sensitive (Dirksen, Hackeng, et al., 2006). A more recent report suggests that *p*-phenylenediamine (*p*-PDA) may be moderately superior to *p*-OMeAn for the catalysis of oxime ligations at neutral pH and was also found to significantly accelerate reactions carried out at lower temperatures and with less concentrated protein samples (Wendeler, Grinberg, Wang, Dawson, & Baca, 2014). However, *p*-PDA was unsuccessful in catalyzing oxime ligation of the HO-4120 spin label in human sulfite oxidase and resulted in significant protein precipitation (Hahn, Reschke, Leimkühler, & Risse, 2014). In this case, catalysis with *p*-OMeAn was effective, suggesting that the choice of appropriate aniline-based catalyst may depend on factors specific to the particular protein of interest. Importantly, the *para*-substituted anilines appear to be effective at catalyzing oxime formation even at 4 °C, with *p*-PDA reportedly effecting the completion of oxime ligations at 4 °C within 6 h when reacted at pH 6 (Wendeler et al., 2014). These newly discovered catalysts might therefore prove useful in expanding the oxime-based labeling techniques to temperature-sensitive proteins such as enzymes.

3.1 Materials

- 37 °C water bath
- 1.5 mL amber microcentrifuge tubes
- Benchtop microcentrifuge

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- Reverse-phase HPLC system
- C4 analytical HPLC column (e.g., Vydac 214MS5415)
- Electrospray ionization mass spectrometer (ESI-MS)
- Labeling buffer 1: 10 m*M* sodium (or potassium) acetate, pH 4.0
- Labeling buffer 2: 10 m*M* 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.0
- HPLC solvent A: H_2O with 0.1% trifluoroacetic acid (TFA)
- HPLC solvent B: Acetonitrile with 0.1% TFA
- *p*-Methoxyaniline (*p*-OMeAn, aka *p*-anisidine) (Sigma Aldrich), as freshly prepared 4.0 *M* stock in acetonitrile.
- 3-Aminooxymethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*–pyrrol-1-yloxyl radical (HO-4120, Toronto Research Chemicals), as 1.0 *M* stock solution in anhydrous methanol, stored under inert atmosphere at −20 °C, protected from light.

3.2 K1 Labeling

The procedures described below are for the reaction of ~1 mg of purified recombinant PrP containing a single pAcPhe substitution in the amino acid sequence. Since full-length PrP is stable at moderately acidic pH in low-salt solutions, we typically carry out spin-labeling reactions in 10 m*M* acetate buffer at pH 4.0 using a 10-fold molar excess of the hydroxylamine spin label HO-4120, and this procedure is presented in Section 3.2.1. Under these conditions, we routinely achieve >95% labeling yields after 18–24 h at 37 °C as judged by HPLC-MS. In previous work, we have also employed aniline-based catalysis at pH 6.0 for the efficient conjugation of hydroxylamine-functionalized fluorescent probes to PrP constructs bearing site-specific pAcPhe (Thompson, Evans, Kasza, Millhauser, & Dawson, 2014). In Section 3.2.2, we describe a similar procedure for the reaction of the HO-4120 spin label with pAcPhe-containing variants of PrP, in which efficient labeling is achieved at room temperature in the presence of 10 m*M* *p*-OMeAn at pH 6.0.

3.2.1 Uncatalyzed Reaction at pH 4.0 (~24 h)

1. Using a thin-gauged needle or narrow glass pipette, bubble a fine stream of nitrogen gas through labeling buffer 1 for 5 min in order to reduce the amount of dissolved oxygen in the solution.
2. Add 430 μL deoxygenated labeling buffer 1 to 1 mg (~43 nmol) lyophilized PrP in an amber microcentrifuge tube at room temperature. Gently flush the headspace of the tube with nitrogen gas, cap, and allow the protein to fully dissolve (~30 min), tapping the tube periodically to mix. Alternatively, if lyophilization is not an option, exchange the purified protein of interest into labeling buffer 1 and concentrate to ~100 μ*M* as calculated from the absorbance and estimated extinction coefficient at 280 nm.
3. To the protein solution, add 0.5 μL HO-4120 spin label from the 1.0 *M* stock solution. Flush the headspace of the tube with nitrogen gas, cap, and mix well by

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gentle inversion of the tube. Seal the cap of the microcentrifuge tube by wrapping with parafilm, and incubate the reaction in a 37 °C water bath for 18–24 h.

4. Dilute the reaction to 1 mL with HPLC solvent A, mix by inverting the tube several times, and centrifuge at 13,000 rpm for 1–2 min.

5. Load the reaction mixture onto a reverse-phase C4 analytical HPLC column equilibrated in 95:5 HPLC solvent A:B and purify with an elution gradient of 80–65% solvent A in 45 min. Alternatively, excess spin label can be removed from the labeled protein by desalting into a buffer appropriate for the protein of interest using a PD-10 Sephadex G-25 (GE Healthcare) or similar size exclusion column, followed by several washes with fresh buffer through a membrane concentrator such as an Amicon Ultra Centrifugal Filter Device (EMD Millipore) with a suitable molecular weight cutoff for the protein of interest.

6. Analyze the HPLC fractions by electrospray ionization mass spectrometry (ESI-MS) operating in positive-ion mode. ESI-MS analysis of K1-labeled proteins will reveal mass additions of ~ 167 amu for each pAcPhe incorporated into the protein. This mass increase corresponds to the addition of HO-4120 (185 amu) and the concomitant elimination of a water molecule, as illustrated in Fig. 3A. Other methods of non-fragmenting ionization mass spectrometry appropriate for the analysis of large macromolecules (e.g., MALDI-TOF mass spectrometry) can also be used.

7. Pool the HPLC fractions containing pure spin-labeled PrP and lyophilize. Lyophilized proteins that will not immediately be used for EPR experiments should be stored at −20 °C, protected from light, in tubes that are capped and sealed with parafilm. If the protein of interest is not amenable to HPLC, buffer exchange into a suitable buffer for long-term storage or immediate analysis by EPR (see Section 4 for DEER EPR sample preparation).

3.2.2 Reaction at Ambient Temperature with *p*-Methoxyaniline Catalysis at pH 6.0 (~24 h)

1. Deoxygenate labeling buffer 2 by purging with nitrogen gas as described in Section 3.2.1.

2. Dissolve ~ 1 mg lyophilized PrP in labeling buffer 2 to a concentration of 100 μ*M*, or buffer exchange and concentrate to 100 μ*M* as described in Section 3.2.1.

3. Add 1.1 μL of *p*-methoxyaniline from a freshly prepared 4 *M* stock solution in acetonitrile (10 m*M* final concentration). Flush the headspace of the microcentrifuge tube with nitrogen, cap, and mix well.

4. Add 0.5 μL HO-4120 spin label from the 1.0 *M* stock solution. Flush the headspace of the tube with nitrogen, cap, and mix well by gentle inversion of the tube. Seal the lid with parafilm and incubate the reaction at room temperature for 12–24 h.

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5. Purify the labeled protein, and determine the success and extent of oxime ligation by mass spectrometry as detailed earlier for the uncatalyzed reaction (Section 3.2.1).

4. INTER-NITROXIDE DISTANCE MEASUREMENT IN DOUBLY K1-LABELED PrP BY DEER EPR

DEER EPR has become the method of choice for the measurement of long-range (~2–8 nm) interelectron distances in biological macromolecules (Jeschke, 2012). Although numerous studies have employed DEER to measure pairwise distances between paramagnetic metal ions in proteins (Astashkin, Rajapakshe, Cornelison, Johnson-Winters, & Enemark, 2012; Martorana et al., 2014; Merz et al., 2014; Yang et al., 2012), by far the most common usage of the DEER technique has been to measure distances between nitroxide spin labels introduced by way of SDSL. Much of the popularity of DEER stems from the ability to gain structural insight into biomolecules that are not amenable to analysis by X-ray crystallography or solution NMR. A key feature of the DEER technique is the ability to determine not only average interspin distances but also entire distance distributions. This feature often allows for the simultaneous detection of multiple conformations existing in dynamic equilibrium within a sample, and also gives an indication of their relative populations. Changes in these conformational distributions that occur with ligand binding or other modulations of protein structure can be readily observed with the DEER experiment (Mchaourab et al., 2011).

An in-depth description of the DEER technique that includes protocols for the collection and analysis of DEER data is beyond the scope of this chapter; however, the reader is referred to recent monographs detailing DEER theory and practice both at X-band (de Vera, Blackburn, Galiano, & Fanucci, 2013) and at the more sensitive and likewise commercially available Q-band (Duss, Yulikov, Allain, & Jeschke, 2015) frequencies. In this section, a detailed protocol for the preparation of spin-labeled protein samples for analysis by DEER at X-band frequency is given, followed by an example of interspin distance measurement by DEER on doubly K1-labeled PrP.

4.1 Materials

- 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, as 0.5 *M* stock solution, pH 7.4, filtered through a 0.2 μm membrane
- Glycerol, spectrophotometric grade (Sigma Aldrich)
- Liquid nitrogen
- Cryogenic Dewar flask or Styrofoam container
- 4 mm OD quartz EPR tubes (Wilmad 707-SQ or 714-PQ)
- E580 pulsed X-band EPR spectrometer with an ER4118X-MD-5 dielectric resonator and a second frequency DEER module (Bruker)

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4.2 Sample Preparation

In the context of spin-labeled proteins, DEER is a frozen-solution technique that is typically performed at cryogenic temperatures ranging from 50 to 80 K. As a consequence, protein samples require the presence of a cryoprotectant in order to avoid the formation of ice crystals upon freezing that can result in protein aggregation. Protein aggregation results in inter-molecular spin-label interactions that lead to dramatically reduced phase memory times (T_m) that preclude distance measurements by DEER. Most commonly, cryoprotection is accomplished by addition of 25–50% glycerol, by volume, to the protein sample prior to freezing. Ethylene glycol and sucrose are also commonly used as cryoprotectants for frozen-solution EPR studies of proteins.

In addition to being affected by protein aggregation upon freezing, the electron phase memory time (T_m) is also affected by the concentration of spin-labeled protein. While the EPR intensity, and hence the signal-to-noise ratio, typically increases with increasing concentration, progressively higher concentrations of spin-labeled protein will eventually begin to decrease the electron T_m owing to intermolecular dipolar interactions. This has the undesirable effect of decreasing the maximum interspin distance measurable by DEER. For X-band DEER on nitroxide-labeled proteins, a 200 μ*M* spin concentration (100 μ*M* doubly labeled protein) is generally considered to be a good compromise between signal-to-noise ratio and T_m effects (Jeschke & Polyhach, 2007). Conversely, increasing the electron T_m will increase the maximum distance observable in the DEER distribution. Such increases in T_m can be achieved by strongly diluting the solvent protons with deuterium using deuterium oxide (D_2O) and deuterated (d8)-glycerol (Cambridge Isotopes) (Jeschke, Bender, Paulsen, Zimmermann, & Godt, 2004). This approach may be worthwhile in systems where longer distance measurements and greater sensitivity are needed.

The procedure given below is typical for the preparation of DEER samples in protonated buffer for the measurement of inter-nitroxide distances in proteins using commercial pulsed EPR spectrometers at X-band (~9.5 GHz) frequencies.

1. Prepare a 1 mL stock buffer solution that is 75% glycerol (v/v) and 75 m*M* MOPS, pH 7.4 by combining 750 μL glycerol, 150 μL of 0.5 MMOPS buffer at pH 7.4, and 100 μL of water in a microcentrifuge tube. Note that glycerol is extremely viscous, and therefore obtaining accurate volumes requires special attention. Accurate volumes are best achieved through the use of a 1-mL glass volumetric pipette, ensuring that the glycerol is drawn up slowly and that the volume has completely equilibrated prior to removing the pipette tip from the liquid. If using a 1-mL micropipette with a disposable plastic tip, the glycerol will take a long time to fill the tip to the correct volume, and therefore the tip should be left below the surface of the liquid for a full minute after the plunger is released. The resulting buffer solution will be quite viscous and care must be taken to ensure that all components are completely mixed.
2. Dissolve the lyophilized, doubly spin-labeled protein in pure water to an approximate concentration of 150 μ*M* and allow to fully resolubilize at room temperature (~30 min). This concentration will be reduced by a third with the

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addition of buffer/glycerol, giving a final protein concentration of approximately 100 μ*M* (200 μ*M* spin).

3. To 70 μL of 150 μ*M* doubly K1-labeled PrP in H_2O in an amber microcentrifuge tube, add 35 μL of the glycerol/MOPS buffer stock, taking precautions to ensure accurate pipetting of the viscous solution, as discussed in step 1. Mix the sample well by pipetting up and down. This procedure will result in final buffer conditions of 25 m*M* MOPS, pH 7.4, and 25% (v/v) glycerol. Alternatively, the protein of interest can be concentrated to 1.25 × the final protein concentration using a membrane concentrator, followed by addition 100% glycerol to achieve a final glycerol concentration of 25% (v/v).

4. Transfer the protein solution into a 4-mm OD quartz EPR tube. This is best achieved using a long, thin glass Pasteur pipette capable of reaching to the bottom of the EPR tube. These can be purchased as “NMR Pasteur pipettes” (Wilmad), or can be fabricated by stretching standard 12.5″ Pasteur pipettes with the use of a Bunsen burner. Alternatively, a syringe fitted with a long piece of Teflon tubing that is thin enough to fit inside of the EPR tube can be used.

5. With the EPR tube uncapped, rapidly freeze the sample by immersing the tube in liquid nitrogen. From this point forward, there is a risk of sudden tube fracture, and protective eyewear should be worn at all times. The risk of tube breakage can be minimized by encouraging the sample to freeze from the bottom of the tube upward, thereby allowing any pressure caused by expansion of the solution during freezing to be relieved. This can be achieved by rapidly “bouncing” the EPR tube up and down in the liquid nitrogen such that the top portion of the sample spends relatively less time submerged in the nitrogen bath than the bottom portion, and thus should take slightly longer to freeze. However, the goal is to freeze the sample as quickly as possible so as not to bias the conformational equilibrium of the protein in solution. As such, the amount of time any portion of the sample spends out of the liquid nitrogen during freezing should be limited to a fraction of a second. It has been suggested that the freezing rate for the method of liquid nitrogen immersion described here is limited by the insulating effect of nitrogen vapor at the outer surface of the sample tube, and therefore vigorous shaking of the immersed sample may result in more rapid freezing (Georgieva et al., 2012). Once the sample is frozen, it should be kept immersed in liquid nitrogen until insertion into the spectrometer for DEER measurement.

4.3 DEER on Doubly K1-Labeled PrP

The K1 label is known to display increased internal mobility as compared to the disulfide-linked R1 spin label, owing to an additional rotatable bond in the K1 side chain (Fleissner et al., 2009). While this property makes K1 somewhat less useful than R1 as a reporter of local backbone dynamics and secondary structures, pairs of K1 labels were shown by Fleissner et al. to give reliable, albeit broadened, interspin distance distributions by DEER. Figure 4 shows DEER EPR data on doubly K1-labeled PrP in which pAcPhe was incorporated at residues 188 and 200, corresponding to the end of helix 2 and the beginning of helix 3, respectively (Fig. 4A). Because of the proximity of these mutations to the native disulfide

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bond of PrP, the combination of these two labeling sites would be difficult, if not impossible, to access using traditional cysteine-based SDSL techniques (see Fig. 1). As is evident from Fig. 4B, the DEER data reveal a well-defined interspin distance distribution that agrees well with predictions based on *in silico* modeling of the spin label positions based on the NMR structure of the globular domain of PrP. These data illustrate the utility of UAA methods for SDSL and confirm the assertions of Fleissner et al. that site-specifically incorporated K1 labels can act as faithful reporters of macromolecular distances in solution. Indeed, we have recently used DEER measurements on dual R1/K1-labeled constructs of PrP to characterize a novel long-range tertiary contact in PrP that is mediated by Zn^{2+} binding (Spevacek et al., 2013).

5. CONCLUDING REMARKS

The expanded genetic code of bacteria, yeast, and mammalian cells by amber suppression, allowing for the site-specific incorporation of a wide variety of UAAs, has seen extensive use in the past decade for the study of biological systems. While the specific application of UAA incorporation to SDSL-EPR was described nearly 6 years ago, the technique has yet to see widespread use. In this chapter, we have described the protocol for efficient incorporation of pAcPhe into bacterially expressed proteins, and subsequent reaction of the ketone moiety with the hydroxylamine spin label HO-4120. We have framed our discussion around recent improvements in UAA incorporation efficiency and new strategies for oxime ligation that allow for reaction under a variety of conditions. These developments, along with the commercial availability of all reagents for pAcPhe incorporation and spin labeling, should increase the appeal of K1-labeling strategies, particularly in protein systems where traditional thiol-based SDSL is not feasible. In addition, for proteins that are tolerant of cysteine substitutions, incorporation of pAcPhe in combination with a cysteine mutation allows for site-specific orthogonal labeling with two distinct spin probes. Potential applications of this strategy might include mixed ^{14}N–^{15}N-nitroxide labels, nitroxide–gadolinium pairs for distance measurements at high field (Kaminker et al., 2012), and combinations of paramagnetic and diamagnetic labels for use in CW lineshape methods of interspin distance measurement (Altenbach, Oh, Trabanino, Hideg, & Hubbell, 2001; Rabenstein & Shin, 1995).

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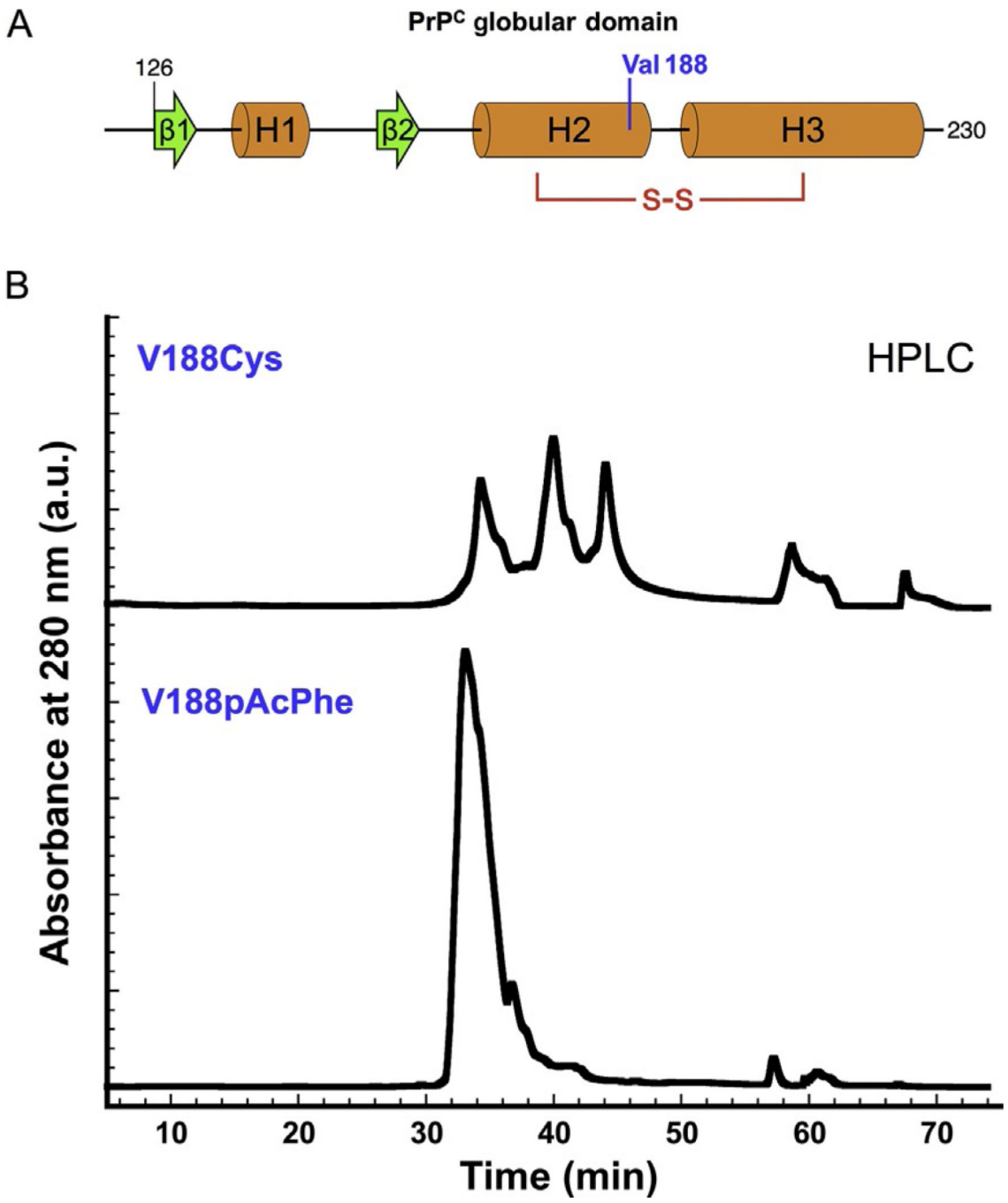

Figure 1.

Difficulties of cysteine-based mutagenesis in PrP in the vicinity of the native disulfide bond. (A) Schematic representation of the globular C-terminal domain of PrP^{C}. Valine 188, shown in blue (black in the print version), is positioned near the end of helix 2, and the native disulfide bond connecting helices 2 and 3 is shown in red (black in the print version). (B) Reverse-phase analytical HPLC traces of PrP constructs in which Val 188 is replaced by cysteine (upper trace) or *p*-acetyl phenylalanine (lower trace). The three peaks between 30

and 50 min in the upper (V188C) trace all possess the same mass, and correspond to different disulfide connectivities.

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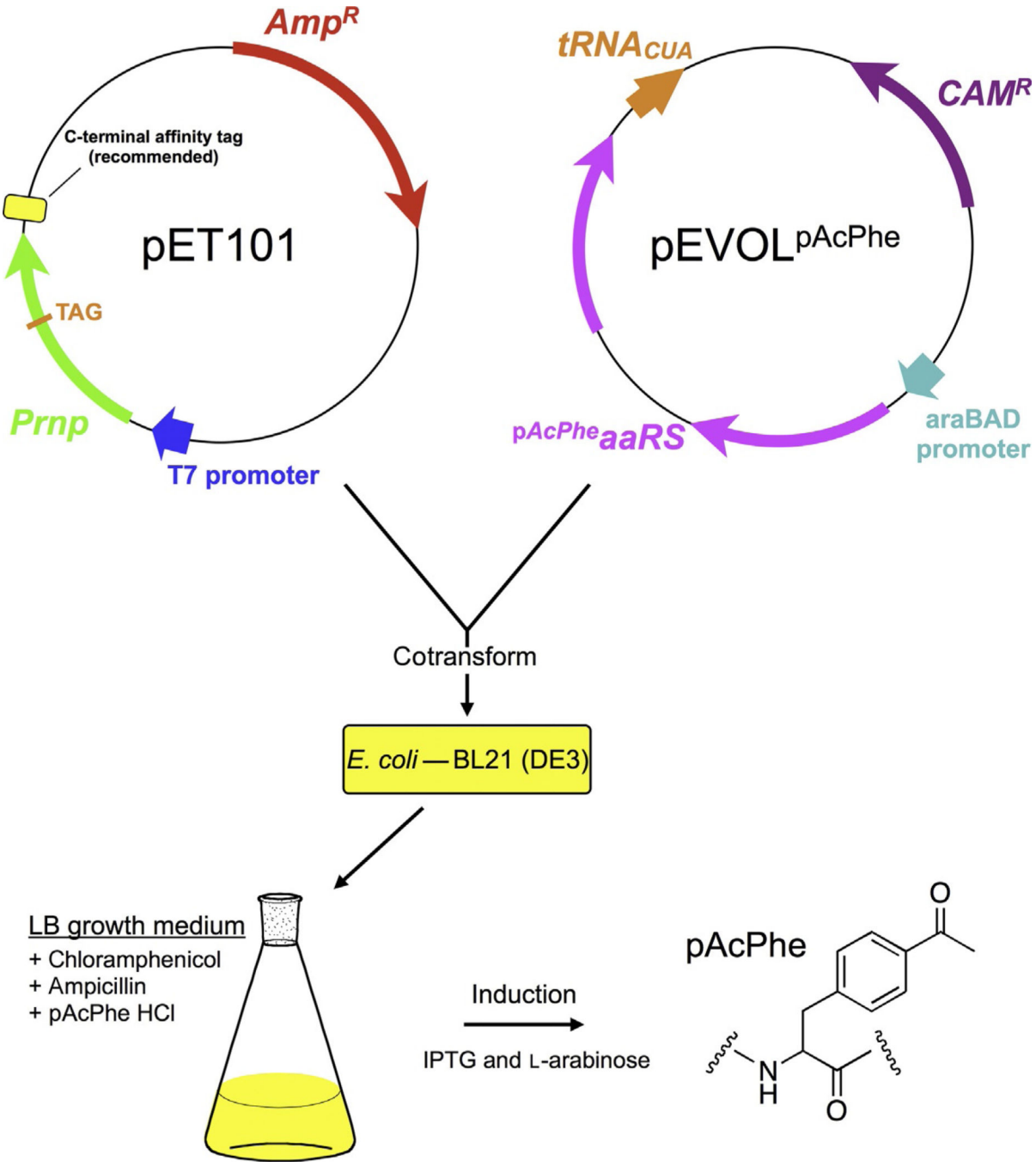

Figure 2.
General scheme of pAcPhe incorporation into recombinantly expressed proteins in *E. coli.*

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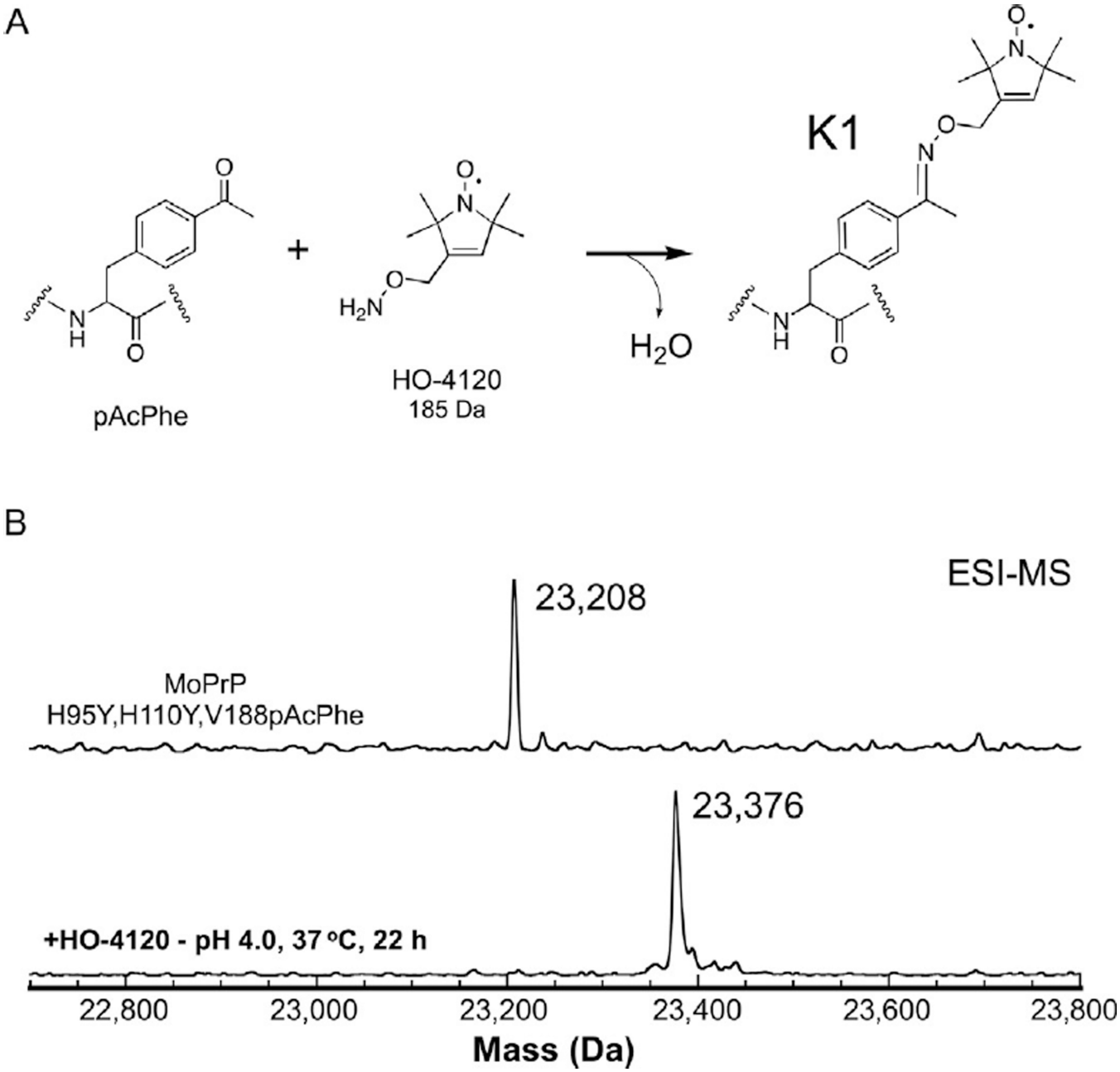

Figure 3.

Reaction of pAcPhe with the hydroxylamine spin label HO-4120 to generate the K1 spin-labeled side chain. (A) K1 reaction scheme. (B) Electrospray ionization mass spectrometry of amouse PrP construct containing a single pAcPhe at residue 188 before (top) and after 22 h reaction with HO-4120 at 37 °C in 10 m*M* sodium acetate, pH 4.0 (bottom). The increase in mass of 168 amu corresponds to addition of HO-4120 (185 amu) with elimination of a water molecule.

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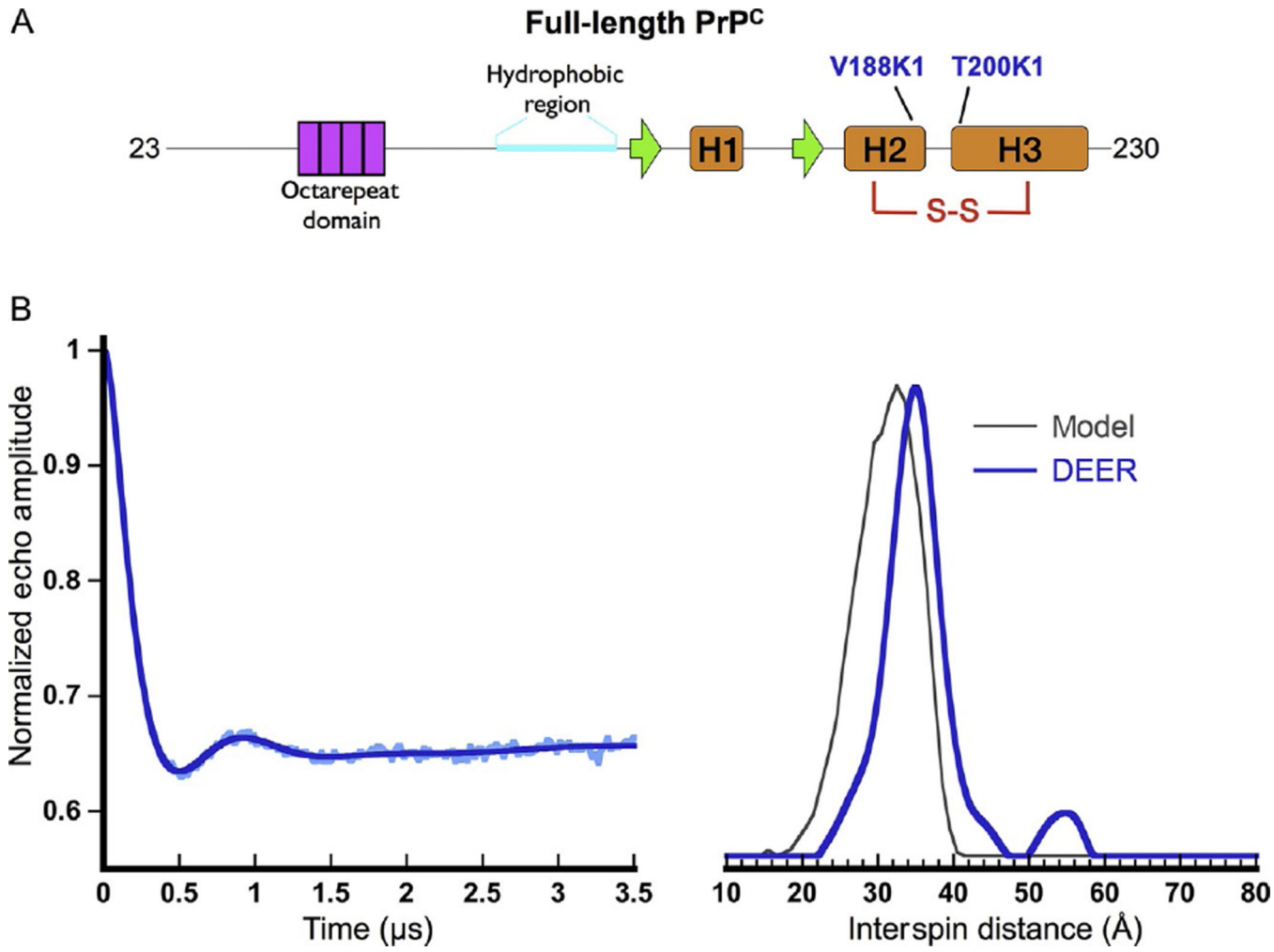

Figure 4.

Interspin distance measurement in doubly K1-labeled PrP by DEER EPR at 50 K. (A) Schematic diagram of full-length PrP showing the positions of key domains and secondary structures. The sites of K1 labeling are indicated in blue (black in the print version). (B) Background-corrected DEER time domain data (left) and calculated distance distribution (right, blue trace, black in the print version) for full-length MoPrP doubly K1-labeled at residues 188 and 200. The DEER data were analyzed with the DeerAnalysis 2011 software package in Matlab (Jeschke et al., 2006). The gray trace in the distance domain is the interspin distance distribution calculated from K1 labels modeled onto the published NMR structure of MoPrP(121–230) (PDB: 1XYX) using mtsslWizard (Hagelueken et al., 2012). *Data from Spevacek et al. (2013).*