Facile Installation of Post-translational Modifications on the Tau Protein via Chemical Mutagenesis

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ABSTRACT: Post-translational modifications of proteins are ubiquitous in living organisms, as they enable an accurate control of the interactions of these macromolecules. For mechanistic studies, it would be highly advantageous to be able to produce in vitro post-translationally modified proteins with site-specificity. Here, we demonstrate one facile way to achieve this goal through the use of post-translational chemical mutagenesis. We illustrate this approach by performing site-specific phosphorylation and methylation of tau, a protein that stabilizes microtubules and whose aggregation is closely linked with Alzheimer’s disease. We then verify the effects of the post-translational modifications on the ability of tau to control microtubule polymerization, revealing in particular an unexpected role for phosphorylation at S199, which is outside the microtubule-binding region of tau. These results show how the chemical mutagenesis approach that we present enables the systematic analysis of site-specific post-translational modifications of a key protein involved in the pathogenesis of Alzheimer’s disease.

KEYWORDS: Post-translational chemical mutagenesis, dehydroalanine, phosphorylation, methylation, protein aggregation, Alzheimer’s disease

INTRODUCTION

The accumulation of aberrant aggregates of the microtubule-associated protein tau is the defining feature of a family of debilitating neurodegenerative disorders known as tauopathies, including Alzheimer’s disease (AD).1–3 These aggregates commonly take the form of highly ordered paired helical filaments or neurofibrillary tangles, where tau is heavily phosphorylated, among other post-translational modifications (PTMs).1–3 While the mechanisms of pathogenesis are yet to be fully elucidated, throughout these diseases the location and abundance of modified tau aggregates strongly correlates with cognitive decline.1–4

Among the physiological functions of tau, the best known is its role in the assembly and stabilization of microtubules.5 In addition, further vital functions are coming to light, such as the regulation of axonal transport,6 DNA maintenance,7 and regulation of synaptic function and plasticity.8 The ability of tau to undergo a plethora of PTMs is key to enabling this functional diversity. The sequence of human tau can be divided into four functional domains (Figure 1a) and is found in six different isoforms depending on splicing events, with varied regions in the N-terminal domain and within the microtubule binding domain (MBD). Alternative splicing variants and PTM patterns also give rise to distinct strain-specific structures within the different tauopathies, such as, for example, the recently elucidated influence of ubiquitination on creating divergent aggregates in AD versus cortico-basal degeneration.4

It is clear that the further investigations of the physiology and pathology of tau would be greatly aided by tools to generate accurate site-specific PTMs.

Phosphorylation of tau has been the most explored PTM by far, as it is the predominant PTM in pathological tau aggregates and is overall the most common PTM on the protein, with over half of the serine, threonine, and tyrosine residues shown to undergo phosphorylation.2,4 To explore the functional consequences of this PTM, in vitro phosphorylation by kinases as well as phosphomimetic mutations using aspartic acid or glutamic acid have been used extensively.9,10 However, these techniques create either heterogeneous products or only partially accurate mimics of the true site-specific modification in question. Such approaches are also not easily amenable to...
exploring the function of more diverse PTMs such as acetylation, methylation, and glycosylation. Recently, a method for the semisynthesis of tau has been reported that is capable of generating full-length tau with highly diverse and exact PTMs. This approach has enabled the incorporation of multiple different PTMs within the same sequence and the installation of nonenzymatic PTMs, providing the ability to study PTM crosstalk and otherwise inaccessible modifications.

While this semisynthesis technique is undoubtedly powerful, and there have been recent advances to extend the practicability of chemical protein synthesis and expressed protein ligation, these approaches require highly specialized training and equipment, which is not readily available to many researchers.

### RESULTS AND DISCUSSION

In this work, we have sought to validate a facile chemical mutagenesis approach to install diverse and accurate site-specific PTM variants on tau using commonly used reagents and techniques. Chemical mutagenesis, the post-translational interconversion of amino acid side chains, has been used extensively to investigate the effects of PTMs on proteins such as kinases and histones. In this work, we report a chemical mutagenesis approach to tau using the synthetically versatile noncanonical amino acid dehydroalanine (Dha) as the conduit to access accurate PTM mimetics (Figure 2). We then validate the physiological relevance of these modified tau variants using a tubulin polymerization assay where we confirm that they reproduce similar activity as observed when created through the semisynthesis method mentioned above and through in vitro phosphorylation. While one limitation of this approach is the likely epimeric mixture of the final PTM (Figure 2), this issue has not caused an issue in the previous studies on other proteins.

To facilitate the installation of Dha, a cysteine mutant precursor is introduced at the target site (Figure 2), and the two native cysteines of tau must also be mutated to serine, which has previously been demonstrated to have no effect on the polymerization activity of tau. We chose three sites and two different PTMs to explore in this study: phosphorylation at S199 in the proline-rich domain (PRD) and at S356 in R4 of the MBD as well as dimethylation at K311 in R3. The
The chemical mutants were then assessed for their influence on the microtubule polymerization activity of tau. The inhibition of tubulin polymerization by our S356pCys mutant is consistent what has previously been observed using both the semisynthesis and in vitro phosphorylation approaches\(^9,12\) (Figure 3b–d). This result supports the accuracy of tau PTMs installed via chemical mutagenesis. With this confidence established, the less characterized PTMs investigated here can now be considered. Thio–ether dimethylation at K311 had a negligible effect on microtubule polymerization (Figure 3c). However, installation of pCys at S199 led to a significant inhibition of polymerization (Figure 3b). At first, this is a surprising result since S199 is deep within the PRD of tau, but a recent study has shown that the PRD is in fact the domain of tau responsible for the initiation of polymerization and early studies of tau function observed that deletion of the PRD results in a total loss of polymerization activity.\(^{22,28}\) Taken together, our results support that phosphorylation at S199 may regulate the role of PRD in microtubule association and could possibly explain its pathological association.

In conclusion, we have described a method of performing site-specific post-translational modifications of tau by chemical mutagenesis and illustrated how this approach enables the systematic analysis of the diverse effects that specific post-translational modifications have on the ability of tau to regulate microtubule polymerization.

### METHODS

**Tau Expression and Purification.** 2N4R tau lacking the endogenous cysteine residues (C291S and C322S) and the relevant cysteine mutants (S199C, K311C, and S356C), created by standard site-directed mutagenesis, were expressed from a pET29b vector in BL21 Gold (DE3) cells (Agilent Technologies). Cultures were grown to an OD600 of 0.6 and then induced with 0.4 mM IPTG and left to express at 18 °C overnight. Cells were harvested by centrifugation, resuspended in 50 mM MES (pH 6.5), 5 mM DTT, 0.1 mM PMSF, and lysed via sonication (1 min 30 s; 5 s on, 10 s off; 40% amplitude) on ice. The lysed mixture was centrifuged, and tau was isolated via cation exchange using a Hitrap CaptoS column (GE Healthcare).
LifeSciences, Little Chalfont, U.K.). Fractions containing tau as determined by gel electrophoresis were pooled and precipitated by the addition of 20% (w/v) ammonium sulfate on ice overnight. The precipitated protein was pelleted by centrifugation and then resuspended in SSPE buffer containing 5 mM DTT. Pure tau was finally isolated via size exclusion chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare LifeSciences, Little Chalfont, U.K.) equilibrated with the aforementioned SSPE buffer, only the purest fractions as assessed by gel were kept for experiments.

**Dha Formation.** The tau cysteine mutants were buffer exchanged into 20 mM NaPi buffer (pH 8) via 7k MWCO Zeba spin desalting columns (Thermo Fisher). Then 200 μl aliquots of 50 μM protein were reacted with 50 mol equiv of methyl 2,5-dibromopentanoate (Sigma-Aldrich) for 12 h at 37 °C and with shaking at 500 rpm. Excess methyl 2,5-dibromopentanoate was removed by passing the reactions through 7k MWCO Zeba spin desalting columns and then conversion to Dha was verified via LC-MS.

**Final Chemical Mutagenesis.** For the creation of the phosphorylation variants, 100 μl aliquots of 50 μM of S199Dha or S356Dha in 20 mM NaPi, (pH 8) were reacted batchwise (5 min intervals) with 30 000 mol equiv of sodium thiophosphate (pH 8.0, 690 mg/mL suspension, 5 × 5000 equiv). The mixtures were left to react for 8 h at 37 °C and with shaking at 500 rpm, excess sodium thiosulfate was removed via two 7k MWCO Zeba spin desalting columns, and reaction completion was verified via LC-MS. For the creation of the dimethyl mimetic at K311, 100 μl aliquots of 50 μM K311Dha in 20 mM NaPi (pH 8) were reacted with 1000 mol equiv of captamine (Sigma-Aldrich) for 12 h at 37 °C and with shaking at 500 rpm. Excess captamine was removed in the same manner as sodium thiophosphate and completion was again verified by LC-MS.

**Protein LC-MS.** Protein LC–MS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 μm, 2.1 mm × 50 mm). Water with 0.1% formic acid (solvent A) and 95% MeCN and 5% water with 0.1% formic acid (solvent B) were used as the mobile phase at a flow rate of 0.2 mL/min. The gradient was programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 min, then a gradient to 95% A over 1.04 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V. Nitrogen was used as the desolvation gas at a total flow of 850 L/h. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on the manufacturer’s instructions.

**Microtubule Polymerization Assay.** The microtubule polymerizations were all performed with reagents from the kit purchased from Cytoskeleton, Inc. (cat. #BK006P) as per the manufacturer’s instructions. Tubulin was added to the reaction mixture at a concentration of 3 mg/mL, and tau and the various chemical mutants were assayed at 15 μM for their ability to polymerize the reaction, which was monitored by OD at 340 nM using a CLARIOstar Plus plate reader (BMG Labtech).

**Circular Dichroism.** Circular dichroism (CD) spectroscopy was used to analyze protein secondary structure in solution. Samples were diluted to 5 μM in PBS, and CD measurements were recorded using a Chirascan spectrophotometer equipped with a Quantum TC125 temperature control unit (25 °C). Data were acquired in a 0.1 cm path length with a response time of 1 s, a per-point acquisition delay of 5 ms, and a pre- and postscan delay of 50 ms. Spectra were averaged over three scans, in the wavelength range from 195 to 250 nm, and the spectrum from a blank sample containing only buffer was subtracted from the averaged data.

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P.R.L., G.J.L.B., and M.V. designed the research. P.R.L. and R.J.T. performed the research. P.R.L., R.J.T., G.J.L.B., and M.V. analyzed the data. P.R.L., R.J.T., G.J.L.B., and M.V. wrote the paper.

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**Notes**

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