***In vitro* AMPylation/Adenylation of Alpha-synuclein by HYPE/FICD**

Ali Camara¹, Anwesha Sanyal^{1, §}, Sayan Dutta^{2, 3},
 Jean-Christophe Rochet^{2, 3, *} and Seema Mattoo^{1, 3, 4, *}

¹Department of Biological Sciences, Purdue University, West Lafayette, USA; ²Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, USA; ³Purdue Institute for Integrative Neuroscience, Purdue University, West Lafayette, USA; ⁴Purdue Institute for Inflammation, Immunology and Infectious Disease, Purdue University, West Lafayette, USA; [§]Present address: Ann Romney Center for Neurological Diseases, Department of Neurology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

*For correspondence: jrochet@purdue.edu; smattoo@purdue.edu

[Abstract] One of the major histopathological hallmarks of Parkinson's disease are Lewy bodies (LBs) –cytoplasmic inclusions, enriched with fibrillar forms of the presynaptic protein alpha-synuclein (α -syn). Progressive deposition of α -syn into LBs is enabled by its propensity to fibrillize into insoluble aggregates. We recently described a marked reduction in α -syn fibrillation *in vitro* upon posttranslational modification (PTM) by the Fic (Filamentation induced by cAMP) family adenylyltransferase HYPE/FICD (Huntingtin yeast-interacting protein E/FICD). Specifically, HYPE utilizes ATP to covalently decorate key threonine residues in α -syn's N-terminal and NAC (non-amyloid- β component) regions with AMP (adenosine monophosphate), in a PTM termed AMPylation or adenylation. Status quo *in vitro* AMPylation reactions of HYPE substrates, such as α -syn, use a variety of ATP analogs, including radiolabeled α -³²P-ATP or α -³³P-ATP, fluorescent ATP analogs, biotinylated-ATP analogs (N6-[6-hexamethyl]-ATP-Biotin), as well as click-chemistry-based alkyl-ATP methods for gel-based detection of AMPylation. Current literature describing a step-by-step protocol of HYPE-mediated AMPylation relies on an α -³³P-ATP nucleotide instead of the more commonly available α -³²P-ATP. Though effective, this former procedure requires a lengthy and hazardous DMSO-PPO (dimethyl sulfoxide-polyphenyloxazole) precipitation. Thus, we provide a streamlined alternative to the α -³³P-ATP-based method, which obviates the DMSO-PPO precipitation step. Described here is a detailed procedure for HYPE mediated AMPylation of α -syn using α -³²P-ATP as a nucleotide source. Moreover, our use of a reusable Phosphor screen for AMPylation detection, in lieu of the standard, single-use autoradiography film, provides a faster, more sensitive and cost-effective alternative.

Keywords: AMPylation, Adenylation, HYPE, FICD, Alpha-synuclein, Post-translational modification

[Background] Deposition of the small (140 amino acids), intrinsically disordered protein alpha-synuclein (α -syn) into Lewy bodies (LBs) is a major hallmark of Parkinson's disease (PD) (Weinreb *et al.*, 1996; Spillantini *et al.*, 1997). On its way to forming the insoluble aggregates found in LBs, monomeric α -syn adopts a β -sheet-rich, amyloid-like conformation, leading to progressive

fibrillation and concomitant insolubility (Iwai *et al.*, 1995). Multiple lines of research describe a compelling correlation between post-translational modifications (PTMs) of α -syn and its oligomeric state. Phosphorylation at Ser 129, for example, is prevalent on aggregated α -syn found within LBs (Chen and Feany, 2005). Tyrosine nitrations of α -syn have been linked to formation of stable oligomers and reduced fibril formation (Yamin *et al.*, 2003), while O-GlcNAcylation of threonine residues reduces α -syn aggregation (Levine *et al.*, 2019). Recently, we described a novel α -syn modification—adenylylation or AMPylation—which reduces α -syn fibrillation *in vitro* (Sanyal *et al.*, 2019). Moreover, AMPylated α -syn displayed diminished disease-linked phenotypes, such as membrane permeabilization (Ysselstein *et al.*, 2017; Sanyal *et al.*, 2019).

We demonstrated that HYPE, the sole Fic protein in humans, carried out AMPylation of α -syn (Worby *et al.*, 2009; Sanyal *et al.*, 2019). Like other members of the conserved Fic domain protein family, HYPE's AMPylation activity is intrinsically regulated by an inhibitory helix called α_{inh} (Engel *et al.*, 2012). Mutation of HYPE's conserved Glu234 to Gly within the α_{inh} renders HYPE constitutively active for its adenylyltransferase activity (Engel *et al.*, 2012; Sanyal *et al.*, 2015). HYPE then covalently attaches the AMP portion of an ATP co-substrate onto target hydroxyl side-chain residues (e.g., serine or threonine) (Ham *et al.*, 2014; Sanyal *et al.*, 2015; Truttmann *et al.*, 2016; Preissler *et al.*, 2017). Indeed, tandem mass spectrometric analysis of purified, recombinant α -syn mapped AMPylation sites to three threonines—T33, T54, and T75—located within regions hypothesized to be crucial for oligomerization (Sanyal *et al.*, 2019).

Several biochemical assays monitoring Fic-mediated AMPylation have been reported (Worby *et al.*, 2009; Mattoo *et al.*, 2011; Yarbrough *et al.*, 2009; Lewallen *et al.*, 2012; Truttmann *et al.*, 2016; Dedic *et al.*, 2016). Most common amongst these are assays using a fluorescent (FI-ATP) or radioactive (α - ^{32}P -ATP or α - ^{33}P -ATP) ATP analogs. While FI-ATP has served as a useful research tool, its large, bulky fluorescent moiety is known to stunt kinetic efficiencies (Lewallen *et al.*, 2012). Moreover, no step-by-step protocol for FI-ATP AMPylation has been documented. A detailed procedure for α - ^{33}P -ATP, however, is available (Truttmann and Ploegh, 2017). This method entails incubation of HYPE and its protein target with radiolabelled α - ^{33}P -ATP, followed by separation via SDS-PAGE, DMSO-PPO precipitation, and then imaging of radiolabeled proteins on autoradiography film. Though effective in detecting AMPylation, this procedure suffers from additional time-consuming steps that are expensive and require hazardous chemicals (e.g., DMSO-PPO and autoradiography exposure and development). Herein, we present an optimized method for monitoring AMPylation *in vitro*, using α -syn as a target and α - ^{32}P -ATP as a nucleotide source. α - ^{32}P -ATP preserves signal sensitivity, while eliminating DMSO-PPO precipitation. Our protocol also replaces the expensive single-use autoradiography film with a reusable Phosphor screen, which allows more sensitive detection of radioactive phosphate-containing proteins under ambient light and at shorter exposure times. Given the broad specificities of Fic proteins and other enzymes for co-substrates including GTP, CTP, phosphocholine, and even ATP used for phosphorylation, this protocol can be adapted to assess other PTMs *in vitro* (Mattoo *et al.*, 2011; Veyron *et al.*, 2018).

Materials and Reagents

1. Kimwipes (Kimberly-Clark Professional, KimTech Science Brand, catalog number: 34155)
2. Set of micropipettes, with volumes ranging from 0.5 to 1,000 μ l (generic)
3. Appropriately sized sterile micropipette tips (generic)
4. 2 ml pipette bulbs (VWR, catalog number: 82024-554)
5. Pasteur pipette (VWR, catalog number: 14672-380)
6. Glass thermometer, 20-100 $^{\circ}$ C range (generic)
7. 1.5 ml tubes (Eppendorf Safe-lock microcentrifuge tubes, catalog number: 022363204)
8. Gel loading pipet tips (Fisher Scientific, Fisher Brand, catalog number: 02-707-181)
9. MTC Stop-Pop locking tubes clips (MTC Bio, catalog number: C2086)
10. Bacterially purified HYPE₁₀₂₋₄₅₆ (WT, E234G, and E234G/H363A) (Sanyal *et al.*, 2019)
11. Bacterially purified full-length α -syn (Sanyal *et al.*, 2019)
12. ddH₂O (ELGA LabWater, PURELAB Flex 2 Dispenser, model number: PF2XXXXM1)
13. HEPES (Sigma-Aldrich, Sigma-Millipore, catalog number: H4034)
14. Manganese (II) chloride tetrahydrate (Sigma-Aldrich, Sigma Millipore, catalog number: M3634)
15. 12.1 N Hydrochloric acid (Fisher Scientific, Fisher Chemical, catalog number: A144-212)
16. 50% (w/w) Sodium hydroxide solution (Fisher Scientific, Fisher Chemical, catalog number: SS254-4)
17. Tris base (Fisher Scientific, Fisher Bioreagents, catalog number: L-15863)
18. Sodium chloride (Fisher Scientific, Fisher Chemical, catalog number: L-11620)
19. Glycerol (Fisher Scientific, Fisher Bioreagents, catalog number: L-13751)
20. α -³²P-ATP 3000Ci/mmol 10mCi/ml, 250 μ Ci (Perkin Elmer, BLU003H250UC)
21. 4-20% Precast SDS-PAGE gel (Bio-Rad, Mini-PROTEAN TGX Gels, catalog number: 456-1091)
22. Protein ladder (Bio-Rad, Precision Plus Protein Dual Color Standards, catalog number: 1610374TGX)
23. 2-Mercaptoethanol (Sigma-Aldrich, Sigma Life Sciences, catalog number: M3148)
24. Sodium dodecyl sulfate (Sigma-Aldrich, Sigma Life Sciences, catalog number: L4390)
25. Bromophenol Blue sodium salt, ACS (Alfa Aesar, catalog number: 32639)
26. Methanol (Fisher Scientific, Fisher Chemical, catalog number: L-21063)
27. Acetic acid, glacial (Fisher Scientific, Fisher Chemical, catalog number: A38C-212)
28. Glycine (Fisher Scientific, Fisher Bioreagents, catalog number: L-15696)
29. Coomassie Brilliant Blue R-250 (Amresco, catalog number: 0472-25G)
30. Phosphor screen (General Electric, Kodak Phosphor Storage Screen, catalog number: 00146931)
31. Protein Storage Buffer (see Recipes)
32. 10x AMPylation Buffer (see Recipes)
33. 4x SDS-PAGE Loading Buffer (see Recipes)

34. 1x SDS-PAGE Running Buffer (see Recipes)
35. Coomassie Stain (see Recipes)
36. Destain (see Recipes)

Equipment

1. Plexiglass or Lucite shield, 0.5 inches thick (Nalgene Acrylic Benchtop Beta Radiation Shield, Thermo Fisher, catalog number: 6700-1812)
2. Geiger counter (Model 3 Survey Meter, Ludlum Measurements, model: 44-7)
3. ddH₂O dispenser (PURELAB Flex 2 Dispenser, ELGA LabWater, model: PF2XXXXM1)
4. Heatblock (Analog Heatblock, VWR, catalog number: 12621-108)
5. pH meter (Seven Easy, Mettler Toledo, catalog number: 1227396066)
6. Centrifuge (Eppendorf, model: Centrifuge 5417C, catalog number: 0023210)
7. Power supply (PowerPac 300/DCode, Bio-Rad, model: PowerPac 300)
8. PAGE system (Mini-PROTEAN Tetra Cell, 4-Gel System, Bio-Rad, catalog number: 1658004)
9. Fluorescent Image Analyzer (Typhoon FLA 9500, General Electric, model: Typhoon FLA 9000)
10. Phosphor screen eraser (FLA Image Eraser, General Electric, model: FLA Image Eraser)
11. Rocker (VWR, Rocking Platform, model: 200)
12. Image scanner (Epson, Epson Perfection V700 Photo)

Software

1. Typhoon FLA 9500 (General Electric)

Procedure

A. AMPylation reaction

Note: This protocol requires use of radioactive α -³²P-ATP. As such, all radioactive materials or materials in contact with them must be handled, stored, and disposed of in accordance with your institution's radioactive safety guidelines. Radioactivity must be handled behind a radiation shield at all times. A Geiger counter should be used to constantly monitor work environment for radioactive contamination. Appropriate personal protective equipment should be worn at all times.

1. Pipette calculated volume of ddH₂O into designated 1.5 ml tubes.
2. Pipette calculated volume of 10x AMPylation Buffer into designated 1.5 ml tubes.

Note: Components of 10x AMPylation Buffer may be modified as needed to accommodate differing experimental conditions, such as enzymes, substrates, etc.

3. Pipette 2 μ g of purified, recombinant HYPE enzyme to designated 1.5 ml tubes.

Notes:

- a. *Reactions were performed at a final volume of 20 μ l. The actual reaction amounts may be*

modified so long as a $\geq 10:1$ molar substrate:enzyme ratio is maintained, and a detectable amount of each protein (i.e., $\geq 0.1 \mu\text{g}$) is loaded onto the gel.

- b. All proteins are purified as previously described (Sanyal et al., 2019) and come dissolved in protein storage buffer.*
- c. It is advisable to prepare twice the amount of required sample in case the gel needs to be rerun.*
4. Pipette 20 μg of purified, recombinant α -syn (or other substrate) into designated 1.5 ml tubes.
Note: When performing kinetic experiments or comparing different substrates side-by-side, molar concentrations should be used in order to ensure that the relative numbers of molecules in the reaction are consistent.
5. Pipette 2.5 μCi α - ^{32}P -ATP into designated 1.5 ml tubes.

Notes:

- a. Reaction start time begins with α - ^{32}P -ATP addition. As α - ^{32}P -ATP is the only radioactive component, the other sample ingredients may be added at a standard lab bench, and samples can then be transported to a designated radioactive area for α - ^{32}P -ATP addition.*
- b. Alternative concentrations of α - ^{32}P -ATP may be used depending on the catalytic efficiencies of the test enzyme and/or the freshness of the nucleotide. These concentrations should be determined empirically. AMPylation reactions should ideally occur within the first half-life (14.3 days) of α - ^{32}P -ATP.*
6. Incubate the samples in a heat block set to 30 °C for 30 min.
Notes:
 - a. Be sure to place the heat block behind a radiation shield.*
 - b. Different AMPylases may require different reaction temperatures or durations for optimal enzymatic activity.*
 - c. Samples may be snap-centrifuged for 5 s at 2,000 x g in order to consolidate any sample components not at the bottom of the tube.*
7. Quench reaction with an appropriate volume of 4x SDS Loading Buffer.
8. Heat samples for 10 min at 95 °C on a heat block.
Note: Ensure that tube lids are firmly closed to avoid lids popping open during heating. A weighted sample tray may be placed flatly over tube lids to prevent opening. Alternatively, MTC Stop-Pop locking tubes clips (MTC Bio, Inc.) could be used.
9. Snap centrifuge samples for 5 s at 2,000 x g.

B. SDS-PAGE separation

1. Carefully remove well comb and green seal tape from precast gel cassette.
2. Assemble gel cassette and tank according to manufacturers' instructions.
Note: Fresh 1x Running Buffer should be used inside of the gel cassette assembly, although used 1x Running Buffer may be used to fill the tank.
3. Using gel loading pipette tips, carefully load 2-4 μl of protein ladder into the first lane.

4. Using gel loading pipette tips, carefully load 10-30 μ l of each sample into lanes adjacent to the first lane.
5. Securely close tank lid.
6. Run gel at constant 200 V for 45 min. Alternatively, better band resolution can be obtained by first running the gel at 80 V for ~30 min (until the samples clear the stacking layer), then ramping up to 120 V for ~1 h (until the dye front reaches the reference line on the gel cassette).
Note: Ensure proper current flow via production of bubbles in the running buffer and migration of the dye front towards the bottom of the gel.

C. Phosphor imaging

1. Carefully remove gel using a cassette lever.
Note: The gel is radioactive at this point and should be handled and disposed of in accordance with your institution's regulations/guidelines for radioactive materials.
2. Place gel in between a clean, plastic cover sheet. Smooth out any air bubbles in the gel.
3. Place cover sheet with gel on top of the imaging side (white) of a clean Phosphor screen.
Notes:
 - a. Phosphor screen should be erased with the Phosphor screen eraser after each use to prevent signal contamination.
 - b. Avoid touching the imaging side of the Phosphor screen, as this can damage the screen.
4. Carefully place Phosphor screen and gel inside of the foam phosphor screen storage container.
5. Expose the screen for 3 h at room temperature.
Note: Duration of Phosphor screen exposure is dependent on the AMPylation reaction parameters and must therefore be determined empirically.
6. Place the Phosphor screen imaging-face down in an imager.
Note: Ensure imager screen is clear of debris before imaging, as these may mask sample signal. Kimwipes and ddH₂O should be used to clean imager screen as needed.
7. Open the Typhoon FLA 9500 software.
8. Under "Mode Settings", select the "Phosphorimaging" tab.
9. Under "Reader Settings," go to the "Method" dropdown tab and select "[Phosphor]" option.
Note: The "[Phosphor]" option will be colored red and correspond to a wavelength of 673 nm.
10. Under "Reader Settings," go to "PMT" (Photo-multiplier Tube) and select a value between 250-1,000 V.
Note: A PMT of 500 V is usually a good starting point to gauge band intensity; the higher the PMT, the stronger the signal.
11. Under "Reader Settings," go to "Area" and select the grid area corresponding to where the gel is placed on the screen. Alternatively, the entire screen may be imaged, but this takes additional time.
12. Under "Reader Settings" go to "Pixel Size" and Select "50 μ m" pixel size.
Note: Lower or higher resolutions may be selected based on specific image quality needs.

13. Select "Scan" to scan image.
14. Select "Save As" to save image as a Tiff file in the appropriate folder.

D. Coomassie staining

1. Once exposure of the Phosphor screen to the gel is complete, incubate the gel in Coomassie Stain with a rocker set to low to medium speed for 10 min.
Note: The amount of stain used will vary depending on the shape and dimensions of the container used for incubation but should be enough to fully immerse the gel.
2. Pour off Coomassie Stain in its original container for reuse.
3. Incubate gel in Destain on rocker for 30 min or until sufficient contrast between protein bands and gel background is established.
4. Discard Destain in appropriate waste container.
5. Scan gel on generic document scanner between cover sheet, or with phone camera or scanning application.

Notes:

- a. Coomassie staining is done to control for protein loading, and should yield bands that validate experimental amounts of protein (see Figure 1, bottom).
- b. If reactions are set up using molar ratios instead of mass-based concentrations, the apparent band sizes may vary with the differing molecular weights of the proteins.

Figure 1. HYPE AMPylates α-syn *in vitro*. AMPylation competent E234G-HYPE robustly AMPylates α-syn and itself (auto-AMPylation) with α-³²P-AMP (top). Intrinsically autoinhibited WT-HYPE and a catalytically dead E234G/H363A mutant show no auto-AMPylation or substrate AMPylation (top). 1 μg of enzyme and 10 μg of substrate from the AMPylation reaction were loaded on the gel (bottom). Phosphor screen was

exposed for 3 h (top).

Recipes

Note: Dissolve the following solutions in ddH₂O. Always add solvent before addition of other components. No pH adjustments are required unless explicitly stated. Adjust pH to appropriate value with 12.1 N HCl or 50% NaOH with pipette and rubber suction bulb. Diluted acid or base may also be prepared to avoid overshooting target pH.

1. Protein Storage Buffer
 - 50 mM Tris base
 - 300 mM NaCl
 - 10% (v/v) glycerol
 - Adjust to pH 7.5
2. 10x AMPylation Buffer
 - 50 mM HEPES
 - 10 mM MnCl₂
 - Adjust to pH 7.5
3. 4x SDS-PAGE Loading Buffer
 - 2.86 M BME
 - 250 mM Tris, pH 6.8
 - 10% (w/v) SDS
 - 0.008% (v/v) bromophenol blue
 - 40% (v/v) glycerol
4. 1x SDS-PAGE Running Buffer
 - 0.3% Tris base
 - 1.4% glycine
 - 0.1% (w/v) SDS
5. Coomassie Stain
 - 50% (v/v) methanol
 - 10% (v/v) acetic acid
 - 0.1% Coomassie Brilliant Blue
6. Destain
 - 50% (v/v) methanol
 - 10% (v/v) acetic acid

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Competing interests

The authors declare no conflicting or competing interests.

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